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Characterization of the Cytokine-Neurotrophic Cascade Associated With Neurotoxin-Induced Plasticity of Dopaminergic Neurons in Young and Middle-Aged Mice

by

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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

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ABSTRACT

Characterization of the Cytokine-Neurotrophic Cascade Associated With Neurotoxin-Induced Plasticity of Dopaminergic Neurons in Young and Middle-Aged Mice

by

Angela Ho

Advisor: Mariann Blum, Ph.D.

The aim of this thesis is to examine the cytokine-neurotrophic cascade associated with plasticity of dopaminergic neurons and factors that may be responsible for age-related decline in axonal sprouting. Striatal implantation of interleukin-1 (IL-1), an immune response-generated cytokine, can stimulate compensatory axonal sprouting in the denervated striatum from remaining dopaminergic afferents of parkinsonian animals. Since IL-1 is thought not to act directly to induce the plasticity of dopaminergic neurons, we investigated whether IL-1's beneficial effects are mediated by the induction of dopaminergic neurotrophic factors derived from astroglia. Among the factors studied, a selective upregulation of basic fibroblast growth factor (bFGF) synthesis by IL-1 was observed suggesting that bFGF could be the putative factor mediating IL-1-induced dopaminergic sprouting. We next sought to determine whether IL-1 and trophic factor activities are associated with spontaneous dopaminergic sprouting in the denervated striatum of young mice following a neurotoxin challenge of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which selectively destroys midbrain dopaminergic neurons. Young mice displayed a greater increase in IL-1α synthesis in the denervated striatum, which remained elevated for several weeks, in contrast to middle-aged mice which exhibited a modest induction for a week. Moreover, the induction of IL-1α synthesis was region specific, for no changes were seen in the midbrain in either age group despite glial activation. Surprisingly, no changes in bFGF or any other astroglia-derived neurotrophic factors

were observed in the denervated striatum subsequent to the induction of IL-1. Unlike a stab injury in which activated microglia are the main source of IL-1α and the induction is detected within several hours, increased levels of IL-1α were found to not only be localized within reactive astrocytes but also not found to induced until several days following MPTP. We postulated that when astrocytes are activated by microglial factors to transcribe IL-1, the ability of astrocytes to respond to IL-1 changes. We therefore, challenged glial cultures first with lipopolysaccharide (LPS), a potent inducer of microglia-derived IL-1α or MPTP, and assessed whether pretreatment with either of these two immune activators differentially affect the ability of astrocytes to respond to IL-1α by inducing bFGF expression. LPS, as predicted, elicited a very rapid induction of IL-1α in microglia and lead to the subsequent induction of astroglial bFGF. However, in response to MPTP, there was a delayed induction of IL-1α in astrocytes and exogenous application of IL-1α to MPTP-treated cultures did not result in the induction of bFGF. Together, these results suggest that different injury-induced inflammatory reactions can alter the molecular profile and responsiveness of astrocytes. To address whether growth-inhibitory substrates may further underlie the age-associated decline in dopaminergic sprouting after MPTP, we examined the expression of the extracellular matrix inhibitory molecule chondroitin-sulfate proteoglycan (CSPG) in the denervated striatum of young and middle-aged mice. We found that while young mice displayed a transient increase of CSPG immunoreactivity, a sustained induction of CSPG was observed in middle-aged mice, which may contribute to the reduced axonal plasticity with age.

DEDICATION

To My Family With Love,

Thank You For Your Neverending Love and Support

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ABBREVIATIONS

aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
CNS	central nervous system
CSPG	chondriotin sulfate proteoglycan
CS-4	chondriotin sulfate-4
CS-6	chondriotin sulfate-6
ECM	extracellular matrix molecule
EGF	epidermal growth factor
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
IL-1	interleukin-1
IL-1α	interleukin-1 alpha
IL-1β	interleukin-1 beta
LPS	lipopolysaccharide
MPP+	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NGF	nerve growth factor
PD	Parkinson's disease
RRA	retrorubral area
SN	substantia nigra
TGF-β1	transforming growth factor-beta 1
TH	tyrosine hydroxylase
TNFα	tumor necrosis factor alpha

CHAPTER 1

Introduction

Selective degeneration of substantia nigra dopaminergic neurons in the midbrain leads to the motor impairments of Parkinson's disease (PD; Bernheimer et al. 1973; Agid et al. 1987; Hirsch et al. 1988). Neural transplants into the denervated striatum, which receives the major axonal projections of these neurons, can stimulate compensatory axonal sprouting from endogenous dopaminergic neurons and promote functional recovery in animal models of PD and Parkinsonian patients, despite limited survival of transplants (Bohn et al. 1987; Fiandaca et al. 1988; Lindvall 1989; Lindvall et al. 1990; Bankiewicz et al. 1991; Kordower et al. 1991; Anglade et al. 1993). This suggests that the transplantation-induced plastic changes may not be specific and do not depend on the survival of the transplants.

Increasing evidence supports that a cytokine-neurotrophic cascade may underlie the transplantation-induced compensatory axonal sprouting in the host denervated striatum (Wang et al. 1991, 1994a; Ewing et al. 1992; Hansen et al. 1995). Transplantation of a neuronal graft initiates an inflammatory reaction (Hansen et al. 1988; Plunkett et al. 1990). This inflammatory reaction is mediated by activation of microglia and infiltrating polymorphonuclear cells and, could release inflammatory cytokines that can stimulate reactive astrocytes (Giulian and Lachman 1985; Giulian et al. 1986, 1988a; Hansen et al. 1988). Astrocytes once activated, hypertrophy and proliferate, can release neurotrophic factors to promote a response in the spared dopaminergic neurons (Manthorpe et al. 1986; Gage et al. 1988; Giulian et al. 1993). Yet, specific cytokines that can regulate glial proliferation, neurotrophic factor expression and neuronal plasticity in animal models of PD have yet to be elucidated. Parkinsonian animals receiving striatal implants of interleukin (IL-1), an immune response-generated cytokine, can demonstrate behavioral

improvement by stimulating compensatory sprouting from spared dopaminergic neurons (Wang et al. 1994a). Since previous evidence have suggested that IL-1 may not act directly to induce the survival and plasticity of dopaminergic neurons (Akaneya et al. 1995; Coelin et al. 1995), we thus asked whether reactive astrocytes induced by IL-1 release dopaminergic neurotrophic factors to promote dopaminergic axonal sprouting.

The dopaminergic cell system has been shown to exhibit compensatory mechanisms in response to injury. However, the degree of neuronal plasticity becomes remarkably reduced with age. Previous studies have shown that young mice have the ability to promote spontaneous sprouting of dopaminergic nerve terminals after a neurotoxin-induced damage, while aging mice do not (Ricaurte et al., 1986, 1987a, 1987b; Date et al., 1990a). To date, the endogenous molecular and cellular mechanisms associated with the sprouting of axonal terminals in the denervated striatum of young mice, but not aging mice is not known. We therefore examined the potential role of IL-1 and trophic factor activities in mediating the spontaneous dopaminergic sprouting of young, but not middle-aged mice. It may be that the responses to injury such as changes in glial reaction, cytokine and trophic factor release and further modulations of the extracellular milieu to support axonal plasticity declines with age.

Astroglial cells are important immune regulators and can ultimately determine, in part, the degree of neural repair following injury (for review, see Eddleston and Mücke 1993). In both the normal adult brain and after injury, astroglial cells are thought to be particularly unsupportive for axonal outgrowth, especially in the vicinity of a glial scar (Reier et al. 1983; Liuzzi and Lasek 1987). However, increasing evidence suggests that astroglial cells contribute beneficial effects on axonal growth by growth factor synthesis (Manthorpe et al. 1986; Gage et al. 1988; Giulian et al. 1993). This indicates functional heterogeneity in the astrocyte population to control recovery of injured brain. We thus sought to examine whether astrocytes respond differently to different types of insults that might influence their functional capacity to promote axonal outgrowth.

Differences in the ability to promote axonal outgrowth after injury may be due to inhibition by the extracellular environment. The extracellular matrix (ECM) plays an important role in regulating growth-promoting and inhibitory functions during development and repair after neuronal injury (for review, see Brodkey et al. 1993). Components of the ECM such as fibronectin, are important for growth and elongation, while chondroitin sulfate proteoglycans (CSPGs) are potent inhibitors of neurite outgrowth (Baron-Van Evercooren et al. 1982; Snow et al. 1990a). Moreover, the ECM molecule tenascin can exhibit both growth-promoting and inhibitory effects on neurite outgrowth (Faissner and Kruse 1990; Lochter et al. 1991). Many of the ECM molecules are expressed during critical periods of development, mediating cell-cell interactions and recognition events, and they reappear following neuronal injury (Steindler et al. 1989a; Snow et al. 1990b; McKeon et al. 1991). Injury-induced expression of these ECM molecules may be key modulators in regulating neuronal plasticity. In the aging brain, dopaminergic axonal sprouting is delayed and reduced in contrast to young adults after a neurotoxin-induced challenge (Ricaurte et al., 1986, 1987a,b; Date et al., 1990a). We therefore investigated whether the presence of potent inhibitory molecules and/or lack of growth-promoting molecules in the ECM may contribute to reduced axonal plasticity with age. In addition, since the cytokine transforming growth factor-beta 1 (TGF-β1) is thought to mediate neuronal repair processes by inducing the synthesis of ECM molecules (Pearson et al. 1988; Baghdassarian et al. 1993; Pasinetti et al. 1993), we also examined whether the effects of TGF-β1 on the expression of ECM molecules are altered with age.

Taken together, our studies will hopefully provide new insights into the cascade of cytokine-growth factor activities that may govern the extracellular milieu to promote survival and plasticity of dopaminergic neurons.

CHAPTER 2

Background

Neuroanatomical Features of the Mesencephalic Dopaminergic Cell System

The mesencephalic dopaminergic cell system is comprised of three groups of dopaminergic neurons (Areas 8, 9 and 10) based on their morphology and connectivity (for review see, Björklund and Lindvall 1984). They form an extensive and fairly continuous cell system throughout the ventral tegmentum of the midbrain. They can be distinquished by substantia nigra (A9), retrorubral area (A8) and ventral tegmental area (A10). The substantia nigra (SN) dopaminergic neurons reside mainly in the pars compacta of the SN. Some scattered SN neurons are also found to reside in the pars reticulata of the SN. The retrorubral area (RRA) and the ventral tegmental area (VTA), often referred to as outlying neurons of the SN, are predominantly found ventrolateral, and medial to the SN, respectively.

The dopaminergic ascending projection system to the telecephalon originating from these dopaminergic cell groups is subdivided into two major subsystems: the mesostriatal and mesolimbocortical dopaminergic systems, which control motor and cognitive behaviors, respectively. The mesostriatal dopaminergic system is comprised of projections to the entire striatal complex which consists of a dorsal and a ventral component. In the rodent, the dorsal striatum (caudate-putamen) is innervated mostly by dopaminergic neurons in the SN pars compacta, while the ventral striatum (nucleus accumbens, and the olfactory tubercle) within the limbic system, receives projections from neurons in the RRA and VTA. Projections to limbic, allocortical and neocortical areas constitute the mesolimbocortical dopaminergic system.

Mesostriatal Dopaminergic System and Parkinson's Disease

The mesostriatal dopaminergic system plays an important role in the control of motor functions. Selective degeneration of the mesostriatal pathway leads to the motor impairments found in PD (Bernheimer et al. 1973; Agid et al. 1987; Hirsch et al. 1988). Neurological symptoms exhibited by parkinsonian patients such as rigidity, bradykinesia, tremor, and loss of postural reflexes occur when more than 80% of SN dopaminergic neurons degenerate (Hoehn and Yahr 1967; Bernheimer et al. 1973; Agid et al. 1987; Hirsch et al. 1988). The dopaminergic cell loss in PD is not universal; dopaminergic neurons that reside in the VTA and RRA are far less affected (Hirsch et al. 1988; German et al. 1989). Thus in PD, the dopaminegic innervation to the dorsal striatum is preferentially depleted, while the dopaminergic terminal fields in the ventral striatum are relatively spared (Hirsch et al. 1988; German et al. 1989). L-DOPA (precursor of dopamine) replacement therapy can temporarily relieve some of the symptoms, but does not prevent further dopaminergic cell loss or alter the natural history of the disease (Koller and Hubble 1990). Grafting dopamine-producing tissues such as adrenal medullary chromaffin cells or fetal mesencephalic tissue into the striatum of animal models of PD and Parkinsonian patients has been shown to induce a compensatory sprouting response in the denervated striatum from endogenous dopaminergic neurons (Bohn et al. 1987; Fiandaca et al. 1988; Lindvall 1989; Lindvall et al. 1990; Bankiewicz et al. 1991; Kordower et al. 1991; Anglade et al. 1993). This suggests that the dopaminergic cell system exhibits compensatory mechanisms. However, the survival of adrenal medullary cells is limited in the intrastriatal grafts (Bankiewicz et al. 1988; Fiandaca et al. 1988; Plunkett et al. 1990) and the use of human fetal dopamine tissue is difficult to obtain and the use is controversial. Possibly the enhancement of the survival and function of residual dopaminergic cells could be a potential mechanism to slow the progression of PD.

Animal Models of Parkinson's Disease

The use of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that selectively destroys the SN dopaminergic cells, while sparing the dopaminergic neurons in the VTA and RRA, has created an animal model of PD (Langston, 1985; Singer and Ramsay, 1990). The neurotoxic effects of MPTP depend on its metabolic conversion to 1-methyl-4-phenylpyridinium ion (MPP^+) by monoamine oxidase-B in astroglial cells followed by the uptake of MPP^+ into dopaminergic neurons (Chiba et al. 1984; Castagnoli et al. 1985; Javitch et al. 1985). The active uptake of MPP^+ in the dopaminergic terminal fields becomes concentrated in the mitochondria which leads to the inhibition of NADH dehydrogenase, blocking the electron transport system (Nicklas et al. 1985; Ramsay and Singer 1986; Sayre 1989). This leads to ATP depletion, free-radical formation, followed by cell death (Di Monte et al. 1986; Adams and Odunze 1991; Chan et al. 1991). While the administration of MPTP has been useful as an animal model of PD in primates (Burns et al. 1983; Langston et al. 1984), the effect of MPTP on rodents, particularly in rats, is less effective (Boyce et al. 1984; Chieuh et al. 1984; Sahgal et al. 1984; Fuller and Steranka 1985). Furthermore, studies have shown that there are strain differences in sensitivity to MPTP in mice (Sundström et al. 1987). It has now been well-documented that C57BL/6 mice given MPTP can produce an almost complete, and relatively selective degeneration of SN dopaminergic neurons similar to that seen in primates (Heikkila et al. 1984; Sundström et al. 1987, 1988). However, the behavioral symptoms have yet to be reported in the mouse model of PD.

Another selective catecholamine neurotoxin that has been widely used is 6-hydroxydopamine (6-OHDA; Ungerstedt 1968; Perese et al. 1989). Neurochemical and behavioral deficits similar to those reported in PD patients can be induced in rats by stereotaxic injections of 6-OHDA into the SN or striatum (Javoy et al. 1976; Sauer and Oertel 1994). It is especially used in rats as an animal model of PD and is widely used

for transplantation studies in the brain.

Compensatory Mechanisms Attributed to Dopaminergic Sprouting

Neural transplants in animal models of PD can stimulate compensatory axonal sprouting in the denervated striatum from endogenous dopaminergic neurons and promote functional recovery (Bohn et al. 1987; Fiandaca et al. 1988; Lindvall 1989; Lindvall et al. 1990; Bankiewicz et al. 1991; Kordower et al. 1991; Anglade et al. 1993). However, the mechanism of recovery has not been fully understood, since survival of transplants have been limited. Several possible mechanisms have been proposed to underlie the transplantation-induced compensatory sprouting in the host-denervated striatum. Neuronal grafts may release neurotrophic factors and initiate a sprouting response in spared dopaminergic axons (Bankiewicz et al. 1990; Hansen et al. 1990, 1995; Plunkett et al. 1990). Another possible mechanism is the activation of a cytokine-neurotrophic cascade, by which neuronal grafts initiate an inflammatory reaction (Plunkett et al. 1990; Wang et al. 1991; Ewing et al. 1992; Wang et al. 1994a; Hansen et al. 1995). This inflammatory reaction mediated by microglia could release inflammatory cytokines that can stimulate reactive astrocytes to release neurotrophic factors, thereby promoting axonal sprouting (Giulian and Lachman 1985; Giulian et al. 1986, 1988a, 1993; Manthorpe et al. 1986; Gage et al. 1988; Hansen et al. 1988). The latter mechanism has been increasingly more likely. Plunkett and his colleages (1990) have shown that parkinsonian animals that underwent surgical trauma (cavitation alone) in the denervated striatum can demonstrate functional and behavioral improvement comparable to those parkinsonian animals that received tissue implants. This indicates that inflammatory cells may play an important role in injury-induced recovery.

Microglial Cells

Microglia are a distinct population of non-neuronal cells in the central nervous system (CNS; del Rio-Hortega and Penfield 1927; del Rio-Hortega 1932). They are specialized cells of the mononuclear phagocyte lineage that infiltrate the CNS during development (del Rio-Hortega 1932; Jordan and Thomas 1988; Ling and Wong 1993). They provide the CNS with its first line of defense against neuronal damage (Giulian 1987; Giulian et al. 1989; Thomas 1992). These cells show a high degree of plasticity, both morphological and functional (Streit et al. 1988; David et al. 1990; Alliot et al. 1991). They can change from a defensive and protective role to involvement in destructive, chronic inflammatory processes. Microglia can be subdivided into three types: (1) ameboid microglia, found in developing brain and characterized by broad flat morphology with pseudopodia, (2) ramified microglia, found in normal adult brain and having a small cell body with several thin processes, and (3) reactive microglia, which arise in adult brain following neuronal injury and possess an enlarged oval or rod-shaped morphology (del Rio-Hortega 1932; Thomas 1992; Davis et al. 1994). While microglia have been known to serve a major role in the removal of cellular debris, they have also been demonstrated to secrete factors which make specific contributions to the wound healing process within the CNS (Giulian and Baker 1985; Giulian et al. 1986, 1987, 1989, 1990).

Astroglial Cells

Astroglia are non-neuronal cells that play an important role in brain function, development and disease (Hatten et al. 1990, 1991). These cells release neurotrophic factors required for the proper maintenance and growth of neurons (Banker 1980; Manthorpe et al. 1986; Gage et al. 1988; Giulian 1993). During brain development, astroglia aid neurite outgrowth, either by providing a scaffold, or trophic support (Silver et

al. 1982; Hatten et al. 1990). In contrast, as the normal brain matures or after neuronal injury, astroglia are thought to be particurlarly unsupportive of axonal growth, especially in the presence of a glial scar (Reier et al. 1983; Liuzzi and Lasek 1987). The reaction of astrocytes to traumatic injury in the CNS, commonly referred to as reactive astrogliosis, is characterized by the increased synthesis of glial fibrillary acidic protein (GFAP; an astrocyte-specific intermediate filament protein), cell proliferation, and extensive hypertrophy of the astrocyte cell body and cytoplasmic processes (del Rio-Hortega and Penfield 1927; Latov et al. 1979; Eddleston and Mücke 1993). The plasticity of the developing brain in response to CNS injury is reflected by an ordered cellular response of astrocytes and other cells which results in a rapid suturing and repair of the wound site (Berry et al. 1983; Rudge et al. 1989; Balasingam et al. 1994). In comparison, the formation of glial scar found at the wound site in the adult CNS has been proposed as a major factor in inhibiting axonal regeneration (Aguayo et al. 1981; Berry et al. 1983; Reier et al. 1983; Reier 1986; Liuzzi and Lasek 1987; Reier and Houle 1988). However, in recent years, it has been debated whether the dense glial scarring directly interferes with neurite outgrowth. The scar may not represent the physical barrier, but rather results in decreased growth-promoting molecules, ECM or soluble factors that must be provided to the external milieu for regeneration to occur, ultimately leading to a non-permissive environment for growth (Tomaselli et al. 1986; Schwartz et al. 1989; McKeon et al. 1991; Thomas and Steindler 1995).

Interleukin-1 and Parkinson's Disease

IL-1 is a cytokine that plays an integral component in the CNS. IL-1 exists in two structurally distinct forms, IL-1α and IL-1β, that share 26% homology in the amino acid sequence by which they both recognize the same receptor and elicit identical biological responses (March et al., 1985). In the developing CNS, IL-1 is an astroglial growth fac-

tor that regulates normal growth and development (Giulian et al. 1988b). When infused into the adult brain, IL-1 can induce reactive gliosis and neovascularization, suggesting that IL-1 act as a regulator of wound healing in response to CNS trauma (Giulian et al. 1988a). However, previous studies have shown that IL-1 does not appear to stimulate gliosis or neovascularization in developing brain suggesting that not only does IL-1 play a role in linking the immune system with brain development, but that there are differences in cellular responses to IL-1 in the developing and adult brain (Sievers et al. 1993).

IL-1 can stimulate the synthesis of certain neurotrophic factors. It was first demonstrated by Lindholm and his colleages (1987) that after sciatic nerve lesion, an increase in nerve growth factor (NGF) mRNA and protein synthesized by non-neuronal cells is induced by IL-1. Moreover, IL-1 can selectively produce an induction of hippocampal NGF and basic fibroblast growth factor (bFGF), but not acidic fibroblast growth factor (aFGF) mRNA after intraventricular injection (Spranger et al. 1990; Rivera et al. 1994). These findings suggest that IL-1 differentially regulates glial hypertrophy, neurotrophic factor expression, and neuroprotection upon CNS injury.

IL-1 has been implicated to play an important role in the survival and plasticity of dopaminergic neurons following neuronal injury. Intrastriatal implantation of IL-1 was shown to enhance compensatory dopaminergic sprouting from residual dopaminergic neurons in the VTA and can induce behavioral improvement in hemiparkinsonian rats (Wang et al. 1994a). This strongly suggests that IL-1 might be the key factor through which tissue implants exert their trophic effects on residual dopaminergic neurons (Wang et al. 1994a). It was further suggested that the effects of IL-1 mediated plastic changes was due to IL-1 induction of neurotrophic factor synthesis (Wang et al. 1994a).

Tranforming Growth Factor-Beta1 and Parkinson's Disease

TGF-β1 is a multifunctional polypeptide that regulates the growth and differenti-

ation of various cells (Massagué 1987; Sporn et al. 1987). In its bioactive secreted form, it consists of a 25 kD disulfide-linked homodimer that binds to specific high-affinitiy receptors (Roberts and Sporn 1990). The involvement of TGF-β1 in wound healing responses is well established in peripheral tissues, where TGF-β1 is a potent mediator of tissue repair by enhancing cell migration, proliferation and production of ECM molecules (Mustoe et al. 1987; Roberts and Sporn 1990). In the normal adult brain, TGF-β1 mRNA is expressed at low levels, found mainly in the meninges and choroid plexus (Heine et al. 1987; Thompson et al. 1989; Unsicker et al. 1991). However, recent evidence suggests that TGF-β1 plays a major role in regulating neuronal repair processes in the brain. When infused into the adult brain, TGF-β1 was shown to induce the synthesis of NGF mRNA in the hippocampus, thus implicating TGF-β1 in regulating trophic functions in response to neuronal injury (Lindholm et al. 1990). Also, TGF-β1 was shown to induce the synthesis of certain ECM molecules like fibronectin and tenascin mRNAs, suggesting a role for TGF-β1 in remodeling of the ECM after neuronal damage (Pasinetti et al. 1993; Smith and Hale 1997).

Members of the TGF-β superfamily such as TGF-β1, 2 and 3 can promote the survival of midbrain dopaminergic neurons *in vitro* and protect them against MPP^+ toxicity (Krieglstein and Unsicker 1994). Since it is unknown whether TGF-β1 has direct effects on dopaminergic neurons, possibly TGF-β1 mediated trophic effects are due to TGF-β1 induction of dopaminergic neurotrophic factors or other growth-promoting molecules in the ECM that facilitate survival and plasticity of dopaminergic neurons.

Astroglia-derived Dopaminergic Neurotrophic Factors

Exogenous administration of several potential dopaminergic neurotrophic factors has been found to reverse the chemical and morphological deficits in animal models of PD (Date et al. 1990c; Otto and Unsicker 1990; Tomac et al. 1995). Two members of the

fibroblast growth factor family, aFGF and bFGF have been found to possess neurotrophic activities on dopaminergic neurons. Both aFGF and bFGF have shown to caused a marked reappearance of tyrosine hydroxylase-immunoreactive fibers indicating morphological recovery of nerve terminals, and were able to enhance recovery of dopamine concentration in the denervated striatum after MPTP-induced injury (Date et al. 1990c; Otto and Unsicker 1990; Chadi et al. 1993; Date et al. 1993). The mechanism by which aFGF and bFGF cause the biochemical and morphological recovery in MPTP-lesioned animals has yet to be elucidated. Previous studies have proposed that aFGF and bFGF have direct effects on dopaminergic neurons (Walicke and Baird 1988; Cintra et al. 1991; Chadi et al. 1994). However, other investigators have claimed that aFGF and bFGF, both being glial mitogens, are acting indirectly by inducing proliferation of astroglial cells which in turn, promote facilitatory recovery by secreting other neurotrophic molecules (Furakawa et al. 1986; Ferrara et al. 1988; Eclancher et al. 1990; Petroski et al. 1991; Yoshida and Gage 1992). Further evidence supports the latter scenario, since studies have demonstrated that mesencephalic astroglial cells play an important role in the survival of dopaminergic neurons and induction of neurite outgrowth (Engele and Bohn 1991; Engele et al. 1991; O'Malley et al. 1991; Takeshima et al. 1994).

Recently, a novel neurotrophic factor was purified and cloned from a glial cell line, termed glial cell line-derived neurotrophic factor (GDNF; Lin et al. 1993). GDNF is a distantly related member of the TGF-β superfamily and was shown to promote the survival and differentiation of dopaminergic neurons and increase high affinity dopamine uptake (Lin et al. 1993). Further studies have reported that GDNF can exert protective and reparative effects in the mesostriatal dopaminergic system in animals after a neurotoxin-induced damage (Hoffer et al. 1994; Kearns and Gash 1995; Tomac et al. 1995).

Extracellular Matrix Molecules

Constituents of the ECM play a crucial role in regulating growth-promoting and inhibiting functions during development and after neuronal injury (for review, see Brodkey et al. 1993). Most of the ECM molecules are developmentally regulated. They appear during critical periods of development and mediate cell-cell interactions and recognition events, and they are down-regulated in the adult brain (Chiquet 1989; Steindler et al. 1989a,b; Crossin et al. 1990; Snow et al. 1990b; Brusno-Bechtold et al. 1992). However, most of these factors are re-expressed after traumatic injury to the adult brain (Egan and Vijayan 1991; McKeon et al. 1991; Laywell et al. 1992). Damage to the adult brain leads to cellular and molecular changes in neural reorganization to promote recovery, and the expression of these ECM molecules can influence the success or failure of sprouting processes. ECM molecules such as fibronectin have been shown to promote axonal growth while CSPGs are inhibitory for neurite outgrowth (Baron-Van Evercooren et al. 1982; Snow et al. 1990a). However, the ECM molecule tenascin can demonstrate both promoting and inhibitory properties for modulating axonal growth (Faissner and Kruse 1990; Lochter et al. 1991). Reactive astrocytes induced by the release of cytokines in response to injury are known to upregulate the expression of ECM molecules (McKeon et al. 1991). However, whether these cytokines influence the expression of ECM molecules to favor or inhibit neuronal plasticity remains unresolved.

Aging and Parkinson's Disease

PD is an age-related neurological disorder. In experimental animals, the neurodegenerative effects of MPTP are age-dependent, that is, young mice show a substantial recovery of striatal dopamine whereas aging mice do not (Ricaurte et al., 1986, 1987a,b; Date et al., 1990a). Aging mice have been shown to be more sensitive to

MPTP, and exhibit extensive damage not only to the mesostriatal dopaminergic system, but also to the mesolimbocortical and other monoaminergic system even with a lower dose of MPTP when compared to young mice (Jarvis and Wagner 1985; Gupta et al. 1986; Saitoh et al. 1987; Date et al. 1990a). Other investigators have reported that aging mice do exhibit recovery processes, but at a much slower rate compared to young mice after neuronal injury (Hoff et al. 1982a,b; Date et al. 1990b). At present, it remains unknown what factors may cause the age-related decline in spontaneous recovery after neuronal injury. It is possible that the presence of growth inhibitors or the lack of growth-promoting molecules in the extracellular environment diminishes the capacity to promote recovery in the aged brain.

CHAPTER 3

Regulation of Astroglia-Derived Dopaminergic Neurotrophic Factors by Interleukin-1β in the Striatum of Young and Middle-Aged Mice

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Ho A and Blum M (1997) Regulation of astroglial-derived dopaminergic neurotrophic factor gene expression by interleukin-1β in the striatum of young and middle-aged mice. *Experimental Neurology* 148:348-359.

Abstract

Interleukin-1 beta (IL-1β) can induce dopaminergic axonal sprouting in the denervated striatum of parkinsonian animals. In order to determine whether IL-1β effects on dopaminergic axonal sprouting are mediated by the induction of astroglia-derived dopaminergic neurotrophic factors, effects of IL-1β treatment on aFGF, bFGF and GDNF gene expression were examined in primary striatal astrocyte cultures and after *in vivo* administration. We found a selective induction of bFGF mRNA synthesis but not aFGF or GDNF mRNA levels after IL-1β treatment both *in vitro* and *in vivo*. This suggests that bFGF may be the putative endogenous dopaminergic neurotrophic factor mediating lesion-induced plasticity of dopaminergic neurons. In addition, to determine why recovery from injury becomes reduced with age, we examined whether there was an aging-associated decline in the ability of IL-1β to induce the synthesis of neurotrophic factors in middle-aged animals compared to young mice. Interestingly, IL-1β promoted a greater induction in bFGF mRNA levels in the middle-aged mice compared to young mice. These results suggest that the regulation of bFGF and possibly its receptor signalling efficacy may vary as the brain ages.

Introduction

Interleukin-1 beta (IL-1β) is a cytokine that plays an important role in mediating cellular responses to injury in the CNS (Bartfai and Schultzberg 1993). IL-1β has diverse actions on the growth and differentiation of cells (Bartfai and Schultzberg 1993; Rothwell and Strijbos 1995). Activated microglia are a principal source of secreted IL-1β; however there is evidence that astrocytes could be another source of IL-1β synthesis (Fontana et al. 1982; Giulian et al. 1986; Nieto-Sampedro and Berman 1987; Hetier et al. 1988; Perry and Gordon 1988; Lieberman et al. 1989). IL-1β can induce reactive gliosis and neovascularization when infused into the adult brain, thus suggesting IL-1β as a regulator of wound healing (Giulian et al. 1988a). In contrast, while IL-1β is an astroglial growth factor in the developing CNS, IL-1β does not appear to stimulate gliosis or neovascularization in immature animals (Giulian et al. 1988b; Sievers et al. 1993). These studies suggest that not only does IL-1β play a role in linking the immune system with the development of the brain, but that there are differences in cellular responses to IL-1β in the developing and adult CNS.

Recent attention has focused on the ability of IL-1β to regulate the synthesis of certain neurotrophic factors. It was first demonstrated that after sciatic nerve lesion, an increase in NGF mRNA and protein synthesized by non-neuronal cells is induced by IL-1β (Lindholm et al. 1987). Moreover, IL-1β can selectively produce an induction of hippocampal NGF and bFGF mRNA, but not aFGF mRNA after intraventricular injection (Spranger et al. 1990; Rivera et al. 1994). These findings suggest that cytokines may differentially regulate glial hypertrophy, neurotrophic factor expression and neuroprotection upon CNS trauma (Spranger et al. 1990).

Parkinson's disease (PD) is a neurodegenerative disorder in which nigral dopaminergic neurons in the mesencephalon selectively degenerate, thus leading to a loss of dopaminergic innervation in the dorsal striatum (Bernheimer et al. 1973; Agid et

al. 1987; Hirsch et al. 1988). Therapeutic approaches such as grafting dopamine-producing tissues into the striatum of animal models of PD and Parkinsonian patients can induce a compensatory sprouting response in the denervated striatum from endogenous dopaminergic neurons (Bohn et al. 1987; Fiandaca et al. 1988; Bankiewicz et al. 1991; Pickel et al. 1992; Blanchard et al. 1995; Hansen et al. 1995). Several possible mechanisms may underlie the transplantation-induced compensatory sprouting in the host denervated striatum. Neuronal grafts may release neurotrophic factors and initiate a sprouting response in spared dopaminergic axons (Bankiewicz et al. 1990; Hansen et al. 1990, 1995; Plunkett et al. 1990). Another possible mechanism is the activation of a cytokine-neurotrophic cascade, by which neuronal grafts initiate an inflammatory reaction (Lawrence et al. 1990; Ewing et al. 1992; Giulian et al. 1993; Wang et al. 1994a; Hansen et al. 1995). This inflammatory reaction mediated by microglia could release inflammatory cytokines that can stimulate reactive astrocytes to release neurotrophic factors, thereby promoting axonal sprouting (Giulian and Baker 1985; Giulian and Lachman 1985; Giulian et al. 1986a,b; Araujo and Cotman 1992a; Giulian 1992; Wang et al. 1994a; Hansen et al. 1995; Sawada et al. 1995).

Recent studies have demonstrated that intrastriatal implantation of IL-1β can enhance compensatory sprouting from residual dopaminergic neurons in the ventral tegmental area of the midbrain, and can induce behavorial improvement in hemiparkinsonian rats (Wang et al. 1994a). Since it is thought that IL-1β may not act directly to induce the survival and plasticity of dopaminergic neurons (Akaneya et al. 1995; Coelin et al. 1995), it is possible that IL-1β stimulates the release of dopaminergic neurotrophic factors from astrocytes. Treatment with aFGF, bFGF or GDNF has been shown to enhance the sprouting of dopaminergic fibers after a neurotoxic lesion (Date et al. 1990c; Otto and Unsicker 1990; Tomac et al. 1995). In addition, these specific dopaminergic neurotrophic factors can be synthesized by striatal astrocytes (Cintra et al. 1991; Strömberg et al. 1993; Chadi et al. 1994; Ho et al. 1995). Therefore, we hypothesized

that the stimulation of dopaminergic axonal sprouting by IL-1β is mediated by the induction of specific growth factor synthesis from astrocytes.

Previous studies have demonstrated an aging-associated decline in compensatory dopaminergic sprouting after neurotoxin-induced injury (Ricaurte et al. 1987a,b; Date et al. 1990a,c). At the same time, other studies reported that there is an aging-associated decline in the ability of astrocytes to support process outgrowth (Smith et al. 1986, 1990; Tomaselli et al. 1986; Rudge et al. 1989). Thus, in the present study, we also examined whether the ability of IL-1β to regulate the synthesis of astroglia-derived dopaminergic neurotrophic factors are altered during the aging process.

Materials and Methods

Preparation of Striatal Astroglial Monolayers. Breeders were paired from C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). Postnatal day 0-3 C57BL/6 mice were decapitated, the striatum dissected and meninges were removed. The tissues were trypsinized (2.5 mg/ml; Gibco, Grand Island, NY) and treated with DNase (10 µg/ml; Sigma, St Louis, MO) for 15 min at 37°C, followed by a series of washes and centrifugations in MEM/Ham's F-12 supplemented with 10% fetal bovine serum. The resulting homogenate (cell suspension) was filtered through a sterile 37 µm nylon mesh screen (Tetko, New York, NY). Cells were plated at ~$1x10^6$ cells per 60 mm dish in poly-L-lysine (100 µg/ml; Sigma) coated culture petri dishes. The cultures were incubated at 37°C in an atmosphere of 8% CO_2 and 95% air.

In Vitro Treatment: Based on conditions previously described (Spranger et al., 1990), cells at 11-13 days *in vitro* were placed into 0.5% serum for 24 h, and treated with IL-1β (50 ng/ml; Intergen, Purchase, NY; dosage was based on experiments previously described in Araujo and Cotman (1992), or vehicle (sterile H_2O) for 0, 2, 4, 6, 8, and 16

h (n=3 for each group). Cells at 21-23 days *in vitro* were treated as above for 6 h (n=5 for each group). Total cytoplasmic RNA was isolated and a nuclease protection assay (Blum, 1989) was used to quantify aFGF, bFGF, and GDNF mRNA from the same treated cultured astrocytes.

In Vivo Treatments. Male C57BL/6 mice (Jackson Laboratory) of three representative age groups were compared: postnatal day 15 (young), 3 months (young-mature) and 8-10 months of age (middle-aged). The 3 and 8 month age groups were anesthetized with 287.5 mg/kg of avertin (stock of 12.5 mg/ml 2,2,2-tribromoethanol; Aldrich, Milwaukee, WI), injected intraperitoneally while the postnatal day 15 age group was anesthetized intraperitoneally with 300 mg/kg of chloral hydrate (Sigma). The mice were placed on a stereotaxic device (David Kopf Instruments, Tunjunga, CA). A burr hole was drilled on the right side of the skull to accomodate injection. Stereotaxic injections of IL-1β (100 Units; Intergen) or vehicle (phosphate buffer saline pH 7.4) in a final volume of 0.5 µl was injected into the right lateral ventricle using a 1 µl Hamilton syringe (Hamilton, Reno, NE). Coordinates for the postnatal day 15 age group were 1.5 mm caudal to the frontal nasal suture, 0.5 mm lateral from the midline suture and 1.5 mm from the surface of the brain. Coordinates for the 3 and 8 month age groups were 2.5 mm caudal to the frontal nasal suture, 1.0 mm lateral from the midline suture and 2.0 mm from the surface of the brain. The injection was at a rate of ~0.05 µl/min, and the needle was left in place for 2-3 min after injection. The needle was withdrawn slowly to prevent backflow of solution along the needle track. Animals were sacrificed at 0, 3, 6, 8, 24 and 48 h by cervical dislocation and decapitated Three to five mice were used for each time point. The brains were removed and chilled in sterile saline. The right dorsal and ventral striatum were dissected, immediately frozen on dry ice, and stored at -80°C until used. Total cytoplasmic RNA was isolated and a nuclease protection assay (Blum 1989) was used to quantify aFGF, bFGF, and GDNF mRNA in dissections from the same animals.

Isolation of cDNA Clones. The aFGF cDNA clone was isolated by polymerase chain reaction (PCR) of mouse striatal cDNA from which a 350 base pair fragment corresponding to nucleotides 33-384 was subcloned into Bluescript II (Stratagene, La Jolla, CA). The bFGF cDNA clone was generously provided by Dr. S. Shimasaki (Shimasaki et al. 1988) from which a 479 base pair fragment corresponding to nucleotides 525-1004 was subcloned into vector Bluescript/SK+ (Stratagene). The GDNF cDNA clone was isolated by PCR of rat genomic DNA as previously described (Blum and Weichert 1995) from which a 414 base pair PCR DNA fragment was subcloned into Bluescript II (Stratagene).

Quantitative Solution Hybridization Nuclease Protection Assay. Unlabeled sense and high specific activity (~$1x10^9$ cpm/µg) ^{32}P-labeled (NEN, Boston, MA) antisense RNA were transcibed according to the manufacturer's recommendations. A standard curve with increasing amounts (0-10 µl of a 100 fg/µl (+) strand) of sense RNA was used for quantification. The standard and known amounts of cytoplasmic RNA isolated were hybridized with ~200 pg of antisense ^{32}P-labeled RNA probe. The samples were heat-denatured at 85°C for 5 min and hybridized overnight at 45°C. After hybridization, the samples were treated with RNase A (5 µg/ml; Sigma) and RNase T1 (2 µg/ml; Sigma) for 1 h at 30°C, followed by proteinase K (0.167 mg/ml; Boehringer Mannheim, Indianapolis, IN) digestion at 37°C for 15 min. Samples were phenol:chloroform extracted, precipitated, resuspended in 1xTE, and electrophoresed on a nondenaturing 5% acrylamide gel. Gels were dried, and quantify by phosphor image analysis. The results were determined by linear regression analysis from the standard curve and presented as amoles mRNA/µg total RNA.

Statistical Analysis. All values given are mean ± SEM. Significant differences in aFGF,

bFGF, or GDNF mRNA levels between control and IL-1β treatment groups were analyzed using analysis of variance (ANOVA) followed by Fisher's protected least significant difference post hoc analysis. The level of significance was set at $p<0.05$. Statistical comparisons between control and IL-1β in mature astrocyte cultures and postnatal day 15 mice that were analyzed at 6 h were made using a two-tailed Student's *t*-test.

Results

Effects of IL-1β on Neurotrophic Factor Gene Expression in Primary Striatal Astrocyte Cultures

Immature animals respond differently to IL-1β compared to mature animals when infused into the brain (Giulian et al. 1988a; Sievers et al. 1993). In addition, it has been observed that the ability of astrocytes to support process outgrowth decreases with time in culture (Smith et al. 1986, 1990; Rudge et al. 1989; Rudge and Silver 1990; Wang et al. 1994b). It has been characterized that as astrocytes mature *in vitro*, a decline in the growth promoting potential is seen after 21 days, in comparison to astrocytes cultured for shorter periods of time (Smith et al. 1986, 1990; Rudge et al. 1989; Rudge and Silver 1990; Wang et al. 1994b). Therefore, we examined whether the *in vitro* maturational state of astrocytes altered their response to IL-1β. Thus, primary striatal astrocyte cultures at 11-13 days *in vitro* (young-immature astrocytes), and cultures at 21-23 days *in vitro* (mature astrocytes) were studied. The characteristics of the cultures were evaluated by staining with an astroglial marker, GFAP. In young and mature astrocyte cultures, more than 90% of the cells were labeled with GFAP (data not shown).

Our results show that IL-1β produced a 34% reduction in aFGF mRNA levels by 6 h, and by 16 h, aFGF mRNA levels returned to control levels in young immature striatal astrocyte cultures (Fig. 3.1*A*). Similarily, IL-1β produced a significant 42% decrease in aFGF mRNA levels within 6 h in mature striatal astrocyte cultures (Fig. 3.1*D*). In con-

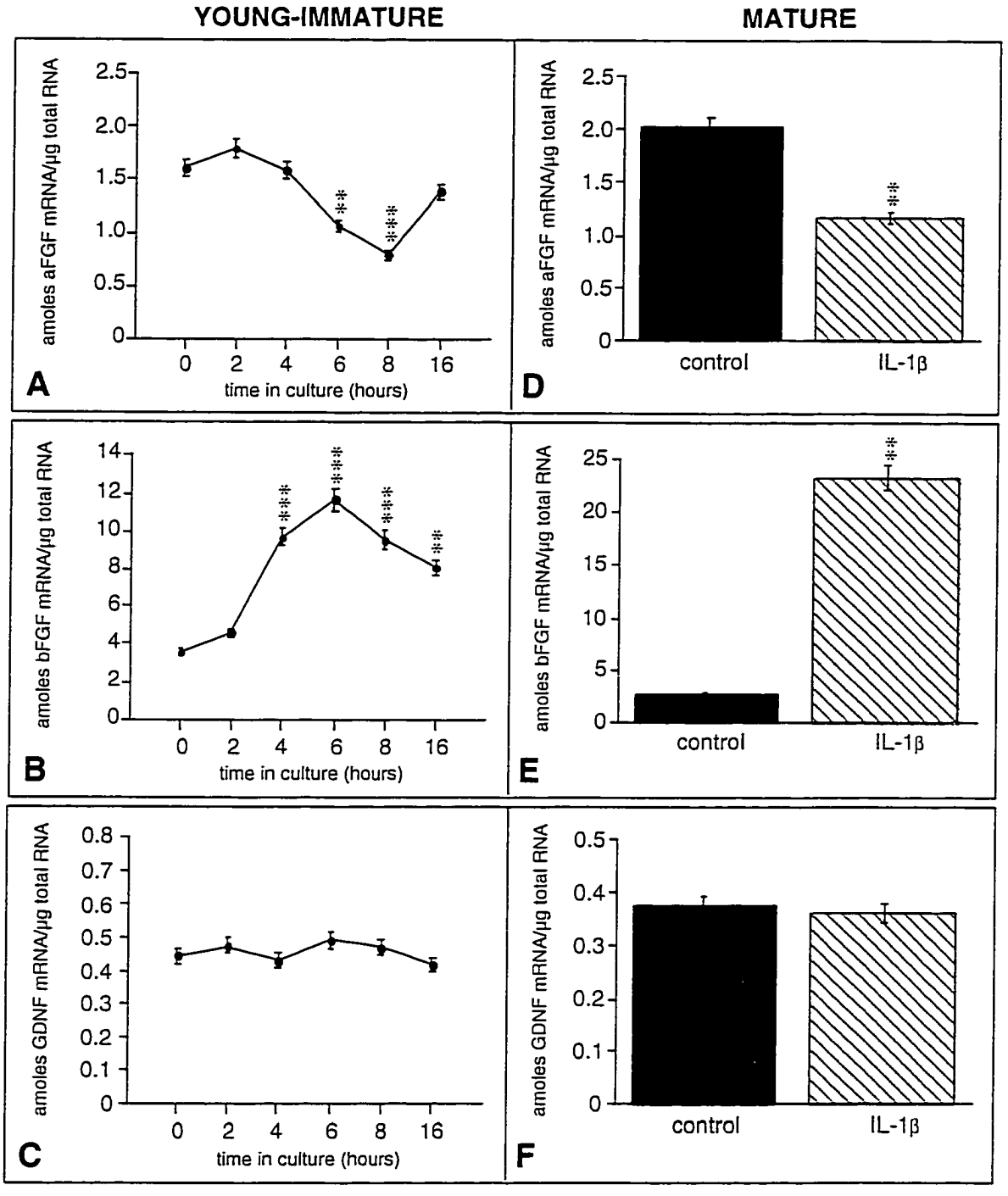

Figure 3.1 Quantitative analysis of astroglia-derived dopaminergic neurotrophic factor gene expression after IL-1β treatment in primary striatal astrocyte cultures. Cells at 11-13 days *in vitro* representing young-immature astrocytes *(A-C)* were placed into 0.05% serum for 24 h, and treated with IL-1β (50 ng/ml) or vehicle for 0, 2, 4, 6, 8 and 16 h. Cells at 21-23 days *in vitro* representing mature astrocytes *(D-F)* were treated as above for 6 h. *A* and *D*, aFGF mRNA levels in young-immature *(A)* and mature *(D)* striatal astrocyte cultures compared to control cultures following IL-1β treatment. *B* and *E*, bFGF mRNA levels in young-immature *(B)* and mature *(E)* astrocyte cultures after IL-1β treatment. *C* and *F*, GDNF mRNA levels in young-immature *(C)* or mature *(F)* striatal astrocyte cultures following IL-1β treatment. Values represent the mean ± SEM for n=3, and n=5 for each group in young and mature astrocyte cultures *in vitro*, respectively. **, P<0.005, ***, P<0.0001 compared to control vehicle

trast, IL-1β maximally induced a 410% increase in bFGF mRNA compared to control at 6 h, and remained elevated by 280% at 16 h in young immature astrocyte cultures (Fig. 3.1*B*). Moreover, there was an even greater increase in bFGF mRNA in mature astrocyte cultures, with an 830% increase in bFGF mRNA levels seen at 6 h after IL-1β treatment (Fig. 3.1*E*). IL-1β did not change GDNF mRNA gene expression in young or mature striatal astrocyte cultures *in vitro* (Fig. 3.1*C* and 1*F* respectively).

Effects of IL-1β on Neurotrophic Gene Expression *In Vivo*

To determine whether IL-1β effects on the induction of dopaminergic neurotrophic factors synthesis is altered with age *in vivo*, IL-1β was injected into the right lateral ventricle in animals of three representative age groups; postnatal day 15 (young), 3 months (young-mature) and 8-10 months of age (middle-aged). To examine if IL-1β can induce the synthesis of neurotrophic factors in the dorsal as well as in the ventral striatum, which has been shown to be the source of dopaminergic axonal sprouting in the denervated dorsal striatum in animal models of PD, both brain regions were dissected and analyzed independently.

In the dorsal striatum of young postnatal day 15 mice, IL-1β injection caused a 41% reduction in aFGF mRNA levels by 6 h (Fig. 3.2*A*). In contrast, IL-1β induced a 46% increase in bFGF mRNA levels by 6 h (Fig. 3.2*B*). Similar to aFGF, IL-1β caused a 36% reduction in GDNF mRNA levels within 6 h compared to control injected mice (Fig. 3.2*C*). IL-1β did not change any of the dopaminergic neurotrophic factors in the ventral striatum of young postnatal day 15 mice (Fig. 3.2*D-F*).

Intraventricular injection of IL-1β into the 3 month age group produced a 34% and 54% reduction in aFGF mRNA levels by 24 h in the dorsal and ventral striatum, respectively (Fig. 3.3*A,D*). In contrast, IL-1β maximally induced a 320% increase in bFGF mRNA levels within 6 h and remained elevated by 24 h in the dorsal striatum (Fig. 3.3*B*). Similarly, IL-1β induced a 220% increase in bFGF mRNA levels by 6 h in the ventral stria-

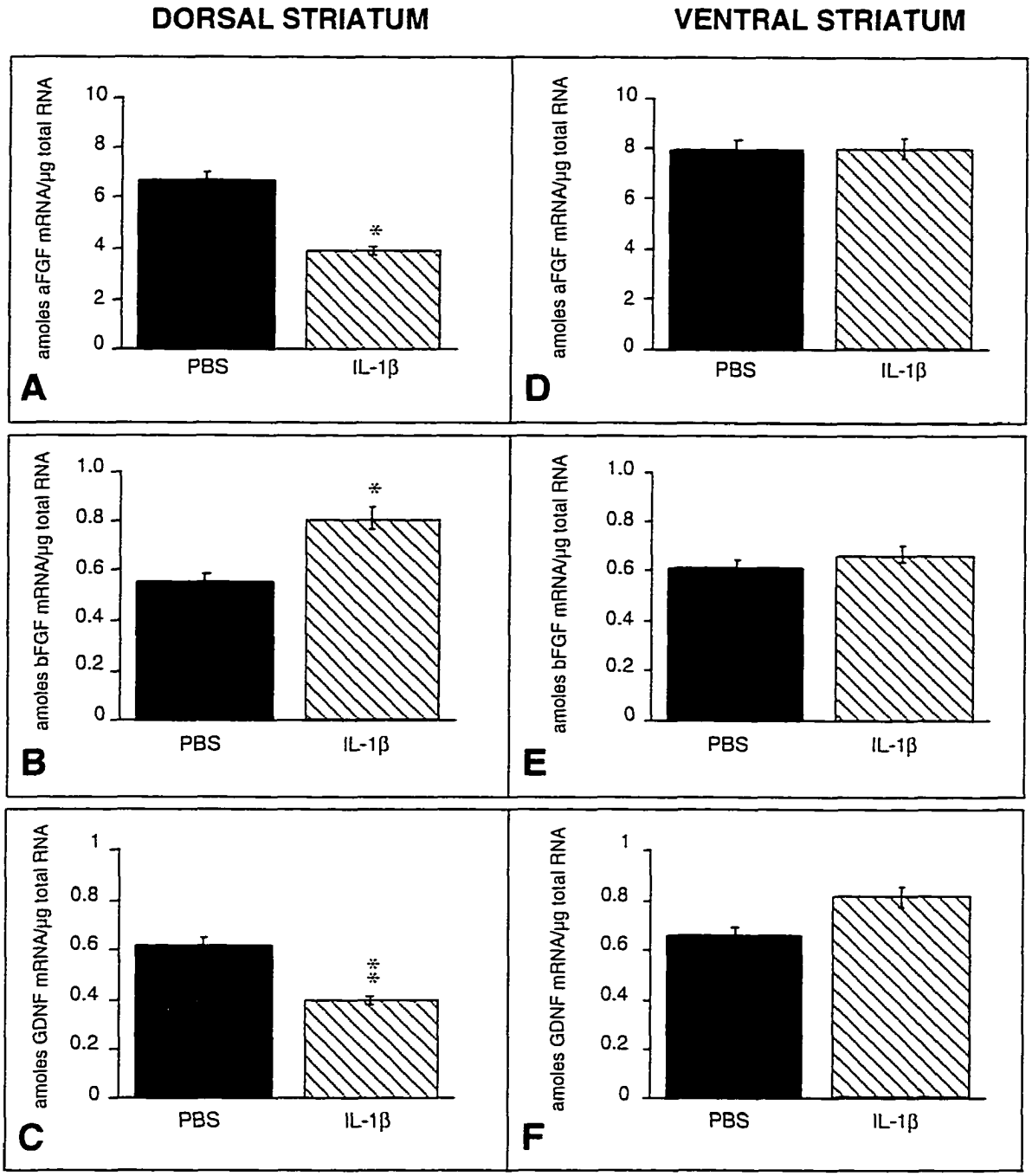

Figure 3.2 Quantitative analysis of astroglia-derived dopaminergic neurotrophic factors in the dorsal *(A-C)* and ventral *(D-F)* striatum after IL-1β injection for 6 h in postnatal day 15 mice. Stereotaxic injections of IL-1β (100 units) or vehicle in a final volume of 0.5 µl were made into the right lateral ventricle using a 1 µl Hamilton syringe. *A* and *D*, aFGF mRNA levels following IL-1β treatment compared to control PBS-injected animals in the dorsal *(A)*, and ventral *(D)* striatum. *B* and *E*, bFGF mRNA levels following IL-1β treatment in the dorsal *(B)*, and ventral *(E)* striatum compared to control PBS-injected animals. *C* and *F*, GDNF mRNA levels in the dorsal *(C)*, and ventral *(F)* striatum compared to control PBS-injected animals. Values represent the mean ± SEM for n=5 *in vivo*. *, $P<0.05$; **, $P<0.0001$ compared to PBS-injected.

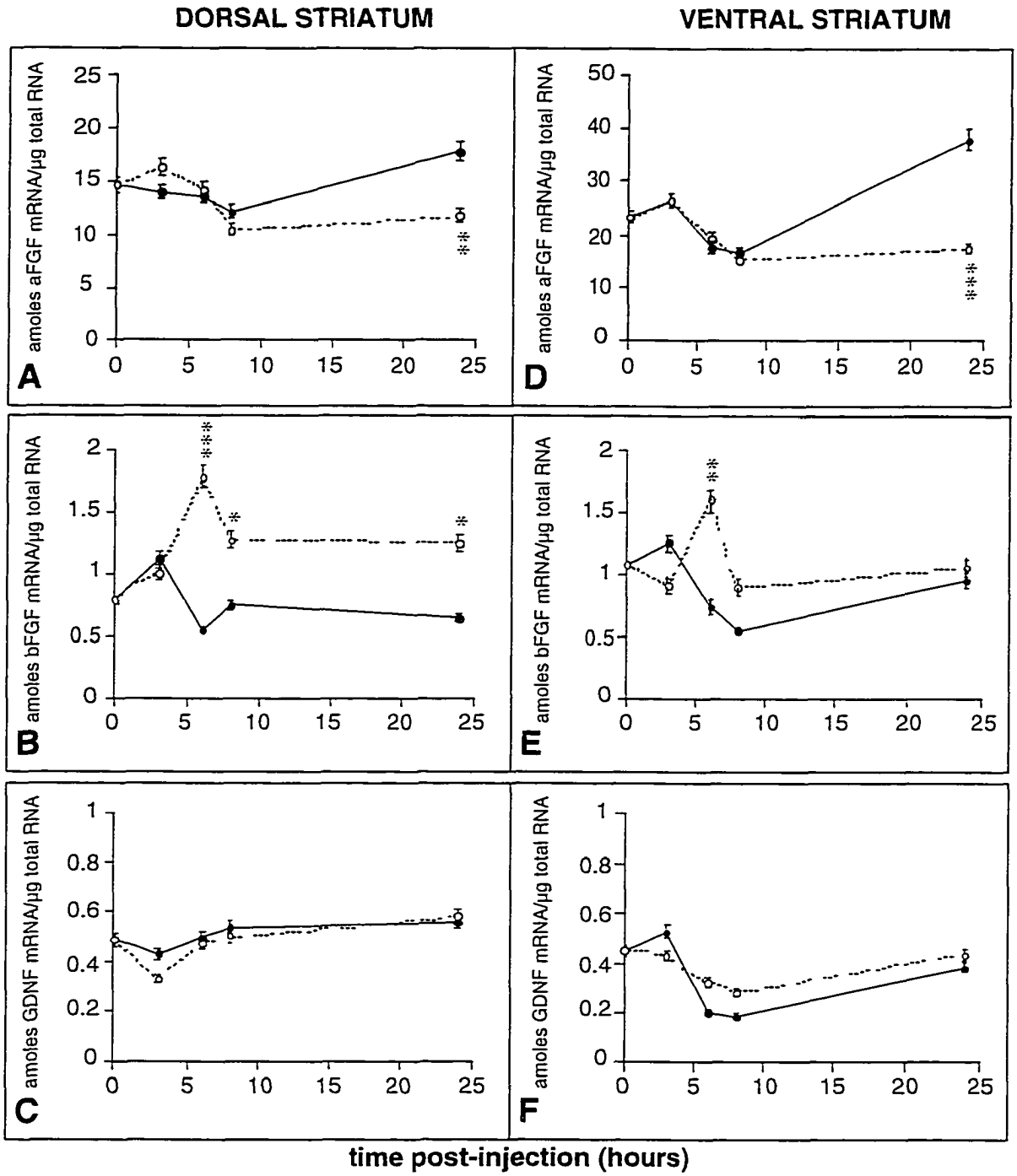

Figure 3.3 Time-course quantitative analysis of astroglia-derived dopaminergic neurotrophic factors in the dorsal *(A-C)* and ventral *(D-F)* striatum at 0, 3, 6, 8, and 24 h after IL-1β treatment in 3 month old mice. Stereotaxic injections of IL-1β (100 units) or vehicle in a final volume of 0.5 µl were made into the right lateral ventricle using a 1 µl Hamilton syringe. The 0 h time point represents non-injected control. The dotted line represents IL-1β-injected, and solid line represents control PBS-injected animals. *A* and *D*, aFGF mRNA levels in the dorsal *(A)* and ventral *(D)* striatum compared to control PBS-injected animals. *B* and *E*, bFGF mRNA levels in the dorsal *(B)* and ventral *(E)* striatum compared to control PBS-injected animals. *C* and *F*, GDNF mRNA in the dorsal *(C)* and ventral *(F)* striatum after IL-1β injection. Values represent the mean ± SEM. *, P<0.05; **, P<0.005, ***, P<0.0001 compared to PBS-injected.

tum (Fig. 3.3*E*). At the same time, we detected no change in GDNF mRNA gene expression in dorsal or ventral striatum of the young 3 month age group (Fig. 3.3*C,F* respectively).

In the 8 month age group, IL-1β did not change aFGF mRNA gene expression in the dorsal and ventral striatum (Fig. 3.4*A,D* respectively). In contrast, IL-1β induced a 530% increase in bFGF mRNA levels by 6 h, and remained elevated by 270% at 24 h (Fig. 3.4*B*). Likewise, IL-1β induced a 220% increase in bFGF mRNA levels by 6 h compared to control in the ventral striatum (Fig. 3.4*E*). Moreover, although it was observed that IL-1β induced a small increase in GDNF mRNA levels in the dorsal striatum within 6 h when compared to control injected, this effect shortly returned to control levels by 8 h (Fig. 3.4*C*). IL-1β did not change GDNF mRNA gene expression in the ventral striatum of middle-aged mice (Fig. 3.4*F*).

Discussion

The present study investigated whether astroglia-derived dopaminergic neurotrophic factors can be regulated by IL-1β. It was observed from the *in vitro* experiments and after intraventricular infusion of IL-1β that bFGF mRNA was upregulated by IL-1β in the striatum. In contrast, there was no sustained induction of aFGF or GDNF mRNA levels after IL-1β treatment. This therefore suggests that bFGF may be the putative dopaminergic neurotrophic factor mediating IL-1β effects on dopaminergic axonal sprouting after a neurotoxin challenge. However, we did not observe an aging-associated decline in the ability of IL-1β to induce bFGF mRNA in the middle-aged group. In fact, IL-1β stimulated a greater induction in bFGF mRNA levels in the middle-aged compared to young mice.

IL-1 and Astroglial cells

Several lines of evidence have indicated that the adult CNS can regenerate after

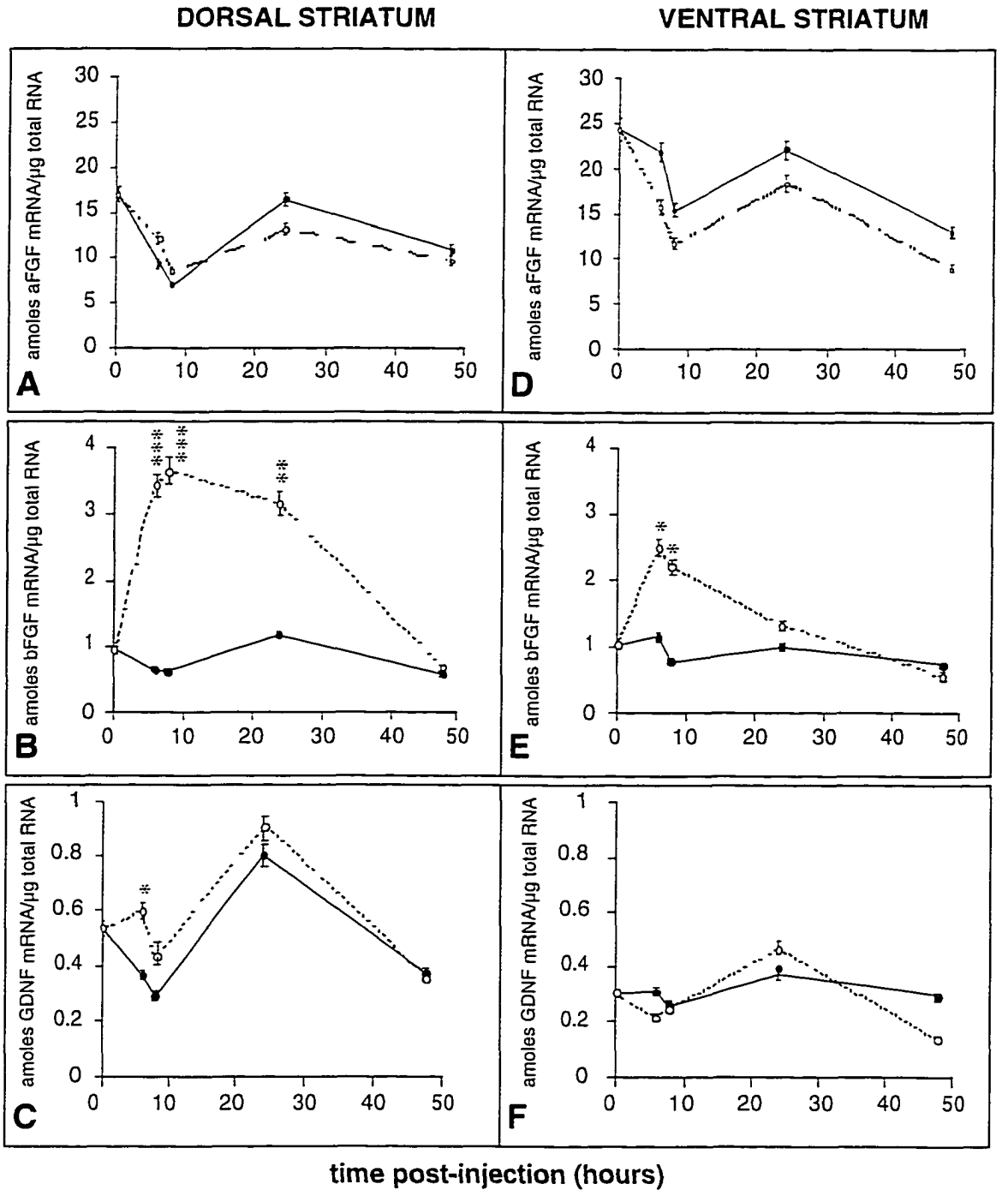

Figure 3.4 Time-course quantitative analysis of astroglia-derived dopaminergic neurotrophic factors in the dorsal *(A-C)* and ventral *(D-F)* striatum at 0, 6, 8, 24, and 48 h after IL-1β treatment in 8 month old mice. Stereotaxic injections of IL-1β (100 units) or vehicle in a final volume of 0.5 µl were made into the right lateral ventricle using a 1 µl Hamilton syringe. The 0 h time point represents non-injected control. The dotted line represents IL-1β-injected, and solid line represents control PBS-injected animals. *A* and *D*, aFGF mRNA gene expression in the dorsal *(A)* and ventral *(D)* striatum compared to control PBS-injected animals. *B* and *E*, bFGF mRNA levels in the dorsal *(B)* and ventral *(E)* striatum compared to control PBS-injected animals. *C* and *F*, GDNF mRNA levels in the dorsal *(C)*, and ventral *(F)* striatum compared to control PBS-injected animals. Values represent the mean ± SEM. *, P<0.05; **, P<0.005, ***, P<0.0001 compared to PBS-injected.

lesion-induced injury. The regenerative capacity of the CNS depends not only on CNS neurons but also on the glial environment (Eddleston and Mücke 1993). Astroglia can release neurotrophic factors required for the proper maintenance and growth of nerve cells (Hatten et al. 1990). During brain development, astroglia can aid neurite outgrowth, either by providing a scaffold or neurotrophic support (Hatten et al. 1990). In contrast, as the normal brain matures, and after injury, astroglia can be unsupportive of axon growth, especially in the vicinity of a glial scar (Berry et al. 1983; Reier et al. 1983, 1989; Reier 1986; Reier and Houle 1988; Rudge and Silver 1990; Hatten et al. 1991). However in recent years, it has been debated whether astroglia directly interfere with outgrowth of neurites in the process of regeneration (Eddleston and Mücke 1993). Previous studies have shown that astrocytes play an important role in the sprouting of NGF-sensitive damaged cholinergic neurons in the basal forebrain after septohippocampal deafferentation (Gage et al. 1988). Furthermore, it was implicated that astroglial synthesis of NGF and other neurotrophic factors which act on damaged neurons to promote regeneration were initially up-regulated by cytokines (Yoshida and Gage 1992).

In the present study, we initially examined whether aFGF, bFGF or GDNF, which specifically have been shown to induce dopaminergic axonal sprouting, can be directly regulated by IL-1β in striatal astrocytes. Our *in vitro* results indicated that IL-1β selectively induced the synthesis of bFGF mRNA but not aFGF or GDNF mRNA. IL-1β induced bFGF mRNA maximally within 6 h and sustained a significant increase for 16 h in young immature astrocytes *in vitro*. Furthermore, it was observed that there was no decrease in the ability of mature astrocytes to respond to IL-1β treatment compared to immature astrocytes. Indeed, mature astrocytes had a relative increase in bFGF mRNA in response to IL-1β treatment.

More recently, it has become appreciated that mature astrocytes can display plasticity in their ability to support neurite extension depending on differences in the expression of cell surface and ECM molecules. Growth-inhibiting versus growth-promoting

affects of astrocytes on neurons can be mediated by the presence of CSPGs and tenascin (McKeon et al. 1991; Meiners et al. 1995). It was shown that astrocytes rich in CSPGs and tenascin are inhibitory to process outgrowth whereas astrocytes that do not express these particular ECM molecules seemed to promote neurite growth (McKeon et al. 1991; Meiners et al. 1995). Others have suggested that the release of cytokines during an inflammatory response to brain injury can upregulate certain ECM molecules which in turn can affect the binding and actions of trophic factors (Meiners et al. 1993; Cotman et al. 1996). For example, an ECM molecule perlecan, has been shown to potentiate the actions of bFGF (Nurcombe et al. 1993; Aviezer et al. 1994). Thus, our findings suggest that it is not a loss of the ability of IL-1β to stimulate neurotrophic factor synthesis in mature astrocytes that compromises their growth promoting potential, but possibly it is due to an alteration in IL-1β regulation of growth-promoting ECM molecules. However further investigation is needed to elucidate the role of cytokines in regulating ECM molecules and their interaction with growth factors after neuronal injury.

IL-1 and the Regulation of Neurotrophic Factor Gene Expression in the CNS

The stimulation of astroglia-derived dopaminergic neurotrophic factor synthesis by IL-1β could be one mechanism by which IL-1β induces the survival and plasticity of dopaminergic neurons after injury. We performed stereotaxic intraventricular injections of IL-1β and examined three representative age groups: postnatal day 15 when astrocytes have been shown to promote neurite outgrowth; and 3 and 8 months of age (young adult and middle-aged, respectively) when neurons and the glial environment of the adult CNS have been shown to be less favorable for regeneration to occur after brain injury (Brodkey et al. 1993). IL-1β was able to induce an increase in bFGF mRNA synthesis in the striatum of postnatal day 15 mice, and this induction was even greater in young adult and middle-aged mice thus demonstrating that there was not an aging-associated loss in the capacity of IL-1β to induce bFGF mRNA. The effects of IL-1β on the induction of

bFGF mRNA but not aFGF or GDNF mRNA suggests that IL-1β effects on bFGF were specific and not the result of generalized mRNA up regulation since the analyses of these neurotrophic factors were from the same animals. In addition, we observed that IL-1β can have opposite actions on the expression of two fibroblast growth factors. IL-1β induced bFGF mRNA synthesis, but at the same time reduced aFGF mRNA. This provides evidence that although aFGF and bFGF have been shown to have similar functions and actions on the brain, cytokines can have distinct influences on neurotrophic factor gene expression in the brain. This is further supported by other studies in which intraventricular injection of IL-1β induced bFGF and NGF mRNA expression but not aFGF mRNA in the hippocampus of adult rat brain (Spranger et al. 1990; Rivera et al. 1994). The present study demonstrates the effects of IL-1β on neurotrophic factor gene expression were examined at the mRNA level, cytokines could alter changes at the protein level such as rate of translation or protein degradation. However, supporting evidence from other studies suggest that IL-1β increase in neurotrophic factor synthesis is reflected by corresponding increase in protein released (Lindholm et al. 1987; Spranger et al. 1990; Araujo and Cotman 1992a).

The properties of IL-1β to stimulate reactive astroglia and neovascularization after infusion of IL-1β that was reported in the adult brain (Giulian et al. 1988a) could be mediated by bFGF. Similar to IL-1β, bFGF has been shown to stimulate astrocyte proliferation as well as to induce new blood vessel growth (Gospodarowicz et al. 1986, 1987). In the present study, we found that IL-1β caused a significant 7 and 13 fold increase of bFGF mRNA in young-mature and middle-aged animals, respectively, when compared to the modest induction observed in the developing postnatal day 15 mice. This enhanced stimulation of bFGF mRNA in the young adult seemed to correlate well with the selective cellular responses of IL-1β effects on the adult brain compared to the developing brain (Giulian et al. 1988b; Sievers et al. 1993), thus suggesting a role of bFGF in mediating the actions of IL-1β in the wound healing process after neuronal injury.

Cytokines frequently act synergistically with other cytokines or neurotrophic factors to elicit an effect (Benveniste 1992). It has been implied by others that the newly synthesized bFGF in the hippocampus after cerebroventricular injection of IL-1β may enhance the initial action of IL-1β to further stimulate bFGF expression (Rivera et al. 1994). Our results of an enhanced IL-1β induction of bFGF mRNA in the striatum of middle-aged mice may be due to a similar synergistic interaction. Since we have shown that it was not the inability of middle-aged animals to synthesize neurotrophic factors, it is possible that receptor trafficking and receptor activation becomes altered with age and might be responsible for the loss of recovery seen in aged animals. Previous studies have reported that in senescent vascular cells, there are aging-associated defects in mitogenic responsiveness to FGF and epidermal growth factor (EGF; Reenstra et al. 1993; Garfinkel et al. 1996). This was accompanied by a parallel decrease in total number of receptors, receptor binding, and signalling of the EGF and FGF receptor system (Reenstra et al. 1993; Garfinkel et al. 1996; Reenstra et al. 1996). However, this still remains to be explored in the aged brain.

IL-1 and Parkinson's Disease

Animal models of PD have shown that young mice can begin spontaneous recovery within one month after a MPTP-induced lesion of the nigrostriatal dopaminergic system compared to aged mice (Ricaurte et al. 1987a,b). Cellular reactions after MPTP involving changes in growth factor gene expression such as bFGF mRNA observed in young mice are a possible factor mediating the recovery of striatal dopaminergic nerve terminals (Leonard et al. 1993). However, it remains to be established what endogenous events may contribute to the age-associated reduction in the ability to recover from neuronal injury. There is a growing body of evidence suggesting that normal repair mechanisms associated with neurodegeneration can also contribute to the disease process if the responses are overstimulated or disregulated (Cotman et al. 1996). For example,

while studies have shown that bFGF can promote neurite sprouting, bFGF may also play a role in plaque formation in Alzheimer's disease (Cotman et al. 1996). Since aging is often accompanied by a decline in immune homeostasis (Hodes 1995), possibly as the brain ages, the responses to injury may lead to a propagation of enhanced inflammatory processes which could be responsible for the aging-associated loss in the ability to recover from injury which is potentially reflected by the greater induction of bFGF synthesis by IL-1β in middle-aged mice when compared to young adults. It is possible that the enhanced stimulation of bFGF by IL-1β observed in middle-aged mice in combination with other uncharacterized factors could be triggering feedback reactions which may lead to alterations in the CNS environment that are not conducive to recovery. Perhaps, by further elucidating how cytokines, ECM molecules, and trophic factors interact and change in response to injury and whether the balance between these interactions becomes altered with age, we can gain a better understanding of how to influence neurodegenerative processes in the adult brain.

CHAPTER 4

Induction of Interleukin-1 Associated With Compensatory Dopaminergic Sprouting in the Denervated Striatum of Young Mice: Model of Aging and Neurodegenerative Disease

Abstract

Young mice challenged with the neurotoxin MPTP, which selectively destroys the SN dopaminergic neurons in the midbrain exhibit spontaneous recovery of dopaminergic nerve terminals. However, such recovery becomes attenuated with age. Here we report that newly sprouted fibers originate from spared dopaminergic neurons in the VTA. We found that IL-1, an immune response-generated cytokine that can enhance dopaminergic sprouting when exogenously applied, increased dramatically in the denervated striatum of young mice compared to middle-aged mice after MPTP. Young mice displayed a maximal 500% induction of IL-1α synthesis that remained elevated for several weeks in the dorsal and ventral striatum while middle-aged mice exhibited a modest 135% induction exclusively in the dorsal striatum for a week. IL-1α immunoreactivity was localized in GFAP-immunoreactive hypertrophied astrocytes as well as in neurons within the denervated striatum of young mice. However, no induction of IL-1α mRNA was seen in the midbrain in either age group despite glial activation. Since we have reported that IL-1 can regulate astroglia-derived dopaminergic neurotrophic factors, it was surprising that no changes were observed in aFGF, bFGF or GDNF synthesis associated with MPTP-induced plasticity of dopaminergic neurons in the striatum of young mice. Interestingly, we found that dopaminergic neurons express IL-1 receptors, suggesting that IL-1α, could directly act as a target-derived dopaminergic neurotrophic factor to initiate or enhance the sprouting of dopaminergic axonal terminals.

Introduction

The mesostriatal dopaminergic system plays an important role in the control of voluntary movement. This system is comprised of midbrain dopaminergic cell groups and their projections to the entire striatal complex which consists of a dorsal and a ventral component (Björklund and Lindvall, 1984). In the rodent, the dorsal striatum (caudate-putamen) is innervated mainly by dopaminergic neurons in the SN pars compacta, while the ventral striatum (nucleus accumbens, and the olfactory tubercle), receives projections from cells in the VTA and the RRA (Björklund and Lindvall, 1984). Selective degeneration of the mesostriatal pathway leads to the motor impairments seen in PD, which is characterized by the loss of dopaminergic neurons in the SN that leads to a preferential depletion in dopaminergic innervation of the dorsal striatum; however, cells in the VTA and the RRA are far less affected (Agid et al. 1987; Hirsch et al. 1988; German et al. 1989; Hornykiewicz 1993).

MPTP, a neurotoxin that selectively destroys the SN dopaminergic cells, has been used to produce an animal model of PD (Langston 1985; Singer and Ramsay 1990). In experimental animals, the neurodegenerative effects of MPTP are age-dependent, in that, young mice show a substantial recovery of striatal dopamine whereas aged mice do not (Ricaurte et al. 1986, 1987a,b; Date et al. 1990a). Thus, the dopaminergic cell system exhibits compensatory mechanisms in response to injury and that the degree of plasticity becomes reduced with age (Hornykiewicz 1993). In this report, we sought to determine the origin of dopaminergic fibers responsible for the spontaneous axonal regrowth in the denervated striatum of young mice. More importantly, we examined the cellular and molecular events associated with the dopaminergic sprouting that occurs selectively in the denervated striatum of young and but not middle-aged mice after MPTP.

Glial cells play an integral role in the brain response to neuronal injury and plasticity (Eddleston and Mücke 1993; Moore and Thanos 1996). Injury to the brain elicits a

sequence of morphological and biochemical events mediated by activated microglia that can release inflammatory cytokines such as IL-1 (Giulian and Baker 1986; Giulian 1987, 1990; Giulian et al. 1989). IL-1, in turn, can stimulate reactive astrocytes and enhance the synthesis of neurotrophic factors from astrocytes, thereby promoting axonal sprouting (Giulian and Lachman, 1985; Giulian et al., 1988; Spranger et al., 1990; Araujo and Cotman, 1992). It was shown that intrastriatal implantation of IL-1 can enhance compensatory sprouting from residual dopaminergic neurons in the VTA and can induce behavioral improvement in hemiparkinsonian rats (Wang et al., 1994a). Recently, we have reported that bFGF may be the putative dopaminergic neurotrophic factor that mediates IL-1 lesion-induced plasticity of dopaminergic neurons since intraventricular administration of IL-1 induces bFGF gene expression (Ho and Blum, 1997).

In the present study, we investigated the potential role of IL-1 and trophic factor activities in mediating the spontaneous dopaminergic sprouting in the denervated striatum of young but not middle-aged mice after MPTP. We hypothesized that perhaps in the aging brain, the ability to induce growth-promoting molecules such as IL-1 and the subsequent induction of trophic factors synthesis declines with age. An understanding of the cascade of cytokine and trophic factor activities could provide insights into basic mechanisms of aging and their relationship to the process of dopaminergic cell death in PD.

Materials and Methods

Drug Administration. Male C57BL/6 mice (Harlan Sprague Dawley, Indianapolis, IN) of two different age groups were used: 8 weeks (young) and 8 months of age (middle-aged). MPTP hydrochloride (Research Biochemicals International, Natick, MA) was administered subcutaneously. Young mice received a single dose of 55 mg/kg and middle-aged mice received a single dose of 40 mg/kg of MPTP. These doses were selected based on titration studies which produced comparable initial depletions of dopamine uptake in the

striatum of young and older mice. Age-matched controls received saline. Animals were sacrificed at 4, 8, 14, 21, and 30 days post-lesion along with their age-matched controls (n=4-5/group).

^{3}H-Dopamine Uptake. Animals were decapitated, and the brain quickly removed and placed into cold sterile saline. The dorsal and ventral striatum were dissected and homogenized in 500 µl of ice-cold pre-lysis buffer (10 mM Tris pH 7.5, 0.32 M sucrose). Homogenized tissue (100 µl) was removed and centrifuged for 10 min at 1000Xg at 4°C to remove nuclei. The supernatant containing the synaptosomes was collected and aliquots were removed for the determination of protein concentration and dopamine uptake (total high affinity and mazindol non-inhibitable). Fifty µl of supernatant was diluted in 450 µl of Krebs-Ringer phosphate buffer (0.1 M) with added EDTA (1.3 mM), glucose (5.6 mM) and ascorbic acid (0.2 mg/ml) and incubated at 37°C in the presence or absence of 10 µM mazindol (Research Biochemicals International), an high affinity dopamine uptake inhibitor. Fifty µl of 24.5 nM [^{3}H]-dopamine (20-40 mCi/mmol, 0.5 µCi/ml; Amersham, Arlington Heights, IL) was added and incubated at 37°C for 6 min. Synaptosomes were collected on presoaked nitrocellulose filters by filtration and non-specific radioactivity was washed with Krebs-Ringer phosphate buffer. The filters were then transferred into scintillation vials of Hionic-fluor and measured by liquid scintillation spectrometry. Specific high-affinity neuronal dopamine uptake was expressed as fmoles of dopamine uptake per µg of protein minus the fmoles of mazindol uptake. Values are presented as change in dopamine uptake (expressed as percent of control).

Immunocytochemistry. Animals were anesthetized with 1:1 Rompun xylazine:Ketaset and sacrificed by intracardiac perfusion with 1% paraformaldehyde in 0.15 M phosphate buffer, pH 7.2 (PBS) followed by 4% paraformaldehyde. Brains were postfixed for 5 h at 4°C and cryoprotected with 30% sucrose. Tissues were frozen with OCT (Tissue Tek,

Torrance, CA) embedding medium immersed in a dry-ice chilled isopentane bath. Thirty μm coronal sections were cut on a cryostat and processed for immunocytochemistry. Sections were incubated in blocking buffer (0.3% Triton X-100, 3% goat serum in PBS) for 30 min, followed by an overnight incubation of primary antibodies to tyrosine hydroxylase (rabbit polyclonal anti-TH, 1:500; Pel-Freeze Biologicals, Rogers, AK), glial fibrillary acidic protein (rabbit polyclonal anti-GFAP, 1:50; Biomeda, Foster City, CA), or microglia (rat monoclonal anti-Mac-1, 1:50; Boehringer Mannheim, Indianapolis, IN) in blocking buffer at 4°C. Sections were then washed 3x10 min with PBS and incubated in biotinylated anti-rabbit IgG to detect TH and GFAP (1:200; Vector Laboratories, Burlingame, CA), or rat IgG to detect Mac-1 (1:200; Amersham, Arlington Heights, IL) for 2 h at room temperature. Sections were washed and then incubated in ExtraAvidin (1:200; Sigma) for 1 h at room temperature. Sections were washed and processed with 0.05% 3,3'-diaminobenzidine tetrachloride (Sigma) with 0.003% H_2O_2. After processing, sections were washed, mounted on coated slides, dried, dehydrated through graded alcohols, cleared in xylene, and coverslipped in DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA).

Double Immunolabeling. Young mice lesioned with MPTP and killed at 8 d along with saline-matched controls were processed for double fluorescence immunocytochemistry for IL-1α (rabbit polyclonal anti-mouse IL-1α, 1:400; Genzyme, Cambridge, MA.) and GFAP (mouse monoclonal anti-GFAP, 1:50; Boehringer Mannheim), IL-1α and Mac-1, IL-1α and Neu N (mouse monoclonal anti-neuronal nuclei, 1:500; Chemicon International, Temecula, CA), and IL-1 receptor (rat monoclonal anti-IL-1 receptor, 1:400; Genzyme) and TH. Sections were incubated in blocking buffer (0.1% saponin, 3% goat serum in PBS) for 30 min, followed by an overnight incubation of primary antibody in blocking buffer at 4°C. IL-1α was visualized by incubation with anti-rabbit IgG directly conjugated to fluorescein (1:200; Vector Laboratories) while GFAP, Mac-1 and Neu N immunoreac-

tivity was reacted to appropriate biotinylated secondary antibodies; anti-mouse IgG to detect GFAP and Neu N (1:200; Vector Laboratories), anti-rat IgG to detect Mac-1 (1:200; Amersham) for 2 h followed by incubation in streptavidin conjugated to rhodamine (1:500; Molecular Probes, Eugene, OR). For IL-1 receptor and TH immunoreactivity, TH was visualized by incubation with anti-rabbit IgG directly conjugated to fluorescein while IL-1 receptor was reacted to biotinylated anti-mouse IgG for 2 h followed by streptavidin conjugated to rhodamine. After processing, sections were mounted on coated slides, dried, and coverslipped in Permafluor (Lipshaw, Pittsburgh, PA) mounting medium.

Retrograde Labeling. To detect degeneration of dopaminergic neurons after MPTP lesion and to identify the cellular origin of the sprouted fibers in the dorsal striatum, young mice prior to MPTP lesion or treated after 30 d with saline and MPTP respectively were used. Animals were anesthetized with 287.5 mg/kg avertin (stock of 12.5 mg/ml 2,2,2-tribromoethanol; Aldrich), injected intraperitoneally. The mice were placed on a stereotaxic device (David Kopf Instruments). A burr hole was drilled on the right side of the skull to accomodate injection. Stereotaxic injections of 0.35 µl of fluorescent latex microsphere "beads", a retrograde neuronal tracer (Lumafluor, Naples, FL), were made into the dorsal striatum using a 1 µl Hamilton syringe (Hamilton). Coordinates were located 2.7 mm caudal to the frontal nasal suture, 2.0 mm lateral from the midline suture and 2.5 mm from the surface of the brain. The injection was made at a rate of ~0.05 µl/min, and the needle was left in place for 5 min after injection. To detect degeneration of dopaminergic neurons, animals were sacrificed 8 d after MPTP lesion (n=3/group), and processed for TH and Mac-1 immunoreactivity. TH and Mac-1 were visualized by incubation with anti-rabbit and anti-rat IgG directly conjugated to fluorescein respectively, and were visualized with a laser scanning confocal microscope (LSM 410, Zeiss, Oberkochen, Germany). To identify the cellular origin of the sprouted fibers in the dorsal striatum after one month post-lesion, animals were sacrificed 7 d after tracer injection (n=5/group) and processed

for TH immunoreactivity which was visualized by incubation with anti-rabbit IgG directly conjugated to fluorescein.

Intrastriatal Stab Wound. Male C57BL6 mice (Harlan Sprague Dawley) at 8 weeks of age (young-adults) and 8 months of age (middle-aged) were anesthetized with 287.5 mg/kg avertin, injected intraperitoneally. The mice were placed on a stereotaxic device. A burr hole was drilled on the right side of the skull and a 1 µl Hamiliton syringe was placed into the dorsal striatum. Coordinates were located 2.7 mm caudal to the frontal nasal suture, 2.0 mm lateral from the midline suture and 2.5 mm from the surface of the brain. The needle was left in place for 5 min. Animals were sacrificed at 4, and 8 days post-lesion (n=4-5/group).

Isolation of cDNA Clones. The IL-1α and IL-1β cDNA clone was generously provided by Dr. A. Shaw of Glaxo from which a 400 and a 200 base pair fragment was subcloned into vector pGEM respectively. The aFGF cDNA clone was isolated by polymerase chain reaction (PCR) of mouse striatal cDNA from which a 350 base pair fragment corresponding to nucleotides 33-384 was subcloned into Bluescript II. The bFGF cDNA clone was generously provided by Dr. S. Shimasaki from which a 479 base pair fragment corresponding to nucleotides 525-1004 was subcloned into vector Bluescript/SK+. GDNF cDNA clone was isolated by PCR of rat genomic DNA from which a 414 base pair PCR DNA fragment was subcloned into Bluescript II.

Quantitative Solution Hybridization Nuclease Protection Assay. Unlabeled sense and high specific activity (~$1x10^9$ cpm/µg) ^{32}P-labeled antisense RNA were transcribed. A standard curve of 0-10 µl of a 100 fg/ µl of sense RNA was used for quantification. The standard and known amounts of cytoplasmic RNA isolated were hybridized with ~200 pg of antisense ^{32}P-labeled RNA probe. Samples were heat-denatured at 85°C for 5 min

and hybridized overnight at 45°C. After hybridization, samples were RNase A (5 µg/ml) and RNase T1 (2 µg/ml) treated for 1 h at 30°C, followed by proteinase K (0.167 mg/ml) digestion at 37°C for 15 min. Samples were phenol:chloroform extracted, precipitated, resuspended in 1xTE, and electrophoresed on a nondenaturing 5% acrylamide gel. Gels were dried, and quantified by phosphor image analysis. Results were determined by linear regression analysis from the standard curve and presented as amoles mRNA/µg total RNA.

Statistical Analysis. Significant differences in IL-1α, IL-1β, aFGF, bFGF and GDNF mRNA levels between control and MPTP treatment groups were analyzed using analysis of variance (ANOVA) followed by Fisher's protected least significant difference post hoc analysis. The level of significance was set at $p<0.05$.

Results

Young Mice Have the Ability to Recover After MPTP-Induced Toxicity of Dopaminergic Neurons While Middle-aged Mice Do Not

Neurochemical Assessment. High affinity synaptosomal dopamine uptake is a sensitive quantitative indicator of dopaminergic axonal terminal density. As shown in Fig. 4.1, determination of dopamine uptake at various times after MPTP revealed an initial comparable loss in dopamine uptake levels in both age groups in the dorsal as well as in the ventral striatum. In the dorsal striatum, we found that MPTP produced a significant reduction of dopamine uptake levels at 4 d after MPTP in young and middle-aged mice (59 and 41 % of control respectively; Fig. 4.1*A*). A reduction of dopamine uptake levels in the dorsal striatum was still observed at 8 and 14 d in both age groups. However, between 14 and 30 d, a significant recovery in dopamine uptake levels was observed in young mice. Dopamine uptake levels increased to 87% of control after MPTP in the dor-

sal striatum of young mice while older mice did not exhibit such recovery. Thus, we found, in accordance with others (Ricaurte et al., 1987a), that young mice but not middle-aged mice showed substantial recovery of striatal dopaminergic nerve terminals in the dorsal striatum within one month.

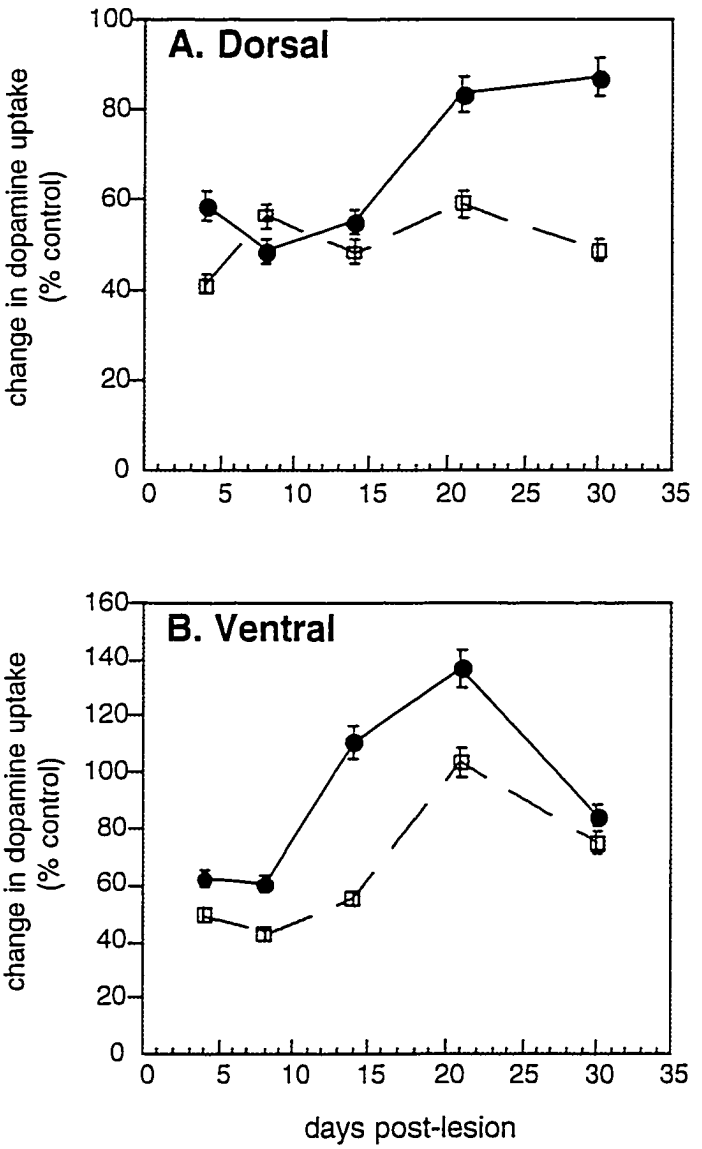

Figure 4.1 ^{3}H-Dopamine uptake after MPTP in the dorsal *(A)* and ventral *(B)* striatum of young and middle-aged mice at 4, 8, 14, 21, and 30 d post-lesion. Values are presented as change in dopamine uptake (expressed as percent of control ± SEM) for n=4-5 animals per group. Solid line represents young mice treated with a single dose of 55 mg/kg of MPTP and the dotted line represents middle-aged mice treated with a single dose of 40 mg/kg of MPTP.

Since it is thought that recovery could arise from spared fibers in the ventral striatum (Gilad and Reis, 1979; Hansen et al., 1995; Blanchard et al., 1996), it was important to determine whether the loss and recovery of dopamine uptake levels in the ventral striatum differs between young and middle-aged animals after MPTP. In the ventral striatum,

we observed that MPTP produced a significant reduction of dopamine uptake levels at 4 and 8 d in both young and middle-aged mice (Fig. 4.1*B*). In young mice, dopamine uptake levels in the ventral striatum recovered between 8 and 30 d after MPTP, increasing to a level that was greater than control values at 14 and 21 d (111 to 137% of control respectively). By 30 d after MPTP, dopamine uptake levels returned to 85% of control in young mice. In middle-aged mice treated with MPTP, recovery of dopamine uptake levels was also seen in the ventral striatum. The recovery occurred 1 week later compared to young mice between 14 and 30 d. Similar to young mice, middle-aged mice showed a greater increase in dopamine uptake levels at 21 d compared to levels observed at 30 d post-lesion. The recovery in dopamine uptake levels in the ventral striatum exhibited by both age groups in which the increase was greater than control levels after MPTP may suggests that there may be a transient increase in the affinity or the number of dopamine uptake sites per terminal.

Morphological Assessment. An antibody against tyrosine hydroxylase (TH), an enzyme involved in dopamine biosynthesis is a marker for catecholamine neurons. TH immunocytochemistry revealed a marked disappearance of TH-immunoreactive fibers in the dorsal striatum of both young and middle-aged mice, as early as 4 d after MPTP (Fig. 4.2*B,G*), compared to age-matched control (Fig. 4.2*A,F*). No marked reduction of TH-immunoreactive fibers was seen in the ventral striatum of young mice after MPTP. However, in middle-aged mice, a modest reduction of TH-immunoreactive fibers was observed in the ventral striatum compared to saline-treated animals at 4 d after MPTP which appeared to recover at later time points. A significant loss of TH-immunoreactive fibers in the dorsal striatum was still observed at 8 d following MPTP in both age groups (Fig. 4.2*C,H*). However, in the dorsal striatum of young mice, a progressive recovery of TH-immunoreactive fibers was observed starting at 14 d (Fig. 4.2*D*), reaching a nearly normal staining pattern by 30 d after MPTP (Fig. 4.2*E*). In contrast, middle-aged mice did not show such an apparent recovery (Fig. 4.2*I,J*).

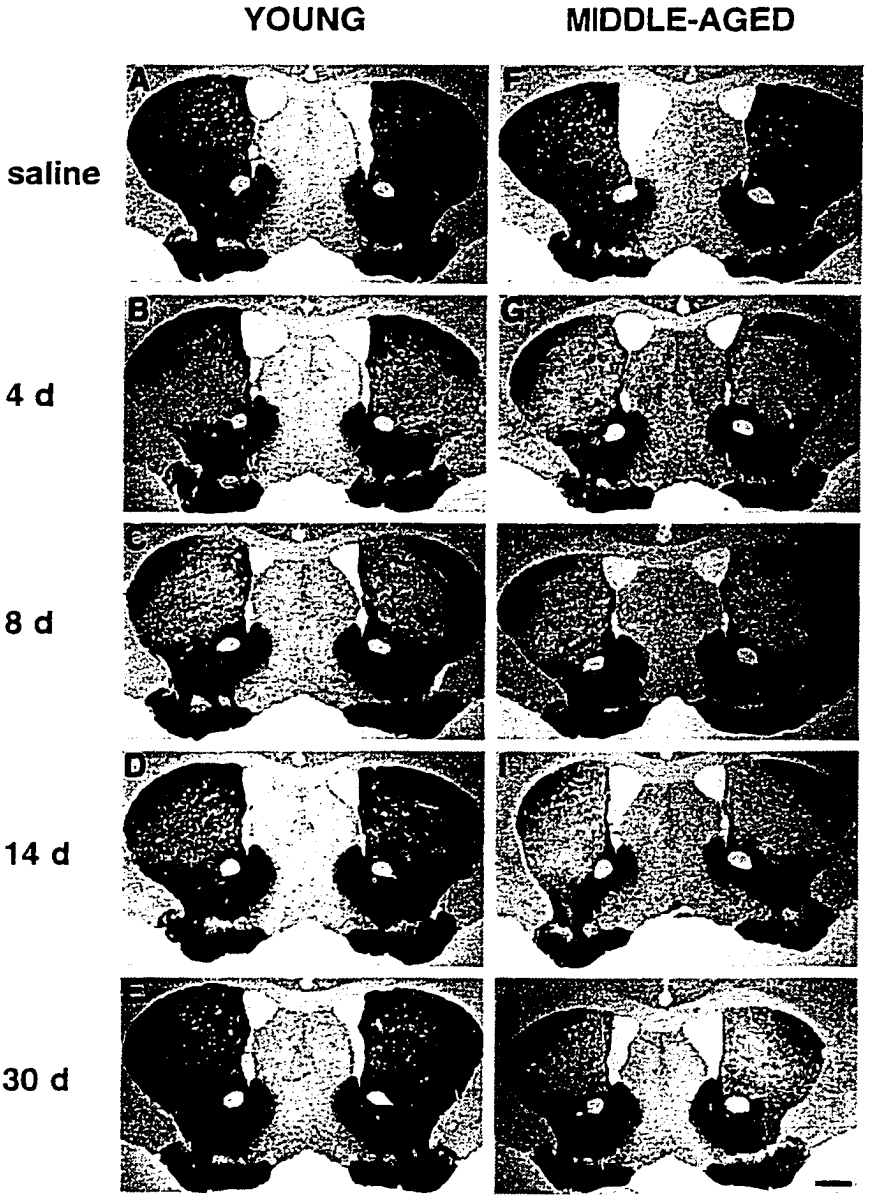

Figure 4.2 TH immunocytochemistry in the striatum of young *(A-E)* and middle-aged mice *(F-J)* following saline and MPTP treatment at 4, 8, 14 and 30 d. MPTP caused a marked disappearance of TH-immunoreactive fibers in the dorsal striatum of both age groups, however a progressive recovery of TH-immunoreactive fibers were observed between 14 and 30 d after MPTP *(D-E)* in young mice, compared to middle-aged mice which did not show such apparent recovery. Scale bar, 500 μm.

Dopaminergic Cell Loss After MPTP

To examine the neurotoxic effects of MPTP on dopaminergic cell bodies in young and middle-aged mice, we assessed midbrain sections immunostained with TH antiserum. TH immunocytochemistry revealed a significant depletion of TH-immunoreactive

cell bodies in the SN of both age groups, as early as 4 d after MPTP (Fig. 4.3*B,G*). In contrast, no marked reduction in TH-immunoreactive cell bodies was seen in the VTA compared to age-matched control (Fig. 4.3*A,F*). A substantial loss of TH-immunoreactive cells in the SN remained evident weeks after MPTP in both age groups (Fig. 4.3*C-J*). At 30 d after MPTP, while a marked depletion of TH-immunoreactive cells was found compared to saline-treated animals, a modest return of TH expression in the SN was observed in young mice (Fig. 4.3*E*). This result indicates that in young mice, some of the dopaminergic neurons may survive the MPTP insult and the transient decrease in TH expression returns as reported by other investigators (Kitt et al., 1987; Sundström et al., 1988).

The use of TH-immunoreactive neuronal counts has not been a reliable method to measure MPTP-induced degeneration of dopaminergic neurons. It was shown that MPTP can cause a loss in TH expression without producing neuronal death (Jackson-Lewis et al., 1995). To determine whether SN dopaminergic neurons are in fact degenerating after MPTP, SN cells were prelabeled with fluorescent microspheres in young mice prior to MPTP administration. Young mice were used since it has been suggested that they are less sensitive to MPTP-induced toxicity than middle-aged mice (Ricaurte et al., 1986). The animals were sacrificed 8 d after saline and MPTP treatment and midbrain sections were reacted with antibodies to TH and Mac-1, a microglia marker specific for the mouse (Springer et al., 1979; Beller et al., 1982). In saline-treated mice, retrogradely transported fluorescent microspheres labeled the cell body of a subpopulation of TH-immunoreactive neurons in the SN (Fig. 4.4*A,B*). In MPTP-treated mice, while some fluorescent microspheres were found in TH-immunoreactive neurons, they were also found to be aggregated and scattered throughout the SN (Fig. 4.4*C*). Mac-1-immunoreactive cells with internalized fluorescent microspheres were found at the level of the SN cell bodies, suggesting that microglia are phagocytosing degenerating SN dopaminergic neurons (Fig. 4.4*D*).

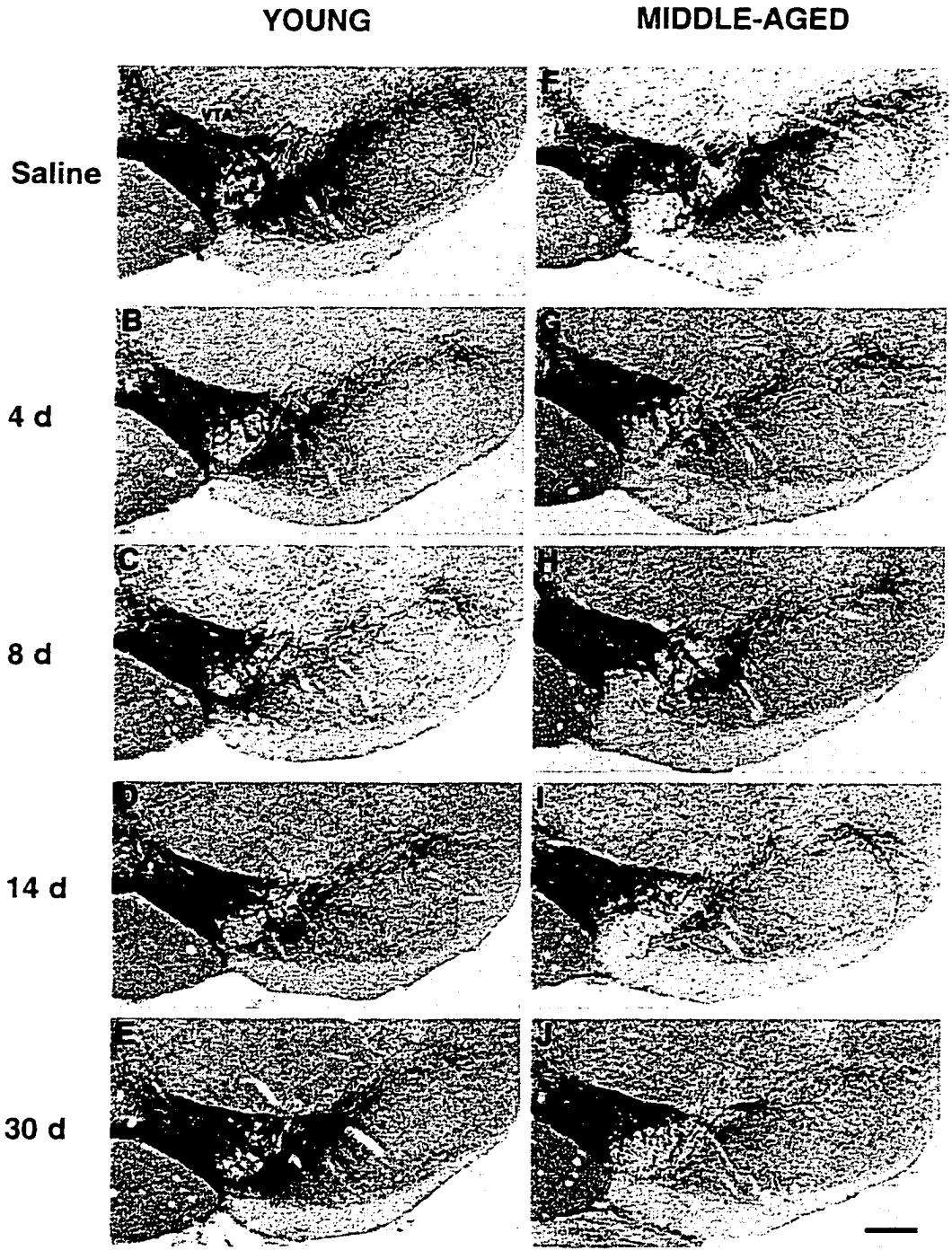

Figure 4.3 TH immunocytochemistry in the midbrain of young *(A-E)* and middle-aged mice *(F-J)* following saline and MPTP treatment at 4, 8, 14 and 30 d. MPTP produced a significant loss of TH-immunoreactive cell bodies in the SN but not in the VTA of both age groups compared to age-matched control *(A,F)*. Scale bar, 200 µm.

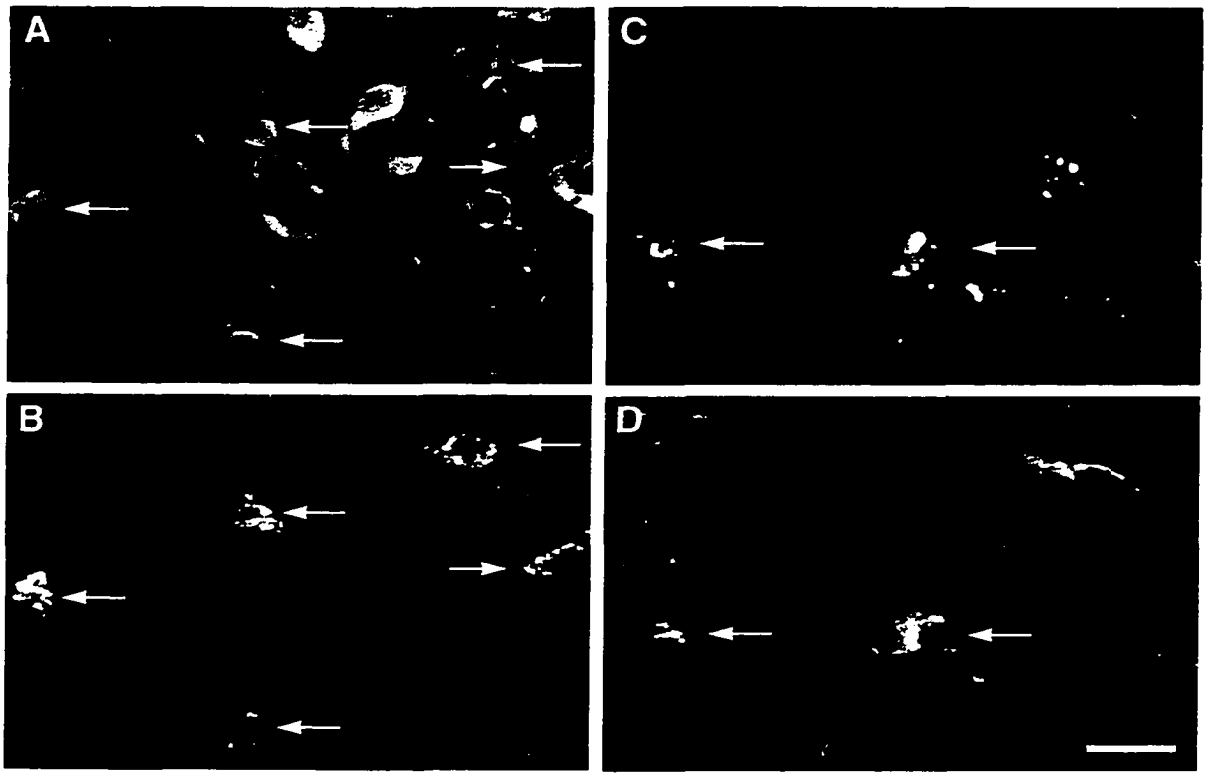

Figure 4.4 Dopaminergic neuronal degeneration in young mice at 8 d after MPTP. *A and B,* Confocal image of a TH-immunoreactive cell bodies *(A)* in the SN retrogradely labeled with microspheres *(B)* in a saline-treated animal. Arrows are in register and show the same location within the same field of view for *A* and *B* indicating that labeled microspheres are within TH-immunoreactive cell bodies. *C and D,* Confocal images showing distribution of labeled microsphere aggregates *(C)* and Mac-1-immunoreactive microglial cells *(D)* within the SN of a MPTP-treated animal. Arrows are in register and show the same location within the same field of view for *C* and *D* indicating that Mac-1-immunoreactive microglial cells are internalizing the labeled microspheres, suggesting that they are phagocytosing degenerating dopaminergic neurons. Scale bar, 25 μm.

Collateral Sprouting of Dopaminergic Terminals Arise from the VTA

To identify the cellular origin of the sprouted fibers in the dorsal striatum of young mice after MPTP-induced injury, fluorescent microspheres were injected into the dorsal striatum of saline and MPTP-treated animals at 30 d post-lesion when recovery of dopamine uptake levels and TH-immunoreactive fibers were observed (Fig. 4.5*A,D*). The animals were sacrificed 7 d after tracer injection and the midbrain was reacted with the antibody to TH. In saline-treated mice, we found a majority of labeled microspheres colocalized with TH-immunoreactive neurons in the SN (Fig. 4.5*B,C*). In MPTP-treated mice, we observed labeled microspheres within TH-immunoreactive neurons in the VTA, sug-

gesting that recovery in the dorsal striatum is mainly due to collateral sprouting of axonal fibers in the ventral striatum (Fig. 4.5*E,F*). However, while the injection of the retrograde tracer was made to a localized area and does not represent the entire dorsal striatum, we can not exclude that remaining SN dopaminergic neurons can also contribute to compensatory sprouting in the denervated striatum.

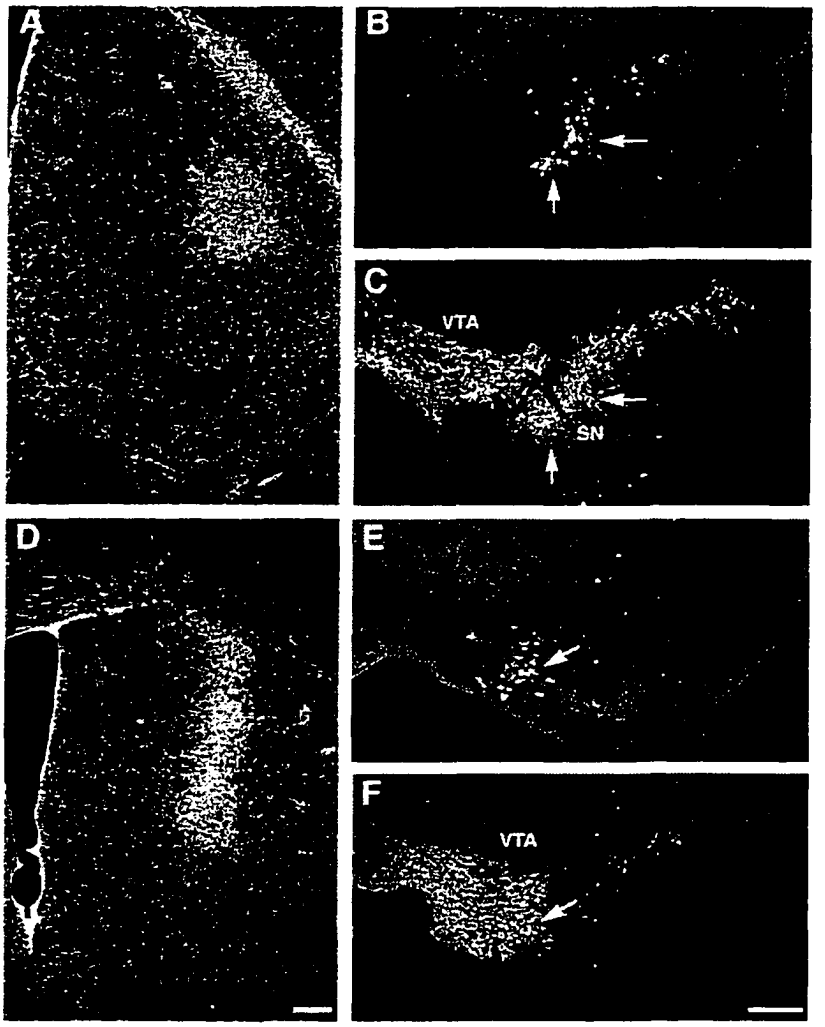

Figure 4.5 Collateral sprouting of dopaminergic axonal fibers arise from the VTA in young mice. *A and D,* Injection site of fluorescent microspheres into the dorsal striatum of saline *(A)* and MPTP-treated mice *(D). B and C,* In a saline-treated animal, fluorescent microspheres *(B)* were found to colocalize with TH-immunoreactive neurons in the SN *(C). E and F,* In a MPTP-treated animal, fluorescent microspheres *(E)* were found to be localized within TH-immunoreactive neurons in the VTA *(F)*; indicating that most of the sprouting fibers were arising from dopaminergic neurons in the VTA. Scale bar, 200 μm.

Glial Activation

MPTP produces a glial reaction in young mice following MPTP treatment (Francis et al., 1995; Czlonkowska et al., 1996). However, glial responses after MPTP have not been investigated in middle-aged mice. Furthermore, studies have shown that reactive

glial response to neuronal injury slows with age (Hoff et al., 1982a,b; Goss et al., 1991). To investigate whether there were differences in glial responses in young compared to middle-aged mice following MPTP treatment, striatum and midbrain sections at various times post-lesion were reacted with antibodies to Mac-1 and GFAP.

Microglial Reaction. Induction of a microglial reaction in the denervated striatum (confined mainly to dorsal striatum) and the midbrain (confined at the level of the dopaminergic cell bodies in the SN) was seen as early as 4 d post-lesion in both age groups compared to age-matched control (Fig. 4.6). A difference in the morphology of microglial cells compared with age-matched control was observed in both young and middle-aged mice. In MPTP-treated mice, the microglia exhibited stronger Mac-1 labeling, larger cell bodies and thicker, less ramified processes compared with saline-treated animals (Fig. 4.9*H*). In young mice, maximal activation of microglial cells in the striatum was observed at 8 d post-lesion and by 14 d, activated microglial cells were no longer found (Fig. 4.6*C,D*). In middle-aged mice, maximal activation of microglial cells in the striatum was observed at 4 d post-lesion (Fig. 4.6F); by 8 and 14 d, a modest activation of microglial cells was still visible (Fig. 4.6*G,H*). In the midbrain, the peak of change in microglial reaction also occurred faster in middle-aged mice, as early as 4 d post-lesion compared to young mice where peak change was observed at 8 d (Fig. 4.6*I-O*). By 14 d, activated microglial cells in the midbrain of young mice were no longer found; however, some scattered activated microglial cells were still seen in the midbrain of middle-aged mice (Fig. 4.6*L,P*).

Astroglial Reaction. In both age groups, immunolabeling for GFAP was dramatically increased in the striatum and midbrain as early as 4 d post-lesion compared to age-matched control (Fig. 4.7). GFAP-immunoreactive astrocytes became hypertrophic and they exhibited an enlarged cell body and shortened, swollen processes (Fig. 4.9*F*). Maximal activation of astroglial cells in the striatum and midbrain occurred faster in middle-aged mice, as early as 4 d post-lesion (Fig. 4.7*F,N*) compared to young mice where

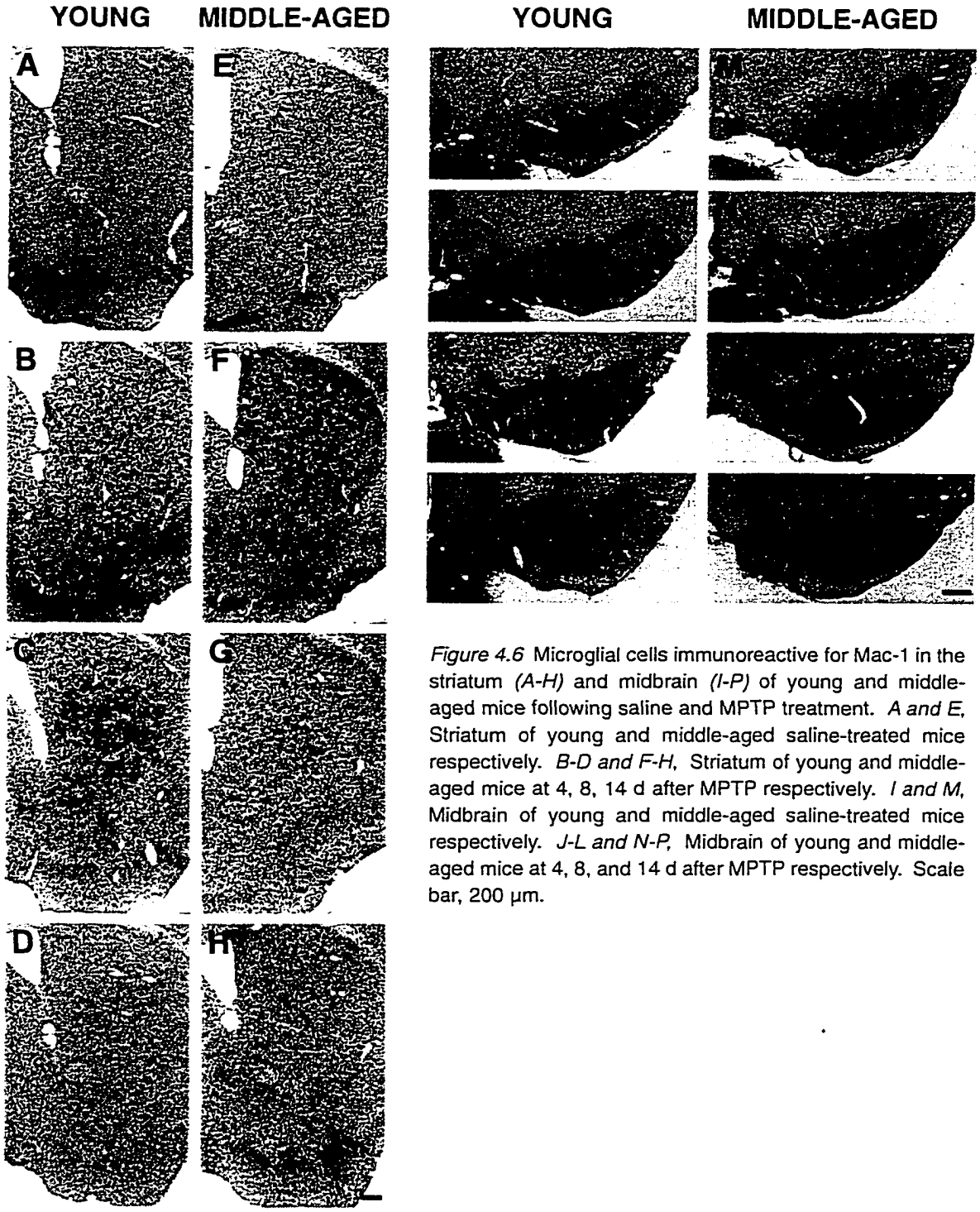

Figure 4.6 Microglial cells immunoreactive for Mac-1 in the striatum *(A-H)* and midbrain *(I-P)* of young and middle-aged mice following saline and MPTP treatment. *A and E*, Striatum of young and middle-aged saline-treated mice respectively. *B-D and F-H*, Striatum of young and middle-aged mice at 4, 8, 14 d after MPTP respectively. *I and M*, Midbrain of young and middle-aged saline-treated mice respectively. *J-L and N-P*, Midbrain of young and middle-aged mice at 4, 8, and 14 d after MPTP respectively. Scale bar, 200 μm.

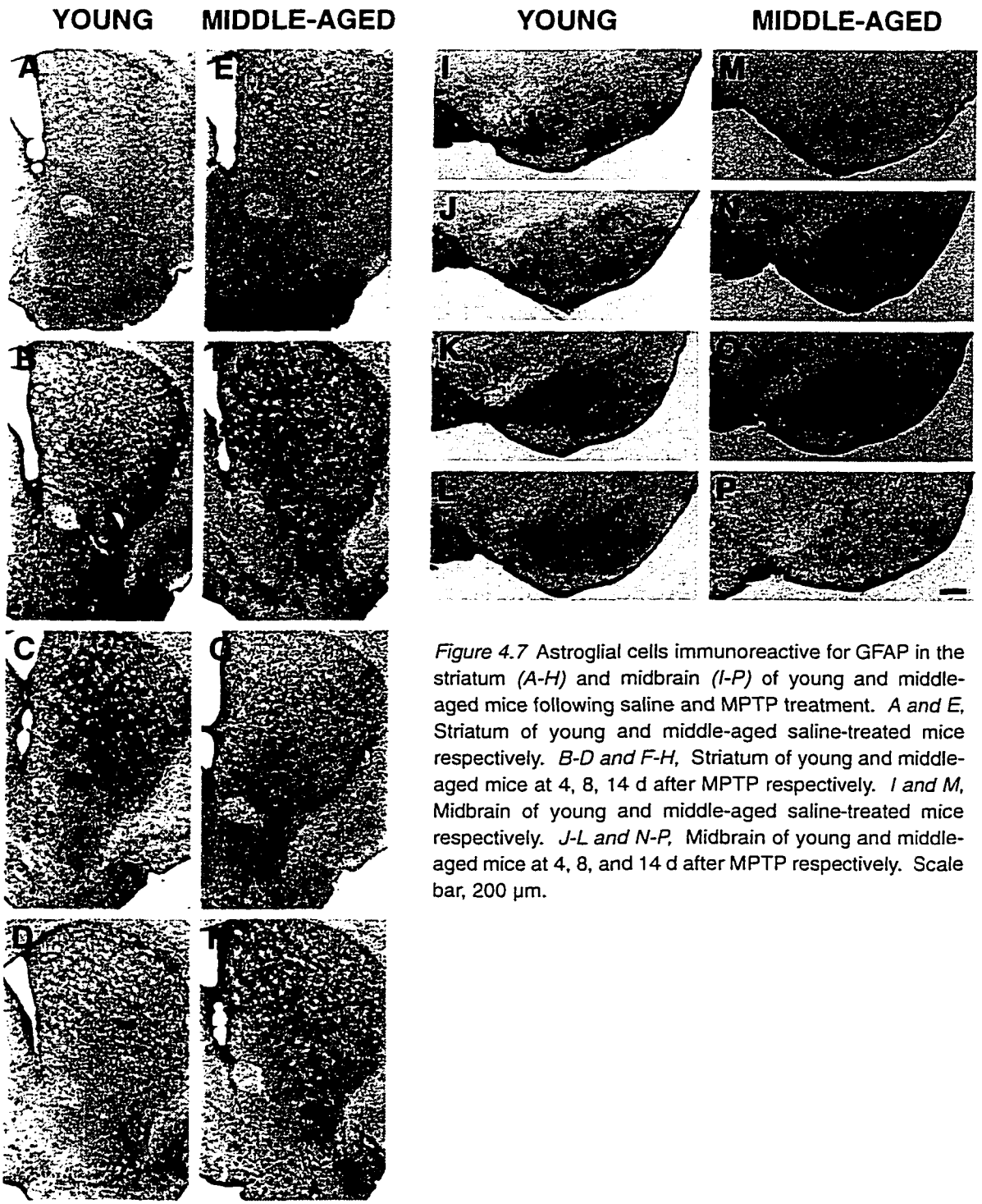

Figure 4.7 Astroglial cells immunoreactive for GFAP in the striatum *(A-H)* and midbrain *(I-P)* of young and middle-aged mice following saline and MPTP treatment. *A and E,* Striatum of young and middle-aged saline-treated mice respectively. *B-D and F-H,* Striatum of young and middle-aged mice at 4, 8, 14 d after MPTP respectively. *I and M,* Midbrain of young and middle-aged saline-treated mice respectively. *J-L and N-P,* Midbrain of young and middle-aged mice at 4, 8, and 14 d after MPTP respectively. Scale bar, 200 μm.

peak changes were observed at 8 d (Fig. 4.7*C,K*). By 14 d, GFAP-immunoreactive cells were no longer observed in the striatum of young mice (Fig. 4.7*D*); however, an increase in GFAP-immunoreactive cells remained distributed in the striatum of middle-aged mice (Fig. 4.7*H*). In the midbrain, a modest induction of hypertrophic astrocytes was still found at 14 d after MPTP in both age groups (Fig. 4.7*L,P*). These results indicate that differences in glial reaction after MPTP exist between young and middle-aged mice and that the reactive glial response was not delayed with age. In fact, middle-aged mice produced a faster and sustained glial response for 2 weeks compared to young mice after MPTP.

Induction of IL-1 mRNA is Correlated With Compensatory Sprouting in the Denervated Striatum of Young Mice

To determine if IL-1 is regulated in response to MPTP and to examine whether age influences the regulation of IL-1, tissues from the same animals used for dopamine uptake analysis were analyzed for IL-1α and IL-1β mRNA changes by RNase protection assay. As a positive control for IL-1α and IL-1β changes after MPTP, total RNA from intrastriatal stab wounds of both age groups were used since both the α and the β form have been shown to increase after stab injury (Giulian and Lachman, 1985).

In the dorsal striatum of young mice after MPTP treatment, IL-1α mRNA was significantly increased at 4 d and was maximally induced at 8 d post-lesion (518% of control; Fig. 4.8*A*). After 14 d, IL-1α mRNA remained significantly elevated and by 30 d, IL-1α returned to control levels in the dorsal striatum of young mice. In the dorsal striatum of middle-aged mice after MPTP treatment, only a modest increase was observed at 4 and 8 d post-lesion (130 and 135% of control respectively; Fig. 4.8*B*). After 14 d, IL-1α returned to control levels in the dorsal striatum of middle-aged mice after MPTP. Activation of IL-1α synthesis in response to intrastriatal stab injury was similar in both age groups. Young mice that received an intrastriatal stab wound injury revealed a 306 and 287% of control induction at 4 and 8 d post-lesion respectively (Fig. 4.8*A*). Similarly, mid-

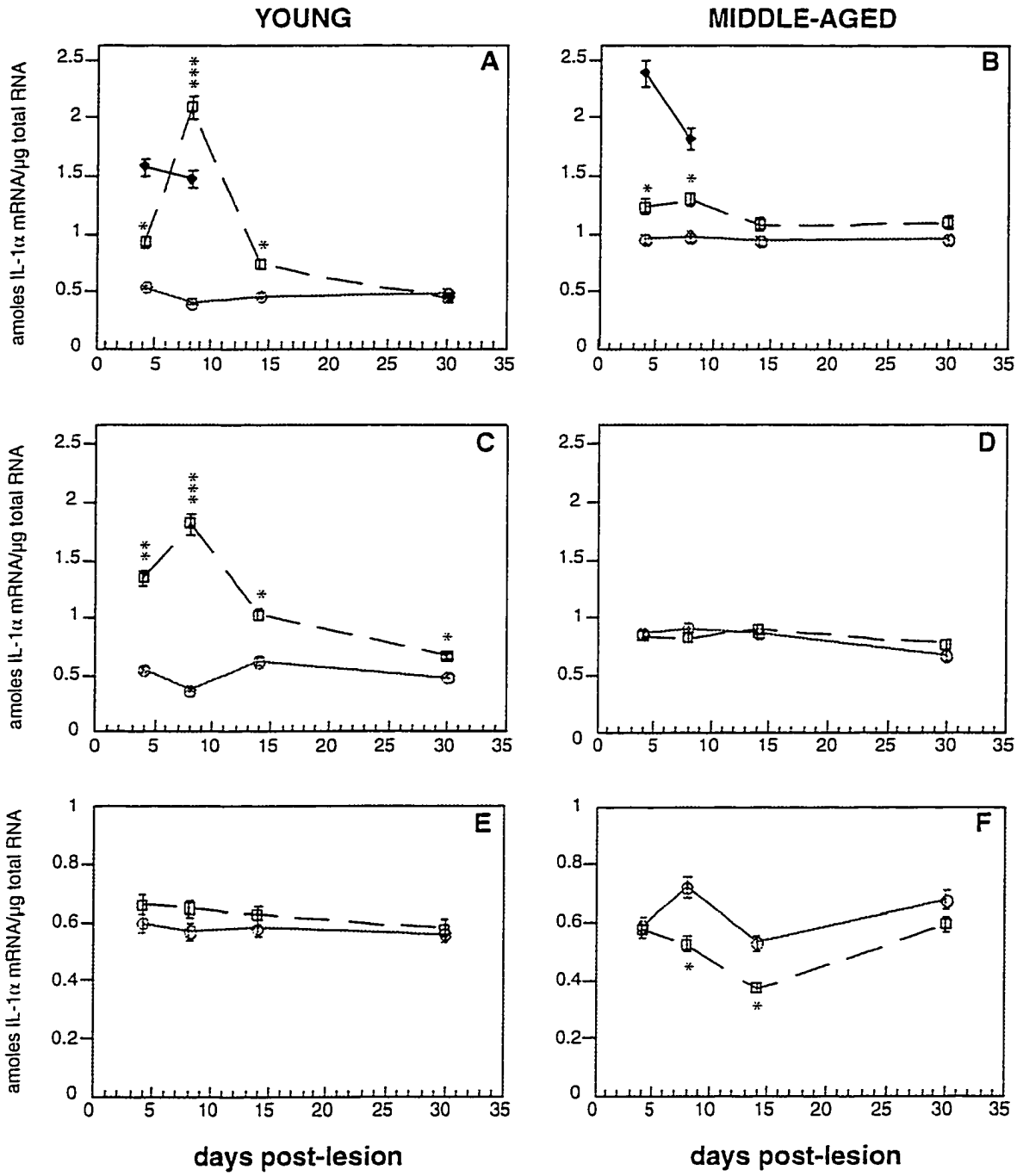

Figure 4.8 Quantitative analysis of IL-1α mRNA in the dorsal *(A and B)*, and ventral striatum *(C and D)* and midbrain *(E and F)* of young and middle-aged mice following saline and MPTP treatment at different days post-lesion. Solid line with open circles represents saline-treated animals, dotted line with open squares represents MPTP-treated animals and solid line with close diamonds represents animals that received intrastriatal stab wounds. Values represent the mean ± SEM for n=4-5 animals per group. Levels of statistical significance were set to *P<0.05, **P<0.005, ***P<0.001 which indicates difference from age-matched control.

dle-aged mice had a 252 and 192% of control increase at 4 and 8 d post-lesion respectively after stab injury (Fig. 4.8*B*). These results indicates that the ability to induce IL-1α synthesis in the dorsal striatum after MPTP dramatically declines with age; however, this strictly depends on the nature of the injury.

Similar to the induction seen in the dorsal striatum of young MPTP-treated mice, IL-1α mRNA was also increased at 4 d in the ventral striatum, and was maximally induced at 8d post-lesion (501% of control; Fig. 4.8*C*). After 14 and 30 d, IL-1α mRNA remained significantly elevated. However, in middle-aged mice, nc changes in IL-1α mRNA were observed in the ventral striatum (Fig. 4.8*D*). The enhanced and extended activation of IL-1α synthesis seen both in the dorsal and ventral striatum of young but not middle-aged mice seems to correlate best with the time when MPTP-induced plastic changes are occurring in young mice.

While significant changes in IL-1α were observed after MPTP, we did not observe any changes in IL-1β mRNA in either the dorsal or ventral striatum of either age group (data not shown). However, IL-1β mRNA was markedly increased in both age groups after intrastriatal stab wound injury. The selective activation seen in the α form but not the β form after MPTP suggests that the type of injury-induced damage to the brain may elicit a difference in the IL-1 response.

Since we found microglial activation and an astrocytic response at the site of degenerating cell bodies, we next evaluated whether there were changes in IL-1α mRNA in the midbrain following MPTP lesion. In both age groups, no induction of IL-1α mRNA was observed in the midbrain after MPTP compared to age-matched control (Fig. 4.8*E,F*). Instead, middle-aged mice exhibited a significant decrease in IL-1α mRNA at 8 and 14 d after MPTP (Fig. 4.8*F*). These results suggest that the activation of IL-1α in response to MPTP is region specific.

Cellular Source of Increased IL-1α Expression

Activated microglia have been shown to be a principal source of IL-1, although there is evidence that astrocytes as well as neurons can synthesize IL-1 (Fontana et al. 1982; Giulian 1987; Breder et al. 1988; Hetier et al. 1988; Lechan et al. 1990; Tchelingerian et al. 1993). To identify the cellular source of IL-1α in our MPTP-lesion model, we performed double labeling immunocytochemistry on the striatum of young mice at 8 d post-lesion when IL-1α mRNA was detected at its maximal level using antibodies to IL-1α in combination with either Neu N, a specific marker of neuronal nuclei, GFAP or Mac-1. In saline-treated young mice, we found faint perinuclear localization of IL-1α in Neu N-immunoreactive cells (Fig. 4.9*A,B*). However, in MPTP-treated mice, we observed perinuclear and cytoplasmic staining of IL-1α in Neu N-immunoreactive cells (Fig. 4.9*C,D*) as well as colocalization of IL-1α and GFAP-immunoreactive hypertrophic astrocytes scattered throughout the denervated striatum (Fig. 4.9*E,F*). Colocalization of IL-1α and Mac-1-immunoreactive microglial cells was not observed in these MPTP-treated animals (Fig. 4.9*G,H*). These results indicate that hypertrophic astrocytes and neurons are most likely the cellular sources of increased IL-1α expression in the striatum of young mice after MPTP-induced lesion.

No Change in Endogenous Dopaminergic Neurotrophic Factor Gene Expression

Since we and others have reported that intraventricular administration of IL-1 can induce neurotrophic factor gene expression (Spranger et al., 1990; Rivera et al., 1994; Ho and Blum, 1997), we wanted to examine whether changes in dopaminergic neurotrophic factor gene expression mediate the MPTP-induced plasticity of dopaminergic neurons. Among the trophic factors expressed in the brain, aFGF, bFGF and GDNF are well-characterized dopaminergic neurotrophic factors that can enhance the sprouting of dopaminergic fibers after a neurotoxic damage and are produced by astrocytes (Date et al., 1990b; Otto and Unsicker, 1990; Tomac et al., 1995). To investigate a potential role

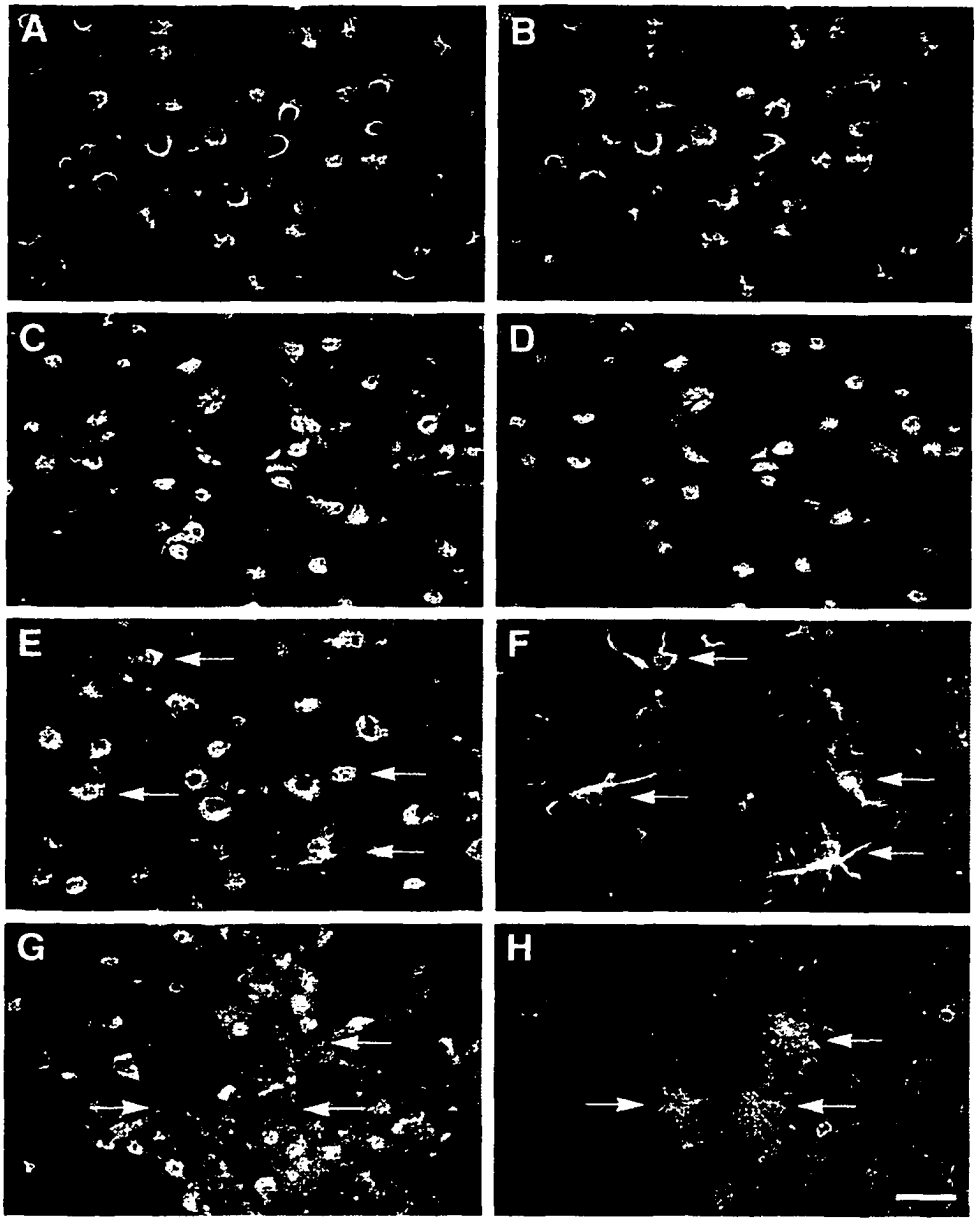

Figure 4.9 Cellular source of IL-1α in the striatum of young mice at 8 d after saline and MPTP treatment. *A and B,* Colocalization of IL-1α *(A)* and Neu N-immunoreactive neurons *(B)* in a saline-treated animal. *C and D,* Colocalization of IL-1α *(C)* and Neu N-immunoreactive neurons *(D)* in a MPTP-treated animal. *E and F,* Colocalization of IL-1α *(E)* and GFAP-immunoreactive astroglial cells *(F; labeled with arrows)* in a MPTP-treated animal. *G and H,* IL-1α *(G)* and Mac-1-immunoreactive cells *(H; labeled with arrows)* showing that IL-1α expression was not present in Mac-1-immunoreactive microglial cells. Scale bar, 25 μm.

for these astroglia-derived dopaminergic neurotrophic factors in MPTP-induced plasticity of dopaminergic neurons, we quantified aFGF, bFGF and GDNF mRNA in the striatum using RNase protection assay. No changes in aFGF and bFGF mRNA were found in the dorsal and ventral striatum of either age group (Fig. 4.10). In addition, no changes in GDNF mRNA were found in the dorsal striatum of young mice after MPTP (Fig. 4.10*C*)

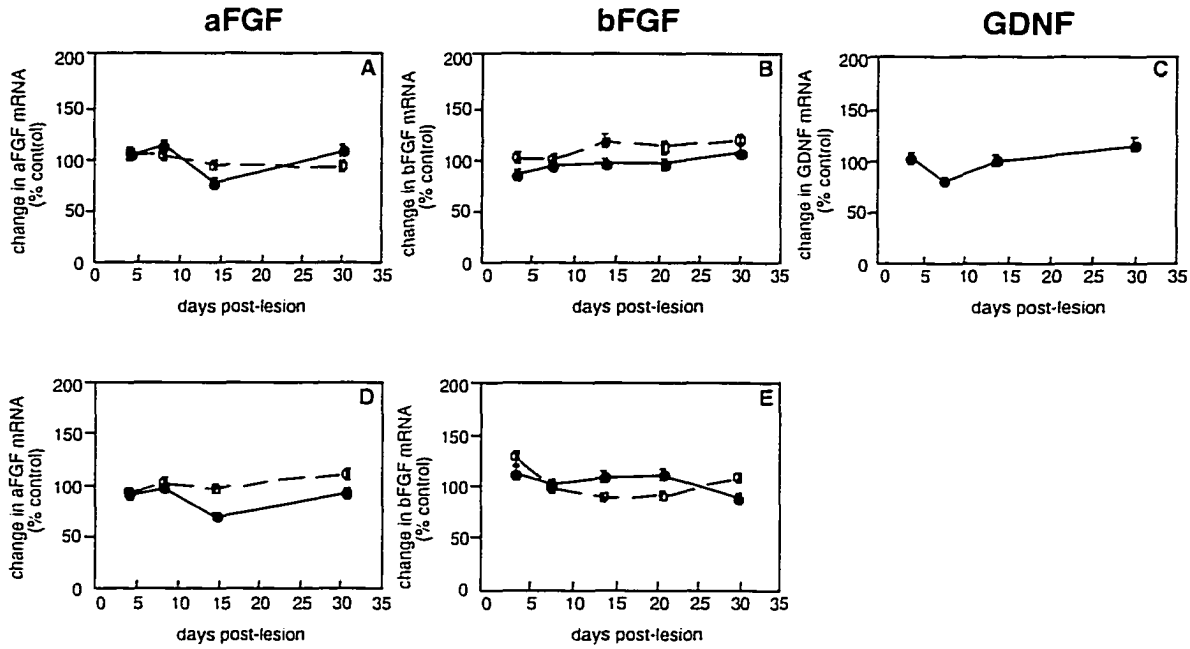

Figure 4.10 Quantitative analysis of dopaminergic neurotrophic factor synthesis in the striatum of young and middle-aged mice at different times after MPTP. Solid line represents young MPTP-treated mice, and dotted line represents middle-aged MPTP-treated mice. Values are presented as change in mean as expressed in percent control ± SEM for n=4-5 animals per group. *A and D,* Changes in aFGF mRNA in the dorsal *(A)* and ventral striatum *(D). B and E,* Changes in bFGF mRNA in the dorsal *(B)* and ventral striatum *(E). C,* Changes in GDNF mRNA levels in the dorsal striatum of young MPTP-treated mice.

Localization of IL-1 Receptor in TH-Immunoreactive Neurons

Since we observed an induction of IL-1α in the striatum of young mice treated with MPTP, and no change in any of the astroglia-derived dopaminergic neurotrophic factors analyzed, we examined whether IL-1α could be acting directly on dopaminergic neurons to induce sprouting of axonal fibers. To assess this, we examined the expression of IL-1 receptor on these neurons. Double labeling immunocytochemistry was performed in

saline and MPTP-treated young mice at 8 d post-lesion with antibodies to IL-1 receptor and TH. In saline-treated mice, IL-1 receptor expression was colocalized with TH-immunoreactive cell bodies in the SN and VTA throughout the midbrain (Fig. 4.11*A,B*). After MPTP treatment, IL-1 receptor expression remained colocalized with TH-immunoreactive neurons in the VTA, and scattered TH-immunoreactive cells in the SN (Fig. 4.11*C,D*). IL-1 receptor-positive/TH-negative profiles within the midbrain were also observed. These results indicate that IL-1α may act as a target-derived neurotrophic factor that enhances the plasticity of dopaminergic fibers after MPTP-induced injury.

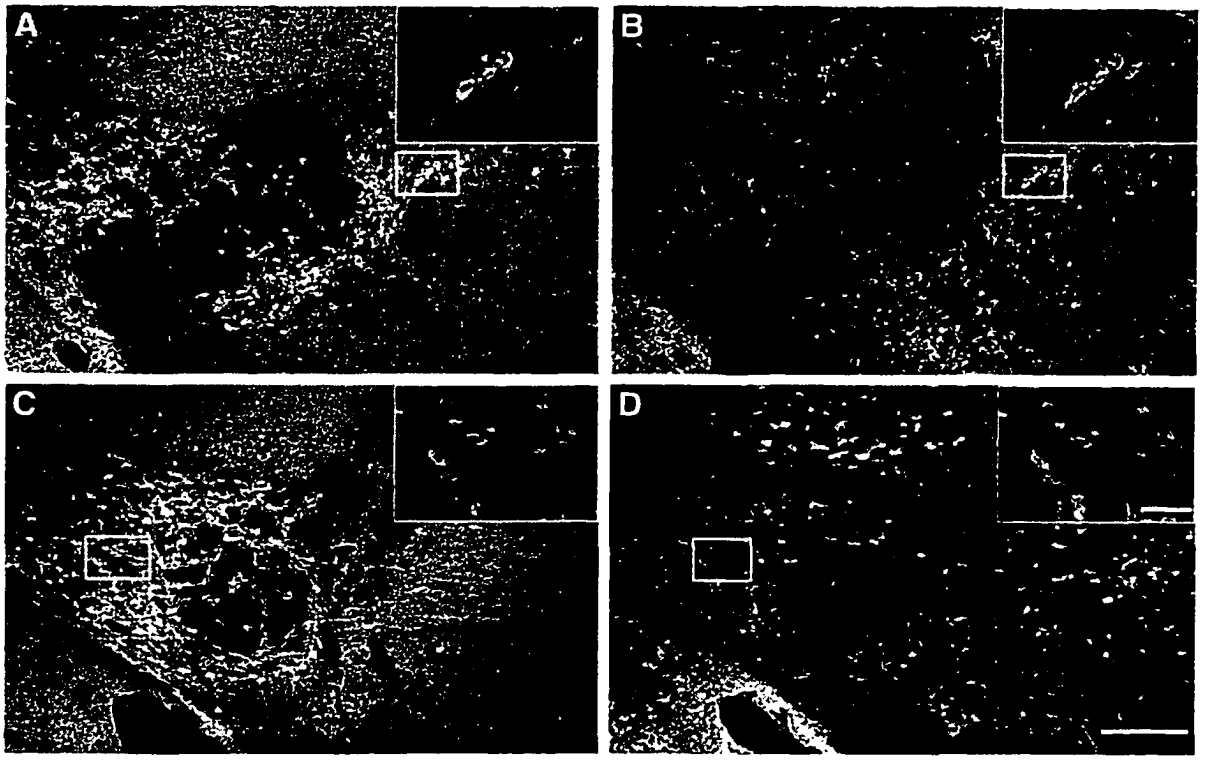

Figure 4.11 TH-immunoreactive cell bodies in the midbrain contain IL-1 receptor in saline and MPTP-treated young mice. *A and B,* Colocalization of TH-immunoreactive *(A)* and IL-1 receptor expression *(B)* in the SN and VTA of a saline-treated animal. Upper right panels are high-power magnification of the representative box outlined in the SN. *C and D,* Colocalization of TH-immunoreactive in the VTA *(C)* and IL-1 receptor expression *(D)* in a MPTP-treated animal. Scale bar, 200 μm; high-power magnification, 50 μm.

Discussion

Our data are consistent with observations that following MPTP treatment young mice show a significant recovery of dopaminergic nerve terminals in the dorsal striatum, whereas middle-aged mice do not (Ricaurte et al. 1986, 1987a; Date et al. 1990a). In this report, we demonstrated that spared dopaminergic neurons in the VTA were the predominant source of axonal afferents re-innervating the denervated striatum of young mice. Moreover, since significant recovery of dopamine uptake levels was observed in the ventral striatum of both age groups, this suggests that the lack of recovery in the dorsal striatum of middle-aged mice is not due to greater damage of the ventral striatum. We found, in fact, that the inability of older mice to support collateral axonal outgrowth after MPTP lesion is best correlated with the lack of sustained induction of IL-1α synthesis in the dorsal and ventral striatum. Young mice displayed a maximal 4-5 fold greater induction of IL-1α mRNA for an extended period of time in both the dorsal and ventral striatum compared to middle-aged mice. These findings strongly suggest that IL-1α plays an important role in MPTP-induced plasticity of dopaminergic neurons.

Role for IL-1α in Compensatory Dopaminergic Sprouting after MPTP

The time course of IL-1α induction seen in the dorsal and ventral striatum of young mice is directly correlated with the time period of MPTP-induced axonal changes. A progressive recovery of dopamine uptake levels and TH-immunoreactive fibers in the dorsal striatum of young mice was seen between 14 and 30 d post-lesion. Interestingly, our results show that IL-1α mRNA upregulation begins 4 and 14 d post-lesion in the dorsal striatum which was just prior to the detectable recovery period. Moreover, a similar induction of IL-1α synthesis was also observed in the ventral striatum of young mice, but lasting for much longer periods, up to 30 d post-lesion. However, in MPTP-treated middle-aged mice, the induction of IL-1α mRNA was only seen for the first 8 d in the dorsal

striatum, and not at all in the ventral striatum. The long-lasting upregulation of IL-1α found both in the dorsal and ventral striatum of young mice after MPTP suggests a role for IL-1α in eliciting, propagating or maintaining dopaminergic axonal sprouting processes.

Our demonstration of IL-1α expression in GFAP-immunoreactive hypertrophied astrocytes and striatal neurons indicates that these cells were the predominant source of increased IL-1α expression in the denervated striatum after MPTP. Since we did not find IL-1α expression in Mac-1-immunoreactive microglial cells, the induction of IL-1α in the denervated striatum after MPTP was unlikely to occur within activated microglia, but rather in astroglia. The astroglial production of IL-1 demonstrates a functional role of reactive astrocytes in repair processes but more importantly, extends their contribution to immunological processes in neurodegenerative disease. We found that striatal neurons also have the capacity to react to MPTP-induced injury by increased IL-1 expression. This expression in striatal neurons could be a consequence of MPTP effects to the denervated afferent projections to the lesioned area. Neurons have been implicated to function as a source of cytokines (Breder et al., 1988; Lechan et al., 1990; Tchelingerian et al., 1993); however, the role of cytokine production in neuronal cell types associated with neurodegenerative diseases has not been described. The present data open a new perspective on neuron-astroglia interactions associated with cytokine network during brain function and neurodegeneration.

A striking observation was that activation of IL-1α in response to MPTP is region specific. We found that although a glial reaction accompanied the dopaminergic cell loss in the midbrain, activation of IL-1α mRNA was not observed in the midbrain in either age group. In fact, middle-aged mice displayed a significant down-regulation of IL-1α mRNA in the midbrain after MPTP. This finding suggests that the midbrain elicits a different inflammatory reaction that is independent of IL-1α activation.

IL-1α effects on dopaminergic axonal sprouting after MPTP may not be indirectly

mediated *via* stimulating astroglial synthesis of dopaminergic neurotrophic factors as originally hypothesized. We have described here that the induction of IL-1α mRNA in young mice after MPTP was not accompanied by a secondary induction of astroglia-derived factors such as aFGF, bFGF or GDNF. This finding suggests that these dopaminergic neurotrophic factors were not critical for the lesion-induced plasticity of dopaminergic neurons. Our results are different from data reported by Leonard and colleages (1993) where both aFGF and bFGF mRNAs were found to increase in the denervated striatum at 1 week post-lesion. This difference in results could be a consequence of strain differences or the dosage of MPTP administration (Sundström et al., 1987). The upregulation of these factors were reported in Swiss-Webster mice which have been shown to be particularly resistant to MPTP toxicity while the C57BL/6 strain which we utilized appears to be more sensitive to MPTP-induced parkinsonism (Leonard et al. 1993).

In contrast to our original hypothesis that dopaminergic trophic factors could mediate IL-1α lesion-induced plasticity of dopaminergic neurons, IL-1α may act directly on dopaminergic cells. It has been suggested that IL-1 may act as a target derived neurotrophic factor since autoradiography studies have shown a distribution of IL-1 receptor binding in the SN (Farrar et al. 1987; Akaneya et al. 1995). Our double labeling experiments revealed that IL-1 receptor expression was found within TH-immunoreactive cell bodies lying both in the SN and VTA. We found that after MPTP, IL-1 receptor expression remained within TH-immunoreactive cell bodies particularly in the VTA and in some TH-immunoreactive cell bodies scattered in the SN. This finding provides further evidence for a direct affect of IL-1α on dopaminergic cells. Inhibiting the action of IL-1α by blocking its receptor through the administration of IL-1 receptor antagonist could be helpful to assess whether IL-1α is responsible for spontaneous dopaminergic sprouting in young mice after MPTP.

Aging and Neurodegeneration

The decline in IL-1α activation in middle-aged mice seems to depend on the nature of the injury considering that IL-1α was found to be induced to a greater extent after intrastriatal stab wound injury. This finding suggests that MPTP propagates a differential immune reaction as the brain ages which could lead to the attenuated recovery. Hence, understanding immunologic responses differing between young and aged brain is of great importance, especially regarding neural transplants for the treatment of PD. Grafting dopamine-producing tissues such as adrenal medullary chromaffin cells or fetal ventral mesencephalon into the striatum of animal models of PD and Parkinsonian patients has been shown to induce a compensatory sprouting response from residual host neurons and ameliorate some motor deficits (Bohn et al. 1987; Fiandaca et al. 1988; Lindvall 1989; Bankiewicz et al. 1991). However, such benefits are greatly diminished in aged animals (Date et al. 1989; Date et al. 1994). The mechanism of recovery has been difficult to interpret due to limited survival of implanted cells and tissues (Bankiewicz et al. 1988; Fiandaca et al. 1988). Interestingly, it was shown that trauma (cavitation) alone in the striatum promotes a similar functional recovery in hemiparkinsonian monkeys suggesting the presence of neurite-promoting factors as a result of the trauma (Plunkett et al., 1990). Inflammatory cells and reactive glia around the grafts have been implicated to mediate the transplantation-induced compensatory sprouting when intrastriatal implantation of microglia or activated leukocytes was shown to promote functional recovery (Wang et al. 1991; Ewing et al. 1992). Further investigation determined that a key mediator of inflammation in the brain, IL-1, was the potential component through which neural tranplants exert their growth-promoting effects in parkinsonian animals (Wang et al., 1994a). In light of these findings, here we report that while middle-aged mice showed a faster glial reaction compared to young mice after MPTP, the ability to induce IL-1α dramatically declined with age. Such age-related alterations in inflammatory reaction could explain the decrease in neurite-promoting activities and functional recovery following transplan-

tation of cells in parkinsonian aged animals.

In conclusion, the present experiments demonstrated that: 1) induction of IL-1α mRNA in the dorsal and ventral striatum is associated with compensatory dopaminergic sprouting, 2) induction of IL-1α mRNA is region specific and varies with age in response to MPTP, and 3) IL-1α neurotrophic actions on axonal sprouting may be directly acting on dopaminergic neurons. By investigating the regulation of IL-1, its interactions with other factors, substrates and ECM molecules could lead to a better understanding of factors attenuating neuronal plasticity in the aging brain and therapeutic approaches to treating neurodegenerative disorders.

CHAPTER 5

Molecular Heterogeneity of Astroglial Response to Different Injury-Induced Inflammatory Reactions

Abstract

Interleukin-1 (IL-1) released by activated microglia regulates inflammation and promotes reactive astrocytes following brain injury. IL-1 when exogenously applied can also exert neurotrophic activity on dopaminergic neurons indirectly by regulating the production of dopaminergic neurotrophic factor bFGF in astrocytes. Previously, we observed that increases in IL-1 production is associated with spontaneous recovery of dopaminergic nerve terminals in young mice damaged with neurotoxin MPTP. Surprisingly, no changes in bFGF were observed subsequent to the induction of IL-1 after MPTP. In comparison to a stab injury in which induction of IL-1α in microglia occurs within hours after injury, increases in IL-1α were not detected until several days after MPTP lesion and astrocytes were the predominant source of IL-1α production. This indicates that the cellular source and time course of IL-1α induction differs upon different type of insults. We postulated that when astrocytes are induced to express IL-1 in response to some types of injuries that this affects the subsequent expression of other astroglia-derived factors such as bFGF. To address this, we first examined the regulation of IL-1α upon bacterial endotoxin lipopolysaccharide (LPS), a potent inducer of IL-1 by microglia and after MPTP in glial cultures. We found that the onset of IL-1α synthesis differs upon LPS or MPTP stimulation. While an early induction of IL-1α mRNA was detected 2 d after LPS stimulation by microglia, increases in IL-1α mRNA were not detected until 8 d after MPTP treatment. Moreover, we found that microglia as well as astrocytes were responsible for IL-1α production following MPTP. We then examined whether astroglial induction of IL-1α after MPTP may prevent astroglial induction of bFGF. Interestingly, we found that bFGF mRNA was dramatically induced, coinciding with IL-1α induction after stimulation by LPS but not after MPTP. Furthermore, astrocytes failed to respond to IL-1α by inducing bFGF synthesis when IL-1α was added after MPTP suggesting that the astroglial response to MPTP is specific.

Introduction

A characteristic response to injury of the adult CNS is the inflammatory reaction. Glial cells play important regulatory and functional roles in immune responses. This reaction consists of activation of microglia and release of inflammatory cytokines such as IL-1 at the site of injury (Giulian and Lachman 1985; Giulian 1987). IL-1, in turn can stimulate reactive astrogliosis which is characterized by cell proliferation, hypertrophy and increased synthesis of the astrocyte-specific intermediate filament protein, GFAP (Giulian et al. 1988a; Eddleston and Mücke 1993). Reactive astrocytes are also capable of cytokine production, contributing to the immune responses after injury (Sawada et al. 1995; Ho and Blum 1998). The functional consequence of whether these induced changes are beneficial or detrimental to recovery has remained controversial. The formation of a glial scar by reactive astrocytes has been proposed as a major factor in inhibiting axonal growth (Reier et al. 1983; Liuzzi and Lasek 1987). However, increasing evidence has suggested that in certain experimental conditions, reactive astrocytes do not inhibit growth, but secrete growth factors that promote sprouting of axons after neuronal damage (Manthorpe et al. 1986; Gage et al. 1988; Giulian et al. 1993). The functional properties of reactive astrocytes, particularly their growth-promoting effects on damaged neurons are not fully understood. It may be that astroglial responses to injury depend on the brain area injured and on the nature of the injury.

Inflammatory reactions around grafts is a universal finding in implantation procedures and have been thought to underlie the functional and behaviorial improvement in animal models of PD since surgical trauma (cavitation) alone in the striatum of parkinsonian animals promotes significant recovery of dopaminergic axonal fibers (Hansen et al. 1988; Plunkett et al. 1990). This suggests the presence of neurite-promoting factors from the immune response. Further investigations demonstrated that a key mediator of inflammation in the brain, IL-1 was possibly the potential component through which neur-

al tranplants exert their growth-promoting effects in parkinsonian animals (Wang et al. 1994a). Striatal implantation of IL-1 in parkinsonian animals has been shown to promote behavioral recovery by stimulating compensatory axonal sprouting in the denervated striatum from remaining dopaminergic afferents (Wang et al. 1994a). Since it was thought that the effects of IL-1 on dopaminergic sprouting were indirect (Akaneya et al. 1995; Coelin et al. 1995), we investigated whether IL-1 induced the synthesis of astroglia-derived dopaminergic neurotrophic factors. We observed that IL-1 specifically induced the synthesis of bFGF, indicating that this factor may mediate the effects of IL-1 on dopaminergic sprouting (Ho and Blum 1997). Previously, we found that an induction of IL-1α was associated with spontaneous recovery of dopaminergic nerve terminals in young mice challenged with the neurotoxin, MPTP which selectively destroys dopaminergic neurons (Ho and Blum 1998). Surprisingly, no subsequent changes in bFGF synthesis were observed following the induction of IL-1 after MPTP treatment (Ho and Blum 1998). Unlike a stab injury in which IL- 1α is found to be upregulated within hours after injury in microglia, induction of IL-1α was not detected until several days after MPTP lesion and astrocytes were the main source of IL-1α production (Rostworowski et al. 1997; Ho and Blum 1998). Thus, this observation that bFGF induction did not follow the endogenous increase in IL-1α synthesis in the denervated striatum following MPTP raised the possibility that astroglial induction of IL-1 after MPTP may prevent astroglial induction of bFGF; for in the immune system, the activation of one cytokine may prevent the induction of another. Therefore, while IL-1 may stimulate astrocytes to transcribe bFGF, the astroglial induction of IL-1 transcription after MPTP may prevent the subsequent induction of bFGF. To address this, we first examined whether the regulation of IL-1α by bacterial endotoxin LPS, a potent inducer of IL-1α in microglia (Sawada et al. 1995; Kong et al. 1997) differs from MPTP stimulation in glial cultures. We then examined whether astroglial induction of IL-1 after MPTP may affect astroglial induction of bFGF. These studies may help to understand the molecular and functional heterogeneity among

population of astrocytes in response to different injury-induced inflammatory reactions.

Materials and Methods

Primary Striatal Mixed Glial Cultures. Postnatal d 0-3 C57BL/6 mice were decapitated, the striatum dissected and meninges were removed. The tissue were trypsinized (2.5 mg/ml; Gibco) and treated with DNase (10 µg/ml; Sigma) for 15 min at 37°C, followed by a series of washes and centrifugations in MEM/Ham's F-12 supplemented with 10% fetal bovine serum. The resulting homogenate (cell suspension) were filtered through a sterile 37 µm nylon mesh screen (Tetko). Cells were plated either at ~333,000 cells per 35 mm or ~$1x10^6$ cells per 60 mm poly-L-lysine coated culture dishes (100 µg/ml; Sigma). The cultures were incubated at 37°C in an atmosphere of 8% CO_2 and 95% air. *Treatment:* Cells at 11-13 d *in vitro* were placed into defined medium for 24 hours, and treated with 250 µM MPTP hydrochloride (dosage was based on experiments previously described from Di Monte et al. 1992; Research Biochemicals International) or 1 µg/ml LPS (Kong et al. 1997; Sigma) or vehicle (phosphate saline; Gibco) with n=4-5 for each group.

Isolation of cDNA Clones. The IL-1α cDNA clone was generously provided by Dr. A. Shaw of Glaxo from which a 400 base pair fragment was subcloned into vector pGEM. The bFGF cDNA clone was generously provided by Dr. S. Shimasaki from which a 479 base pair fragment corresponding to nucleotides 525-1004 was subcloned into vector Bluescript/SK+.

Nuclease Protection Assay. Unlabeled sense and high specific activity (~$1x10^9$ cpm/µg) ^{32}P-labeled antisense RNA were transcibed according to the manufacturer's recommendations (NEN). A standard curve with increasing amounts (0-10 µl of a 100 fg/ µl (+)

strand) of sense RNA was used for quantification. The standard and known amounts of cytoplasmic RNA isolated were hybridized with ~200 pg of antisense ^{32}P-labeled RNA probe. The samples were heat-denatured at 85°C for 5 min and hybridized overnight at 45°C. After hybridization, the samples were treated with RNase A (5 µg/ml) and RNase T1 (2 µg/m) for 1 h at 30°C, followed by proteinase K (0.167 mg/m) digestion at 37°C for 15 min. Samples were phenol:chloroform extracted, precipitated, resuspended in 1xTE, and electrophoresed on a nondenaturing 5% acrylamide gel. Gels were dried, and quantified by phosphor image analysis. The results were determined by linear regression analysis from the standard curve and presented as amoles mRNA/µg total RNA.

Double Immunolabeling. Cultures treated with LPS or MPTP at 2 and 8 d respectively along with saline-treated control were processed for combined fluorescence immunocyto-chemistry for IL-1α (rabbit polyclonal anti-mouse IL-1α, 1:400; Genzyme) and GFAP (mouse monoclonal anti-GFAP, 1:50; Boehringer Mannheim), and IL-1α and Mac-1 (rat monoclonal anti-Mac-1, 1:50; Boehringer Mannheim). Cultures were fixed with ice-cold 4% paraformaldehyde for 30 min followed by a wash in 0.15 M phosphate buffer, pH 7.2 (PBS) for 5 min. Cultures were incubated in blocking buffer (0.1% saponin, 3% goat serum in PBS) for 30 min, followed by an overnight incubation of primary antibody in blocking buffer at 4°C. IL-1α was visualized by incubation with anti-rabbit IgG directly conjugated to fluorescein (1:200; Vector Laboratories) while GFAP, and Mac-1 immunoreactivity was reacted to appropriate biotinylated secondary antibodies; anti-mouse IgG to detect GFAP (1:200; Amersham) and anti-rat IgG to detect Mac-1 (1:200; Amersham) for 2 h followed by incubation in streptavidin conjugated to rhodamine (1:500; Molecular Probes). After processing, cultures were coverslipped in Permafluor mounting medium.

Results

Production of IL-1α mRNA After LPS or MPTP Treatment in Primary Striatal Glial Cultures

Microglia in culture can be stimulated with LPS to produce IL-1α (Kong et al. 1997). We have previously shown that astrocytes were the predominant source of IL-1α production following MPTP-induced lesion in the denervated striatum of young mice (Ho and Blum 1998). To examine whether glial cultures respond differently to induce IL-1α synthesis upon different immune insults, cultures were treated with either LPS (1 μg/ml) for 2 d or MPTP (250 μM) at various lengths of time in culture. We observed that while LPS produced a 3-fold induction of IL-1α mRNA after 2 d exposure, induction of IL-1α mRNA was not observed until 8 d after MPTP treatment compared to control-treated cultures (Fig. 5.1). Moreover, MPTP-induced IL-1α was 5-fold greater than LPS-treated cultures. These results indicate that the time course of IL-1α induction differs upon MPTP or LPS stimulation.

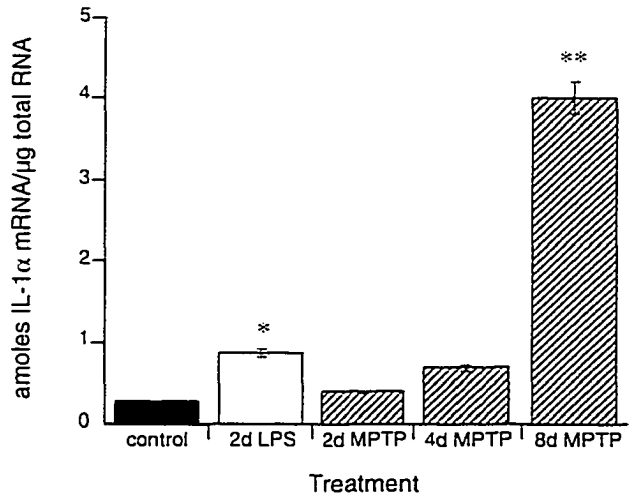

Figure 5.1 Quantitative analysis of IL-1α mRNA after LPS and MPTP treatment in primary striatal glial cultures. Cells at 11-13 days *in vitro* were placed into defined medium for 24 h and treated with LPS (1 μg/ml), MPTP (250 μM) or control. Values represent the mean ± SEM for n=4-5 for each time group. Levels of statistical significance were set to *P<0.05, **P<0.001 which indicates difference from control vehicle.

To identify the cellular source of IL-1α in our glial cultures, cultures were exposed to LPS or MPTP for 2 and 8 d respectively when IL-1α mRNA was induced and processed for double labeling immunocytochemistry using antibodies to IL-1α in combination with either Mac-1, a microglia marker specific for the mouse (Springer et al., 1979; Beller et al., 1982) or GFAP. In control-treated cultures, while more than 90% of the cells were labeled with GFAP, localization of IL-1α was only found in a small population of Mac-1-immunoreactive microglial cells (Fig. 5.2*A,B*). Following LPS stimulation, we observed an increased in the number of Mac-1 immunoreactive cells and localization of IL-1α in Mac-1-immunoreactive microglial cells were significantly higher than those in control cultures (Fig. 5.2*C,D*). However, 8 d after MPTP treatment, while significant loss of cell viability was observed compared to control-treated cultures (Fig. 5.3*A-D*), we found induction of IL-1α was highly expressed in both Mac-1 and GFAP-immunoreactive cells (Fig. 5.3*E-H*). These results indicate that while microglial cells were the main source of increased IL-1α following LPS stimulation, microglial as well as astroglial cells were the cellular source of increased IL-1α after MPTP.

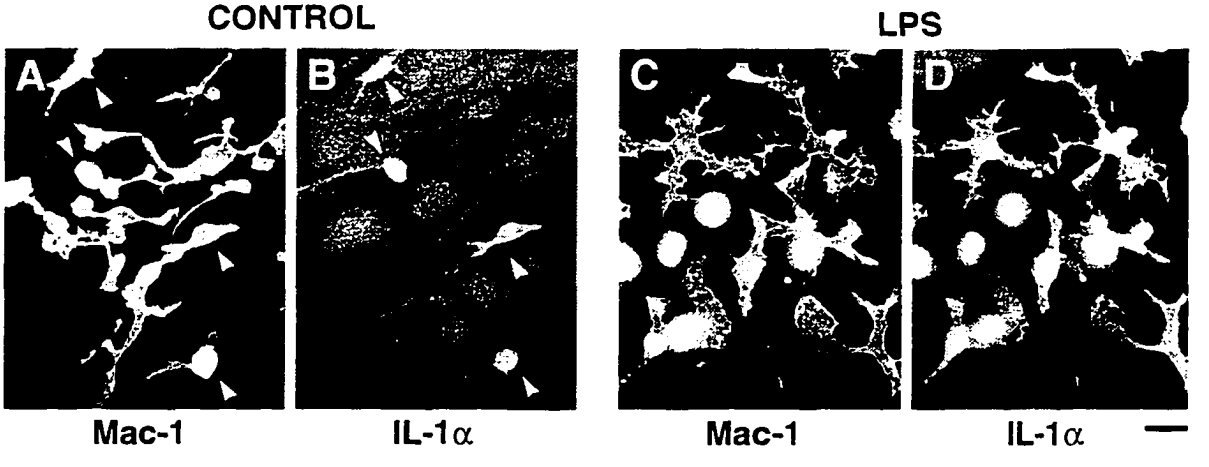

Figure 5.2 Stimulation of IL-1α production by Mac-1 immunoreactive cells at 2 d after LPS treatment in primary striatal glial cultures. *A and B,* Colocalization of Mac-1 immunoreactive *(A)* and IL-1α expression *(B)* in a control-treated glial culture. C and D, Colocalization of Mac-1 immunoreactive *(C)* and IL-1α expression *(D)* in a LPS-treated glial culture. Scale bar, 20 μm.

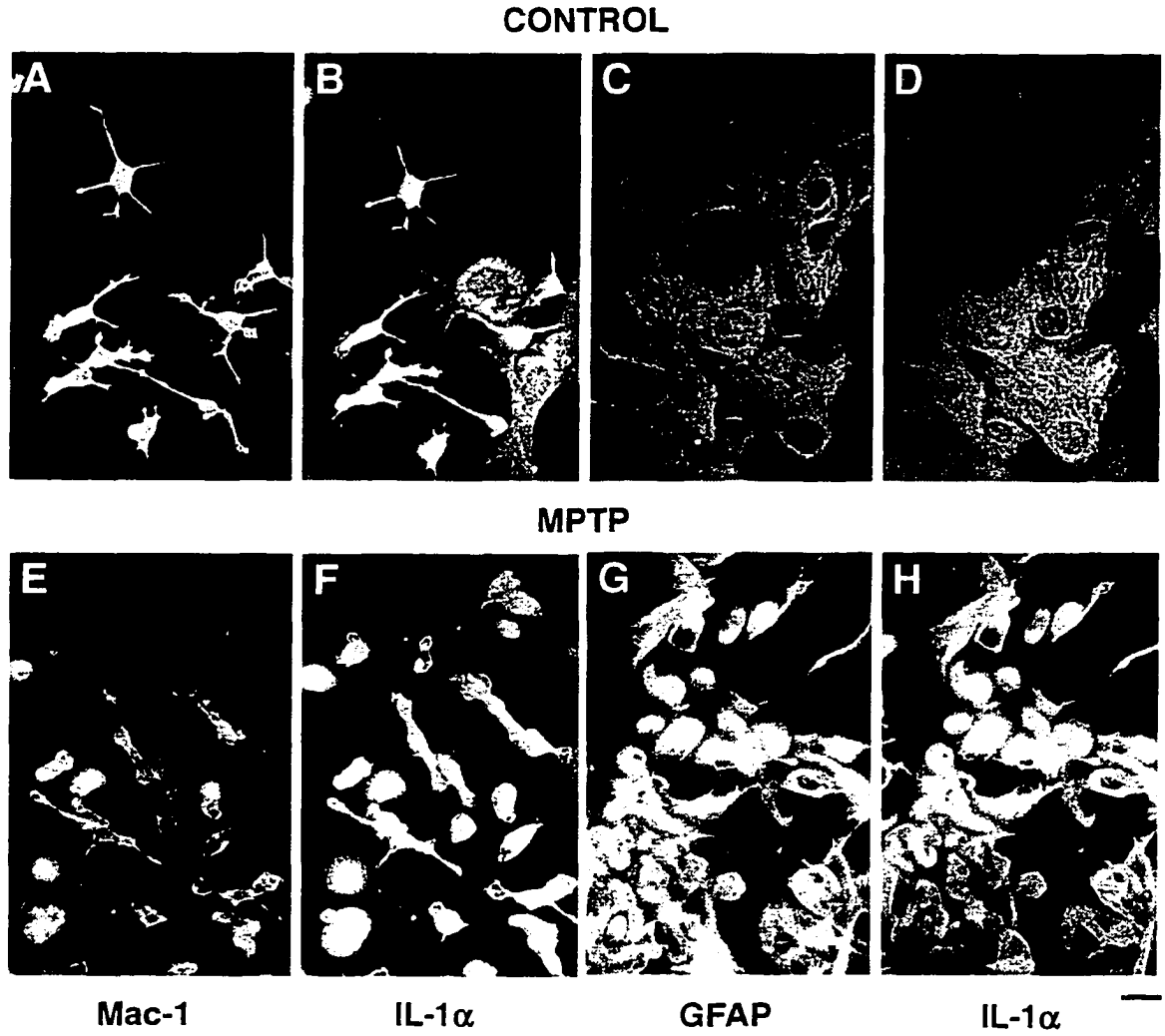

Figure 5.3 Stimulation of IL-1α production by Mac-1 and GFAP immunoreactive cells at 8 d after MPTP treatment in primary striatal glial cultures. *A and B,* Colocalization of Mac-1 immunoreactive *(A)* and IL-1α expression *(B)* in a control-treated glial culture. *C and D,* Colocalization of GFAP immunoreactive *(C)* and IL-1α expression *(D)* in a control-treated glial culture. *E and F,* Colocalization of Mac-1 immunoreactive *(E)* and IL-1α expression *(F)* in a MPTP-treated glial culture. *G and H,* Colocalization of GFAP immunoreactive *(G)* and IL-1α expression *(H)* in a MPTP-treated glial culture. Scale bar, 20 μm.

Selective Induction of bFGF mRNA After LPS But Not After MPTP Treatment in Primary Striatal Glial Cultures

To examine whether astroglia-derived IL-1α affect the response of astrocytes to induce bFGF in glial cultures, cultures were treated with either LPS (1 μg/ml) for 2 d or MPTP (250 μM) at various lengths of time in culture. We observed that LPS induced a

2.7-fold increase in bFGF mRNA after 2 d exposure compared to control-treated cultures, coinciding with IL-1α induction (Fig. 5.4). In contrast, changes in bFGF mRNA were not observed following MPTP treatment compared to control, even at 8 d when IL-1α mRNA was shown to be greatly increased. In fact, we found that bFGF mRNA was significantly reduced in MPTP-treated cultures compared to control. We next wanted to examine whether astrocytes could respond to the exogenous application of IL-1α by increasing bFGF mRNA if IL-1α was added at 8 d following MPTP treatment. We observed that bFGF mRNA remained significantly reduced even with the addition of IL-1α. These results suggest that induction of IL-1α and the subsequent synthesis of bFGF by striatal astrocytes is specific and not reversible.

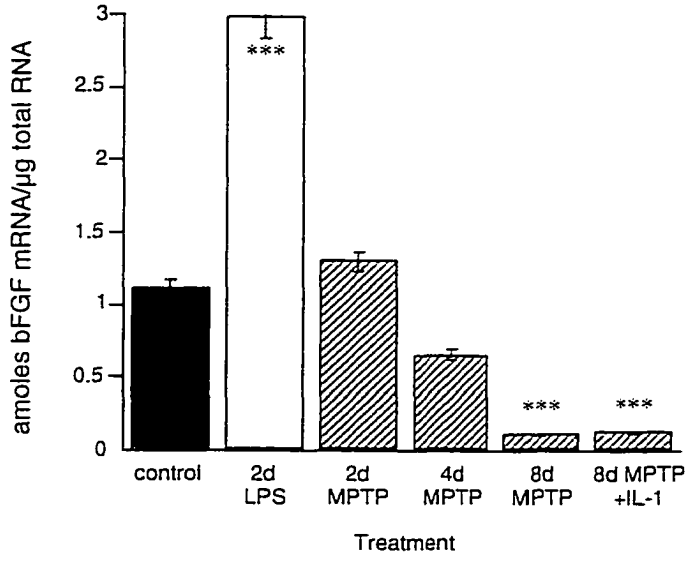

Figure 5.4 Quantitative analysis of bFGF mRNA after LPS and MPTP treatment in primary striatal glial cultures. Cells at 11-13 days *in vitro* were placed into defined medium for 24 h and treated with LPS (1 µg/ml), MPTP (250 µM) or control. Values represent the mean ± SEM for n=4-5 for each time group. Levels of statistical significance were set to ***P<0.001 which indicates difference from control vehicle.

Discussion

The present study characterizes the molecular profile of cytokine expression in cultured glial cells in response to different injury-induced inflammatory reactions. Glia exposed to LPS elicited a more rapid induction of IL-1α synthesis in contrast to a slower onset of IL-1α production after MPTP treatment. Increases in IL-1α mRNA were 5-fold greater after MPTP compared with LPS stimulation. Moreover, while microglial cells were

the predominant cellular source for IL-1α production following LPS, both microglia and astrocytes begin to express IL-1α after MPTP treatment. We found that increases of bFGF synthesis coincided with IL-1α induction after LPS, but not after MPTP treatment. The specificity in the onset of IL-1α induction and the selectivity in the regulation of bFGF by astrocytes in response to LPS or MPTP suggests that different injury-induced inflammatory reactions can alter the molecular profile and responsiveness of astrocytes.

The cascade of molecular events suggested by these results indicate that different immune activators can differentially affect the ability of glial cells to induce IL-1 expression which may ultimately affect the subsequent expression of astroglia-derived factors such as bFGF. We observed that LPS stimulated the proliferation of microglia and induce the synthesis of IL-1α. IL-1α may, in turn act on astrocytes to stimulate the synthesis of bFGF. However, this cytokine-neurotrophic cascade changes when glial cells are exposed to MPTP. It appears that the neurotoxic challenge of MPTP to glial cells is inducing an increase in IL-1α production by astrocytes. Astroglial induction of IL-1α transcription may represent a shift in the cytokine cassette expressed by astrocytes which may preclude the synthesis of bFGF by astrocytes.

In peripheral tissues, immunological reactions can be clearly defined by the nature of cytokine profile (for review, see Abbas et al. 1996). Immune cells upon LPS or other immune-activating signals can induce or inhibit different cytokines which can significantly alter the outcome of the immune responses. In the CNS, such cytokine profiles in modulating different immune responses have not been fully explored. Recent studies have shown that microglia under natural culture conditions express specific immune suppressive cytokine profiles, and upon LPS stimulation, a shift to a proinflammatory cytokine profile prevails (Xiao et al. 1996). This shift in the cytokine expression cassette may modulate regulatory mechanisms to control microglial cytotoxic and phagocytic activity and further act to orient astrocyte function after injury (Xiao et al. 1996; Zahn et al. 1997). However, it remains unknown what signals or their source within the brain

which determines the specificity of the astroglial response to injury. Since microglial cells provide the CNS with its first line of defense against neuronal damage by initiating inflammatory reactions mediated by cytokines to exhibit phagocytic activity and removing degenerating elements (for review, see Gebicke-Haerter et al. 1996; Moore and Thanos 1996), possibly the different cytokine cassettes elicited by microglia may determine the profile that astrocytes will produce. For astroglial cells have been shown to contribute to the intermediate/late responses expanding the immune reactions and secreting growth factors to support neuronal survival and axonal outgrowth after injury (Gage et al. 1988; Giulian et al. 1993; Sawada et al. 1995). In light of this fact, it is possible that the early induction in IL-1α by LPS-stimulated microglial cells most likely reflects the role of IL-1α in regulating the acute-phase inflammatory response and promoting astroglial proliferation. In comparison, the delayed changes in IL-1α synthesis exhibited by astroglial cells after MPTP may support the role for IL-1 as a neurotrophic factor in promoting neuronal survival and sprouting of dopaminergic axonal fibers rather than a regulator of immune responses. Nevertheless, what signals and whether they are released by microglia to regulate the astroglial immune response leading to the expression of IL-1 is not known. A number of cytokines released by activated microglia have been shown to have diverse actions on astrocytes. Previous studies reported that microglia-derived interferon-γ, IL-1, IL-2, IL-6 and tumor necrosis factor-α (TNF-α) can modulate astroglial reactivity, and induce the expression of major histocompatibility complex antigens and adhesion molecules involved in immune responses on astrocytes *in vitro* (Fontana et al. 1989; Liu et al. 1989; Hertz et al. 1990). It may be that certain cytokine profiles participate in determining the nature and specificity of the astroglial immune response, thereby influencing the functional properties of reactive astrocytes to promote or inhibit sprouting of axons after neuronal damage. Perhaps, elucidating the molecular cytokine profile regulating microglia-astroglia functions may provide a greater understanding in the mechanism underlying injury-induced reparative processes.

CHAPTER 6

Expression of Chondroitin-Sulfate Proteoglycan Associated With Reduction of Compensatory Dopaminergic Sprouting in the Striatum of Middle-Aged Mice After MPTP: Role of TGF-β1

Abstract

Dopaminergic denervation to the adult striatum after MPTP treatment leads to compensatory sprouting of remaining dopaminergic afferents. In middle-aged mice, such recovery is dramatically reduced. ECM molecules secreted by reactive glia following brain lesions can contribute to the failure of axonal outgrowth. Here, we investigate whether the presence of inhibitory and/or lack of growth-promoting ECM molecules is associated with the inability of middle-aged mice to support dopaminergic sprouting following MPTP. Our results demonstrate an age-associated increase in growth-inhibitory ECM molecule CSPG immunoreactivity following MPTP. While MPTP caused a transient localized increase of CSPG in the denervated striatum of young mice, a broad staining pattern of CSPG remained significantly elevated for several weeks in middle-aged mice. In contrast, the growth-promoting molecule fibronectin was transiently reduced in both age groups, while tenascin, a molecule that has both inhibitory and facilitatory effects on axonal outgrowth, did not change in the denervated striatum in either age group. Interestingly, we found that TGF-β1, a cytokine that can induce the synthesis of neurotrophic factors and ECM molecules, increased to a greater extent in the denervated striatum of young mice compared to middle-aged mice after MPTP. When TGF-β1 was injected into the brain, an induction of CSPG immunoreactivity in the striatum of both age groups was detected; however, changes in fibronectin and tenascin were not observed. These results suggest that the attenuated sprouting response exhibited by middle-aged mice is most likely reflected by the sustained induction of CSPG immunoreactivity in the denervated striatum.

Introduction

ECM molecules play an important role in regulating growth-promoting and inhibitory functions during development and after neuronal injury (for review, see Brodkey et al. 1993). ECM molecules such as fibronectin promote axonal growth while CSPGs inhibit neurite outgrowth (Baron-Van Evercooren et al. 1982; Snow et al. 1990a). However, an ECM molecule tenascin demonstrates both promoting and inhibitory properties for modulating axonal growth (Faissner and Kruse 1990; Lochter et al. 1991). Most ECM molecules are developmentally regulated and appear during critical periods of development (Chiquet 1989; Snow et al. 1990b; Brusno-Bechtold et al. 1992). They have distinct spatial and temporal expression that help guide neurons and their processes to their appropriate targets (Steindler et al. 1989a,b; Crossin et al. 1990). In the adult brain, ECM molecules are detected at very low levels; however, these molecules are re-expressed after traumatic injury and can alter the facilitation or failure for compensatory sprouting processes (Egan and Vijayan 1991; McKeon et al. 1991; Laywell et al. 1992).

After an experimental brain lesion, young animals have a greater ability to promote and support compensatory sprouting than older animals. Studies have shown that young mice lesioned with MPTP, a neurotoxin that selectively destroys SN dopaminergic neurons, can recover striatal dopaminergic nerve terminals whereas such recovery is significantly reduced in the aging brain (Ricaurte et al. 1986, 1987a,b; Date et al. 1990a). It may be that the presence of potent inhibitory molecules and/or lack of growth-promoting molecules reduces the capacity to promote recovery in the aging brain. Therefore, in the present study, the expression of ECM molecules CSPG, fibronectin and tenascin in the denervated striatum of young and middle-aged mice following MPTP was examined.

Factors that can regulate the expression of ECM molecules after neuronal injury are important in understanding reparative processes in the adult CNS. In peripheral tissues, TGF-β1 has been shown to play an important role in inflammation and tissue repair

after injury by promoting cell migration, cell proliferation and inducing the synthesis of ECM components (Mustoe et al. 1987; Roberts and Sporn 1990). In the adult CNS, TGF-β1 mRNA is expressed at low levels, found mainly in the meninges and choroid plexus (Heine et al. 1987; Thompson et al. 1989; Unsicker et al. 1991). However, TGF-β1 mRNA rapidly increases in the adult rodent brain in response to brain injury (Lindholm et al. 1992; Logan et al. 1992; Pasinetti et al. 1993). The upregulation of TGF-β1 in the lesioned brain has been suggested to mediate neuronal repair processes by controlling astrocyte proliferation and stimulating neurotrophic factor synthesis such as NGF and bFGF (Labourdette et al. 1990; Lindholm et al. 1990; Vergelli et al. 1995). Moreover, TGF-β1 has been found to induce the synthesis of certain ECM molecules such as fibronectin and tenascin suggesting that TGF-β1 may be involved in remodeling of the extracellular matrices after brain injury (Pearson et al. 1988; Baghdassarian et al. 1993; Pasinetti et al. 1993).

In order to understand the role of TGF-β1 in relation to the age-associated decline in spontaneous recovery after MPTP-induced injury, we examined the physiological changes of TGF-β1 synthesis in the denervated striatum of young and middle-aged mice. Furthermore, we investigated whether the effects of TGF-β1 on the expression of ECM molecules are altered with age, by intraventricular administration of TGF-β1 in young and middle-aged mice. Characterization of TGF-β1 synthesis in the denervated striatum after injury and interactions of TGF-β1 with ECM molecules could provide insights in facilitating plasticity of dopaminergic neurons in the aging brain.

Materials and Methods

Drug Administration. Male C57BL/6 mice (Harlan Sprague Dawley) of two different age groups were used: 8 weeks (young) and 8 months of age (middle-aged). MPTP hydrochloride (Research Biochemicals Internationa) was administered subcutaneously.

Young mice received a single dose of 55 mg/kg and middle-aged mice received a single dose of 40 mg/kg of MPTP. These doses were selected based on previous titration studies which produced comparable initial depletions of dopamine uptake in the striatum of young and older mice (Ho and Blum, 1998). Age-matched controls received saline. Animals were sacrificed at 4, 8, 14, 21 and 30 days post-lesion along with their age-matched controls (n=4-5/group).

Animals and Surgical Procedures. Male C57BL6 mice (Harlan Sprague Dawley) at 8 weeks of age (young) and 8-12 months of age (middle-aged) were anesthetized with 287.5 mg/kg avertin (stock of 12.5 mg/ml 2,2,2-tribromoethanol; Aldrich), injected intraperitoneally. The mice were placed in a stereotaxic device (David Kopf Instruments). For intrastriatal stab wound: a burr hole was drilled on the right side of the skull and a 1 µl Hamiliton syringe was placed into the dorsal striatum. Coordinates were located 2.7 mm caudal to the frontal nasal suture, 2.0 mm lateral from the midline suture and 2.5 mm from the surface of the brain. The needle was left in place for 5 min. Animals were sacrificed at 4, and 8 days post-lesion (n=4-5/group). For intraventricular injection of TGF-β1: stereotaxic injections of TGF-β1 (10 ng; Genzyme) or vehicle (PBS) in a final volume of 0.5 µl was injected into the right lateral ventricle using a 1 µl Hamilton syringe. Coordinates were located 2.5 mm caudal to the frontal nasal suture, 1.0 mm lateral from the midline suture and 2.0 mm from the surface of the brain. The needle was was left in place for 5 min and withdrawn slowly to prevent backflow of solution along the needle track. Animals were sacrificed at 24 h post-injection (n=4-5/group).

Immunocytochemistry. Animals were anesthetized and sacrificed by intracardiac perfused with 1% paraformaldehyde in 0.15 M phosphate buffer, pH 7.2 (PBS) followed by 4% paraformaldehyde. Brains were postfixed for 5 h at 4°C and cryoprotected with 30% sucrose. Thirty µm coronal sections were cut on a cryostat and processed for immuno-

cytochemistry. Sections were incubated in blocking buffer (0.3% triton X-100, 3% goat serum in PBS) for 30 min, followed by an overnight incubation of primary antibodies to chondroitin sulfate types A and C (but not B, dermatan sulfate) proteoglycan (mouse monoclonal anti-CS-56; 1:200, Sigma), fibronectin (rabbit polyclonal anti-FN; 1:400, Sigma), tenascin (mouse monoclonal anti-mTn-12; 1:200, Sigma), or TH (rabbit polyclonal anti-TH; 1:500, Pelfreeze Biologicals) in blocking buffer at 4°C. The next day, sections were washed 3x10 min with PBS and incubated in biotinylated anti-mouse IgM to detect CS-56 (1:200; Amersham), rabbit IgG to detect fibronectin and TH (1:200; Vector Laboratories) or mouse IgG to detect tenascin (1:200; Vector Laboratories) for 2 h followed by washes. Sections were then incubated in ExtraAvidin (1:200; Sigma) for 1 h and washed. Sections were processed with 0.05% 3,3'-diaminobenzidine tetrachloride/0.003% H_2O_2.

Western Blot. Striatal tissue from saline and MPTP-treated mice of both age groups were homogenized (wt:vol) in 50 mM Tris buffer, pH 8, 150 mM NaCl, 2 mM EDTA, 2 µg/ml leupeptin, 0.5% aprotinin, 1 mM PMSF, 10 µg/ml pepstatin A. Homogenized tissue was spinned at 60,000 rpm for 30 min at 4°C and the supernatant was saved. Protein concentrations were determined by Bradford assay. Ten µg of protein were treated with 0.1 U/ml chondroitinase ABC (ICN Biomedicals) at 37°C for 1 h. Samples were dried and resuspended in 20 µl of Laemmli's sample buffer and subjected to a 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose paper and the blots were incubated in blocking solution containing 5% dry milk (carnation brand) in Tris-buffered saline (TBS: 20 mM Tris-HCl pH 7.5, 0.5 M NaCl) for 2 h at 37°C. Monoclonal antibodies against chondroitin-4-sulfate proteoglycans (CS-4-PG) and chondroitin-6-sulfate proteoglycans (CS-6-PG) of chondroitase ABC-treated proteoglycans were used (ICN Biomedicals). Mouse anti-CS-4-PG diluted at 1:400 and anti CS-6-PG diluted at 1:200 in 3% dry milk/TBS was incubated overnight at 4°C and washed 4x10

min with 0.05% Tween-20/TBS. The blots were then incubated with [^{125}I]-iodinated-labeled anti-mouse IgG (Amersham) and washed as above. Blots were quantified by phosphor image analysis.

Isolation of cDNA Clones. The TGF-β1 cDNA clone was generously provided by Dr. H.L. Moses from which a 527 base pair fragment corresponding to nucleotides 868-1395 was subcloned into vector Bluescript. For quantitative nuclease protection assay, unlabeled sense and high specific activity (~1x10^9 cpm/μg) ^{32}P-labeled antisense RNA were transcribed according to the manufacturer's recommendations. A standard curve with increasing amounts (0-10 μl of a 100 fg/ μl (+) strand) of sense RNA was used for quantification. The standard and known amounts of cytoplasmic RNA isolated were hybridized with ~200 pg of antisense ^{32}P-labeled RNA probe.

Statistical Analysis. Significant differences in TGF-β1 mRNA levels between control and MPTP treatment groups were analyzed using analysis of variance (ANOVA) followed by Fisher's protected least significant difference post hoc analysis. The level of significance was set at $p<0.05$.

Results

Age-associated Reduction in Spontaneous Recovery of Dopaminergic Terminals After MPTP

We have previously performed a detailed biochemical and morphological analysis the time course of dopaminergic loss and recovery of axonal terminals in young but not middle-aged mice following MPTP-induced lesion (Ho and Blum, 1998). We found that young mice have the capacity to promote sprouting of dopaminergic axonal terminals in the denervated striatum after MPTP within a month while middle-aged mice do not

(Ho and Blum, 1998). To illustrate the age-associated reduction in spontaneous recovery of dopaminergic terminals after MPTP treatment, we evaluated TH immunoreactivity in the denervated striatum of young and middle-aged mice. We found, in accordance with our previous observations that TH-immunoreactivity was markedly reduced in the dorsal striatum of both young and middle-aged mice at 8 d after MPTP compared to age-matched control (Fig. 6.1*A,B,D,E*). At 30 d post-lesion, young mice displayed a consistent recovery of TH-immunoreactive fibers in the dorsal striatum (Fig. 6.1*C*). In contrast,

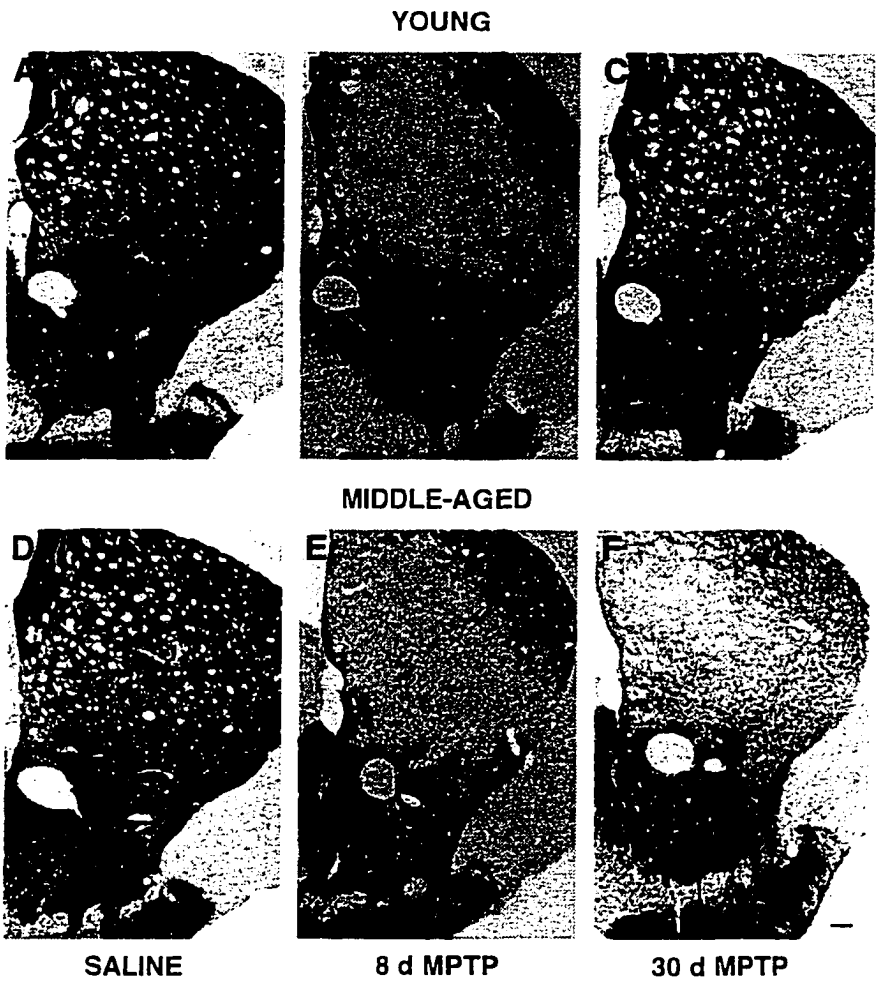

Figure 6.1 TH immunocytochemistry in the striatum of young *(A-C)* and middle-aged mice *(D-F)* at 8, and 30 d following saline and MPTP treatment . MPTP caused a marked disappearance of TH immunoreactive fibers in the dorsal striatum of both age groups compared to saline-treated mice, however recovery of TH immunoreactive fibers were observed at 30 d after MPTP in young mice *(C)*, compared to middle-aged mice which did not show such apparent recovery *(F)*. Scale bar, 200 µm.

middle-aged mice did not show such recovery, suggesting an age-associated decline in the ability to promote spontaneous recovery (Fig. 6.1*F*).

Expression of ECM Molecules in the Denervated Striatum After MPTP

To examine whether the presence of putative growth-inhibitory molecules or the lack of growth-promoting molecules may influence the attenuated sprouting response in the denervated striatum of middle-aged mice after MPTP, immunolabeling for CSPG, fibronectin and tenascin were assessed in the denervated striatum of young and middle-aged mice after MPTP.

CSPG. Increased labeling for CSPG immunoreactivity was apparent in the denervated striatum of both age groups at 8 days post-lesion compared to age-matched control (Fig. 6.2*A,B,D,E*). We observed that young mice displayed a localized CSPG staining in the denervated striatum, while a more intense and generalized increase was observed throughout the denervated striatum of middle-aged mice following MPTP (Fig. 6.2*B,E*). After 30 days post-lesion, no significant difference from control was observed in the denervated striatum of young mice (Fig. 6.2*C*). However, CSPG immunoreactivity remained increased at 30 days post-lesion in the denervated striatum of middle-aged mice compared to control (Fig. 6.2*F*).

Since the antibody to CSPG recognizes both chondroitin-sulfate-4 (CS-4) and chondroitin-sulfate-6 (CS-6) glycosaminoglycan moieties of a variety of proteoglycans, immunoblots specific for CS-4, or CS-6 proteoglycans were performed. A selective increase in CS-6 (Fig. 6.3), but not CS-4 (data not shown) was observed in the denervated striatum of both age groups at 8 d post-lesion compared to age-matched control. Immunoblots quantitated by phosphor image analysis revealed a 2.8-fold increase of ~245 kD core proteoglycan (indicated by arrowhead) in the denervated striatum of young, but not middle-aged mice following MPTP, which most probably corresponds to a 1D1 CS-6-PG proteoglycan reported by Rauch and colleages (1991), a form only expressed

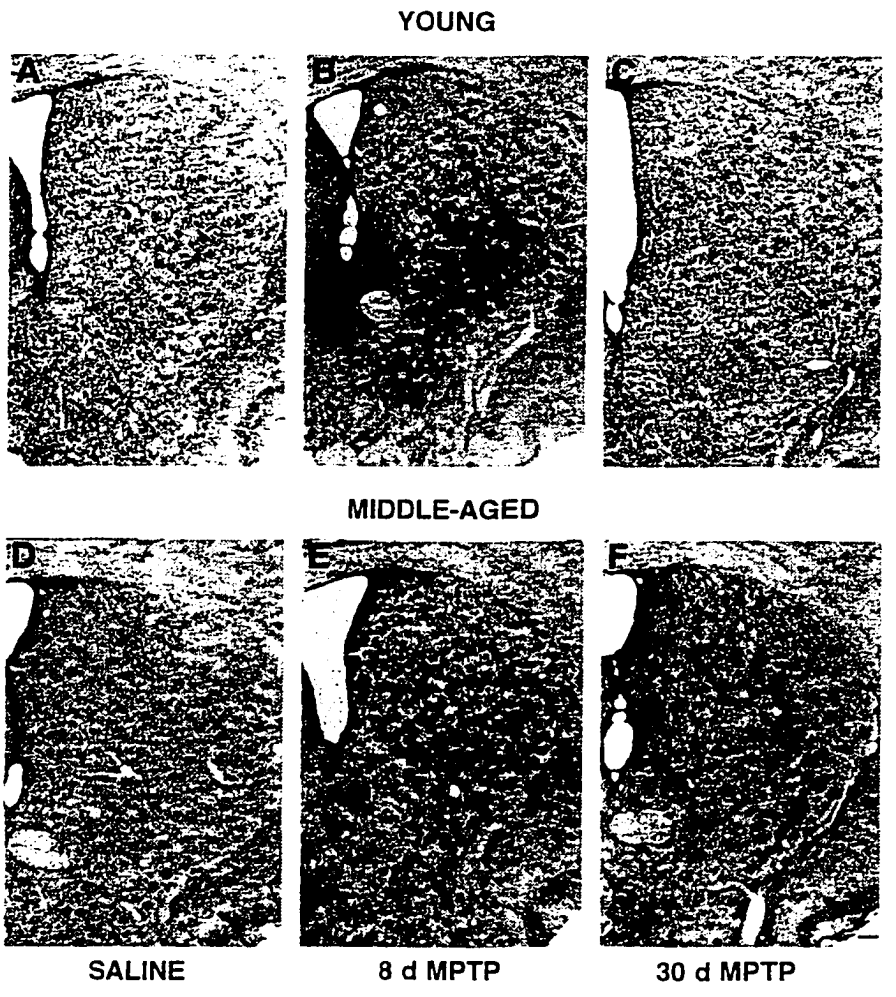

Figure 6.2 CSPG immunocytochemistry in the striatum of young *(A-C)* and middle-aged mice *(D-F)* at 8, and 30 d following saline and MPTP treatment. MPTP caused a transient and localized increase in CSPG immunoreactivity in the denervated striatum of young mice at 8 d *(B)*; by 30 d *(C)*, no significant differences from control were observed. Meanwhile, middle-aged mice displayed a more intense and generalized increase throughout the denervated striatum at 8d *(E)* which remained increased, up to 30 d post-lesion *(F)*. Scale bar, 200 μm.

middle-aged mice did not show such recovery, suggesting an age-associated decline in the ability to promote spontaneous recovery (Fig. 6.1*F*).

Expression of ECM Molecules in the Denervated Striatum After MPTP

To examine whether the presence of putative growth-inhibitory molecules or the lack of growth-promoting molecules may influence the attenuated sprouting response in

Figure 6.3 Western blot analysis of CS-6-PG production (10 µg/lane) isolated from the striatum of young *(lanes A-D)* and middle-aged mice *(lanes E-H)* at 8 d following saline *(lanes A,B,E,F)* and MPTP treatment *(lanes C,D,G,H)*. *Lanes A, C, E, G,* untreated proteoglycans; *lanes B, D, F, H,* chondroitinase-treated proteoglycans prior to extraction for CS-6-PG immunoblots. Samples were resolved on SDS-PAGE 6% acrylamide gel, transferred to nitrocellulose paper, and probed with CS-6-PG. Antibody binding was visualized using [^{125}I]-iodinated-labeled anti-mouse IgG and quantitated by phosphor image analysis.

the denervated striatum of middle-aged mice after MPTP, immunolabeling for CSPG, fibronectin and tenascin were assessed in the denervated striatum of young and middle-aged mice after MPTP.

CSPG. Increased labeling for CSPG immunoreactivity was apparent in the denervated striatum of both age groups at 8 days post-lesion compared to age-matched control (Fig. 6.2*A,B,D,E*). We observed that young mice displayed a localized CSPG staining in the denervated striatum, while a more intense and generalized increase was observed throughout the denervated striatum of middle-aged mice following MPTP (Fig. 6.2*B,E*). After 30 days post-lesion, no significant difference from control was observed in the denervated striatum of young mice (Fig. 6.2*C*). However, CSPG immunoreactivity remained increased at 30 days post-lesion in the denervated striatum of middle-aged mice compared to control (Fig. 6.2*F*).

Since the antibody to CSPG recognizes both chondroitin-sulfate-4 (CS-4) and chondroitin-sulfate-6 (CS-6) glycosaminoglycan moieties of a variety of proteoglycans, immunoblots specific for CS-4, or CS-6 proteoglycans were performed. A selective increase in CS-6 (Fig. 6.3), but not CS-4 (data not shown) was observed in the denervated striatum of both age groups at 8 d post-lesion compared to age-matched control.

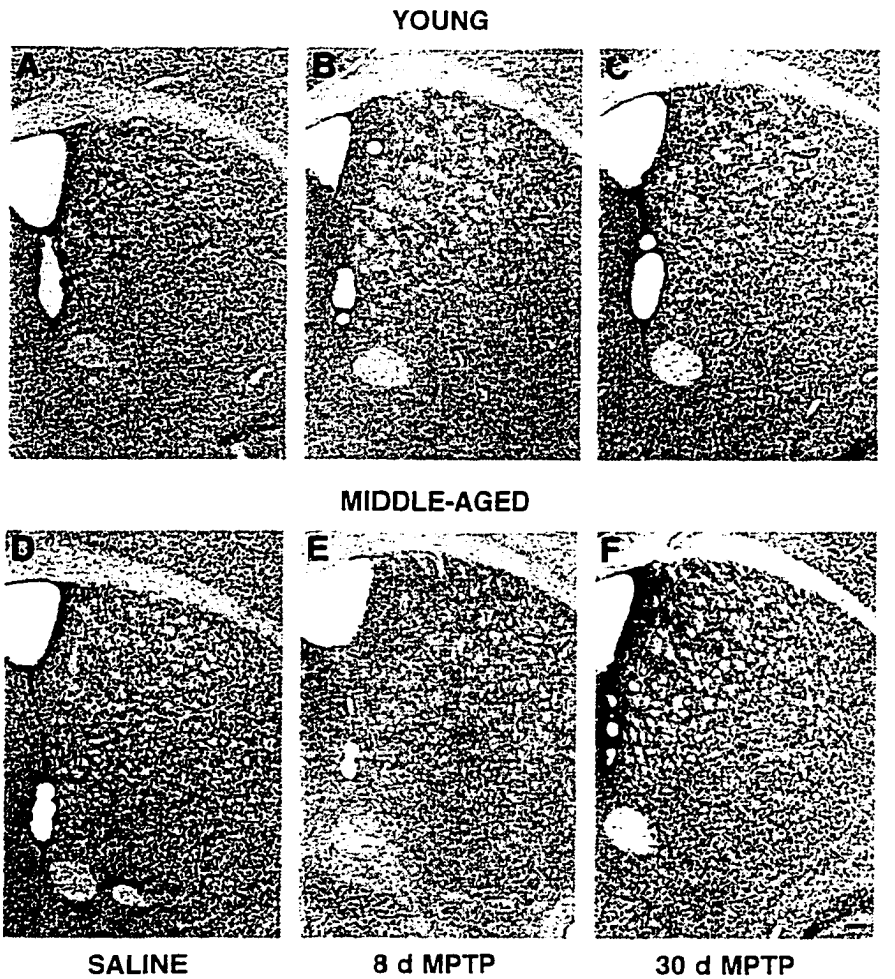

Figure 6.4 Fibronectin immunocytochemistry in the striatum of young *(A-C)* and middle-aged mice *(D-F)* at 8, and 30 d following saline and MPTP treatment. MPTP caused a marked reduction in fibronectin immunoreactivity in the denervated striatum of both age groups at 8 d post-lesion *(B, E)* compared to age-matched control *(A, D)*. After 30 d *(C, F)* , no marked differences from control were observed in either age group. Scale bar, 200 µm.

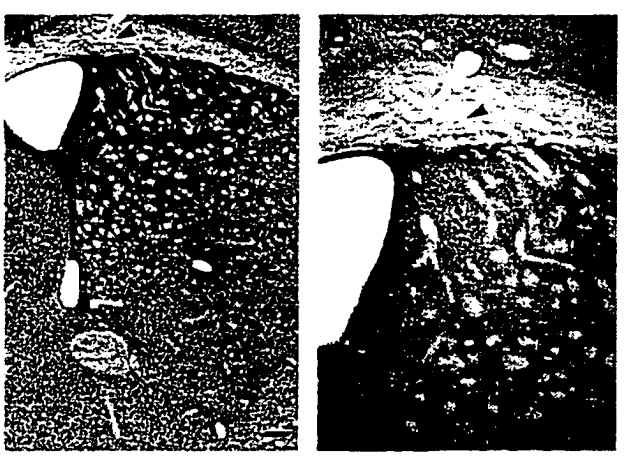

Figure 6.5 Tenascin immunocytochemistry in the striatum of a middle-aged animal at 8 d following MPTP treatment. *B,* High-power magnification of *(A)* outlining the corpus callosum. Scale bar, 200 µm; high-power magnification, 100 µm.

Immunoblots quantitated by phosphor image analysis revealed a 2.8-fold increase of ~245 kD core proteoglycan (indicated by arrowhead) in the denervated striatum of young, but not middle-aged mice following MPTP, which most probably corresponds to a 1D1 CS-6-PG proteoglycan reported by Rauch and colleages (1991), a form only expressed during development. Interestingly, smaller core proteoglycan of ~148 kD and ~105 kD (indicated by arrowhead), correspond most likely to 1D1 and 3H7 CS-6-PG respectively described by Rauch and colleages (1991) to be found exclusively in adult animals

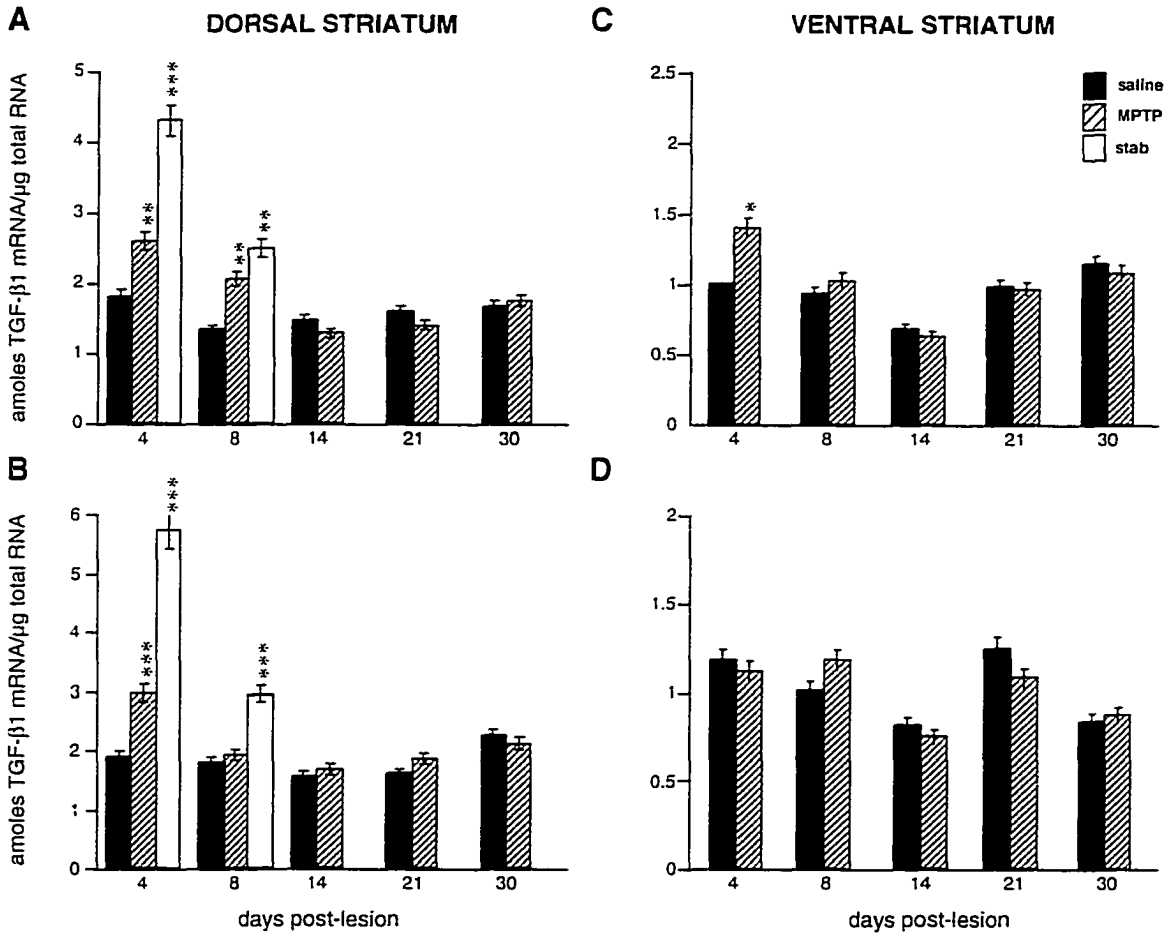

Figure 6.6 Quantitative analysis of TGF-β1 mRNA in the dorsal *(A and B)*, and ventral striatum *(C and D)* of young and middle-aged mice following saline and MPTP treatment at different days post-lesion. Solid black bars represent saline-treated animals, dashed bars represent MPTP-treated animals and solid white bars represent animals that received intrastriatal stab wounds. Values represent the mean ± SEM for n=4-5 animals per group. Levels of statistical significance were set to *P<0.05, **P<0.005, ***P<0.001 which indicates difference from age-matched control.

showed an increased ranging from 1.5 to 2-fold in the denervated striatum of both age groups.

Fibronectin. Fibronectin immunoreactivity was observed in a number of cells throughout the striatum of controls (Fig. 6.4*A,D*). In contrast to CSPG immunoreactivity, MPTP caused a marked reduction in fibronectin immunoreactivity in the denervated striatum of both age groups at 8 d post-lesion compared to age-matched control (Fig. 6.4*B,E*). After 30 d post-lesion, no marked differences from control were observed in either age group (Fig. 6.4*C,F*).

Tenascin. Immunolabeling for tenascin revealed no significant differences after MPTP-induced lesion in the denervated striatum of either age group at 8 d post-lesion compared to age-matched control. However, tenascin immunoreactive material was observed in the corpus callosum of both age groups after MPTP (Fig. 6.5; middle-aged animal shown).

Induction of TGF-β1 mRNA in the Denervated Striatum After MPTP

To examine whether TGF-β1 is regulated in response to MPTP and to determine whether the regulation of TGF-β1 is altered during aging, the dorsal striatum of young and middle-aged mice were analyzed by RNase protection assay. Since recovery has been observed to arise mainly from spared fibers in the ventral striatum of young mice, it was important to also determine whether TGF-β1 synthesis differs in the ventral striatum between young and middle-aged mice after MPTP. Since TGF-β1 has been shown to increase after stab injury, total RNA from intrastriatal stab lesions of both age groups were collected to serve as a positive control for the regulation of TGF-β1 expression.

In the dorsal striatum of young mice after MPTP, TGF-β1 mRNA was induced at 4 d (143% of control) and remained significantly elevated at 8 d post-lesion (Fig. 6.6*A*). By 14 d after MPTP, TGF-β1 returned to control levels in the dorsal striatum of young

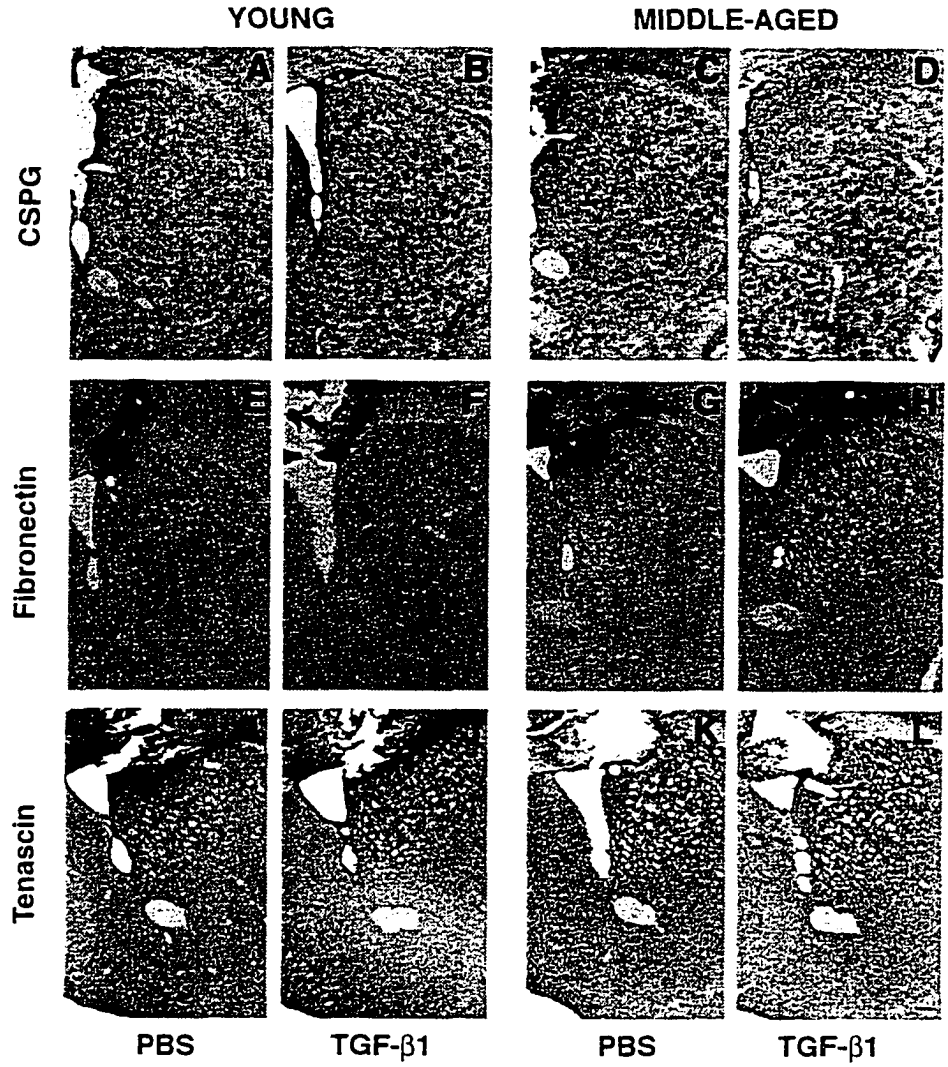

Figure 6.7 Immunocytochemistry for CSPG, fibronectin, and tenascin in the striatum of young and middle-aged mice at 24 h following TGF-β1 treatment. Stereotaxic injections of TGF-β1 (10 ng) or PBS in a final volume of 0.5 µl were made into the right lateral ventricle using a 1 µl Hamilton syringe. *A-D*, CSPG immunoreactivity of young *(A, B)* and middle-aged mice *(C, D)*. *E-F*, Fibronectin immunoreactivity of young *(E,F)* and middle-aged mice *(G,H)*. I-J, Tenascin immunoreactivity of young *(I, J)* and middle-aged mice *(K,L)*. Scale bar, 200 µm.

mice. In middle-aged mice lesioned with MPTP, a significant induction of TGF-β1 mRNA was observed only at 4 d post-lesion in the dorsal striatum (158% of control; Fig. 6.6*B*). Young mice that received intrastriatal stab lesion had a greater induction of TGF-β1 mRNA (272 and 160% of control at 4 and 8 d respectively; Fig. 6.6*A*) than after MPTP treatment. Similarly, middle-aged mice that received stab wounds resulted in a 340 and 160% of control increase at 4 and 8 d post-lesion respectively (Fig. 6.6*B*).

In the ventral striatum of young mice following MPTP treatment, TGF-β1 mRNA

observed at the boundaries of the transplants, axons fail to grow (Davies et al. 1997). This suggests that the formation of a glial scar found at the lesion site in the adult CNS does not represent a physical barrier, but can result in the expression of growth-inhibitory molecules that ultimately lead to a non-permissive environment for axonal growth. In the present study, we demonstrated an age-associated extended increase in growth-inhibitory ECM molecule CSPG in the denervated striatum following a MPTP-induced lesion. We observed that although elevated levels of CSPG immunoreactivity were initially found in the denervated striatum of young and middle-aged mice after MPTP, middle-aged mice displayed a more intense and sustained induction of CSPG immunoreactivity lasting for several weeks. The prolonged expression of CSPG immunoreactivity could play an important role in the attenuated sprouting response in the denervated striatum of middle-aged mice following MPTP. We have previously characterized the time course of dopaminergic reinnervation process in the denervated striatum of young mice and found it to occur between 14 and 30 d post-lesion (Ho and Blum 1998). Here, we demonstrated that a transient upregulation of inhibitory CSPG molecules was observed at 8 d post-lesion in the denervated striatum of young mice. Based from our results, we found that by 14 d post-lesion, the inhibitory barrier was not present in the denervated striatum of young mice (data not shown). This observation is interesting in light of the fact that the induction of CSPG immunoreactivity occurs prior to the reinnervation process.

Glycosaminoglycan moieties associated with CSPG are required for the inhibitory functions of neurite outgrowth (Snow et al. 1990a). The CSPG antibody, CS-56, used in the present immunocytochemical study, recognizes undigested chondroitin sulfate chains recognizing both CS-4 and CS-6 glycosaminoglycan moieties, but not dermatan sulfate (Avnur and Geiger 1984). Studies have shown that neutralization with CS-56 antibody promotes neurite outgrowth *in vitro* (Gates et al. 1996). Our further examination for CS-4, and CS-6 stubs following chondroitase ABC-digestion by western blot analysis

revealed a selective induction of CS-6, but not CS-4 in the denervated striatum of both age groups following MPTP. Studies have reported that the CS-6 elicits a more potent inhibition of neurite outgrowth than equivalent concentration of CS-4, suggesting that glycosaminoglycan moieties do not all function equivalently with respect to neurite inhibition (Snow et al. 1990a). Further studies have described that cultured astrocytes rich in CS-6 and tenascin, but not CS-4 seem to be repulsive for neurite growth while astrocytes that do not express these inihibitory molecules seem to support process outgrowth (Meiners et al. 1995). These studies therefore suggest that the induction of CSPG immunoreactivity, most likely by CS-6 may contribute to the age-associated decline in dopaminergic axonal sprouting after MPTP.

In this study, we observed that fibronectin immunoreactivity was present in the striatum of control mice. However, after MPTP treatment, a transient reduction of fibronectin immunoreactivity was observed in the denervated striatum of both age groups. Furthermore, we did not detect any changes of tenascin immunoreactivity in response to MPTP. These results are markedly different from the increased fibronectin and tenascin expression in reactive astrocytes following direct damage to the adult brain that other investigators have reported (Egan and Vijayan 1991; Laywell et al. 1992). However, our results indicate that the lack of increased expression of these ECM molecules in the denervated striatum could be specific to the nature of the injury or more importantly to the brain region which is damaged. Studies have shown that while tenascin immunoreactivity was abundantly increased after cortical stab lesions (Laywell et al. 1992), an absence of tenascin immunoreactivity was observed in the striatum following either aspiration or thermocoagulatory cortical lesions (Szele et al. 1995). At present, it is unclear why there are varying degrees of expression for these ECM molecules between different brain structures following injury. Increasing evidence suggests that by varying the ratios of certain growth-promoting to inhibitory molecules can influence the degree of inhibition for neurite outgrowth. For example, increases in the growth promot-

ing molecule laminin can significantly reduce or override the inhibitory effects of CSPG on neurite growth (Snow et al. 1990a; Snow and Letourneau 1992). Fibronectin, like laminin, has also been shown to promote neurite outgrowth (Baron-Van Evercooren et al. 1982; Williams and Varon 1985). The distribution of fibronectin has been found to coincide with tenascin expression during neural crest migration (Epperlein et al. 1988; Mackie et al. 1988). However, when tenascin is coupled to CSPG, migrating neural crest cells become inhibited, thus suggesting that tenascin can exhibit both facilitatory and inhibitory effects on axonal growth depending on the combination of molecules tenascin is associated with (Newgreen et al. 1986; Tan et al. 1987; Newgreen et al. 1990). Possibly, variations in the expression of injury-induced ECM molecules may explain the selective effects on neurite outgrowth in different brain regions following injury.

TGF-β1 and ECM molecules

TGF-β1 has been shown to display multifunctional properties in the CNS (for review, see Finch et al. 1993). It was shown to promote survival of both sensory and motor neurons, and stimulate sprouting of hippocampal neurons in culture *via* induction of neurotrophic factor synthesis (Martinou et al. 1990; Chalazonitis et al. 1992; Ishihara et al. 1994). Moreover, TGF-β1 can promote the survival of midbrain dopaminergic neurons *in vitro* and protect them against MPP^+ toxicity (Krieglstein and Unsicker 1994). Similar effects were also observed with cortical neurons after treatment with glutamate at neurotoxic levels (Prehn et al. 1993). Here, we report that TGF-β1 mRNA was significantly elevated at 4 d after MPTP lesion in the dorsal striatum of both young and middle-aged mice. However, the ability to maintain this induction declines with age. Young mice exhibited a sustained increase after 8 d post-lesion whereas middle-aged did not. Moreover, young mice also displayed a significant increase in TGF-β1 mRNA in the ventral striatum, a region that can contribute to MPTP-induced sprouting responses. In contrast, middle-aged mice did not display such induction in the ventral striatum. However,

this age-associated difference is specific to MPTP-induced lesion considering that TGF-β1 was found to increase to a greater extent following intrastriatal stab lesion. These results may suggest an association of TGF-β1 with sprouting and an impairment of TGF-β1 induction could a component leading to reduced axonal plasticity with age.

Modulation of ECM is a critical step during remodeling of tissue after injury, as the ECM can facilitate or inhibit sprouting processes. TGF-β1 has been involved in reactive synaptogenesis after lesion by regulating the synthesis of ECM molecules like fibronectin (Pasinetti et al. 1993). It can synergistically act with bFGF regulating the synthesis of tenascin (Smith and Hale 1997). Moreover, in mesenchymal and epithelial cell lines, TGF-β1 can increase the expression of CSPG, up to 20-fold (Bassols and Massagué 1988). Here, we report for the first time, that when TGF-β1 is injected into the brain, it stimulates CSPG immunoreactivity in the striatum of both young and middle-aged mice, but not fibronectin or tenascin immunoreactivity. The absence of increased fibronectin and tenascin immunoreactivity in our *in vivo* model is surprising due to the fact that TGF-β1 has been shown to induce the synthesis of both these molecules in astrocyte cultures as well as in *in vivo.* Several reasons could account for these differences. First, previous studies have shown that when TGF-β1 is injected into the adult brain, changes in fibronectin are detected at the mRNA level in the striatum (Pasinetti et al. 1993). While our observations were based on changes in fibronectin protein in the striatum after TGF-β1 injection, it is possible that increased mRNA levels may not translate into protein expression. However, it is possible that measuring mRNA is a more sensitive method for detecting changes in expression. Second, recent studies have shown that the regulation of tenascin requires synergistic actions of TGF-β1 and bFGF (Smith and Hale 1997). Thus, this could explain why TGF-β1 injection alone did not induce tenascin immunoreactivity.

In conclusion, the present study demonstrates that age-associated alterations of growth-promoting versus inhibitory molecules could influence the degree of sprouting fol-

lowing injury. We found that middle-aged mice lesioned with MPTP showed a decline in the ability to induce the favorable factor TGF-β1 and a prolonged induction of growth-inhibitory ECM molecule CSPG. This pattern of expression could contribute strongly to the limited axonal sprouting responses with age. Furthermore, TGF-β1 regulation of CSPG immunoreactivity in the striatum of both young and middle-aged mice, suggests the existence of broad influence of TGF-ß1 involvement during responses to neurodegeneration. By discovering how to maintain growth-promoting factors while manipulating inhibitory factors which can impede axonal outgrowth within the denervated site, this may provide therapeutic strategies to enhance surviving neurons and the restoration of function after injury.

CHAPTER 7

Summary and Discussion

In contrast to the classical view of failed regeneration in the adult CNS, significant mechanisms exist that promote structural and functional recovery in response to injury. In the classic injury cascade, glial cells are the principal cellular elements in the response to injury. Stereotypically, microglia are the first cells in the brain to sense not only changes in the brain's structural integrity but also any subtle alterations such as imbalances in ion homeostasis that often precedes pathological conditions (Giulian 1987; Giulian et al. 1989; Thomas 1992). They exhibit phagocytic activity by removing degenerating elements and they are an important source of cytokines, which are crucial to regulate astrocyte reactivity in the injury response (Giulian 1987; Giulian et al. 1989; Thomas 1992). Among the cytokines, IL-1 plays an important role in the earliest immune events regulating inflammation and promoting reactive astrocytes (Giulian et al. 1986; Giulian 1987, 1989; Thomas 1992). Aside from IL-1's potential role as a mediator of astrogliosis, it can also exert neurotrophic activity either directly or indirectly by regulating the production of neurotrophic factors in astrocytes (Lindholm et al. 1987; Spranger et al. 1990; Rivera et al. 1994). As neurotrophic factors are released, they can promote axonal sprouting and neuronal restabilization (Manthorpe et al. 1986; Gage et al. 1988; Giulian et al. 1993). However, it has become increasingly evident through the observations of these studies that this stereotypical cascade of cellular events and the molecular mechanisms controlling these signals into cellular responses can be dramatically different depending on the type of neuron and area injured, the nature of the injury, and more importantly, the age of the experimental animal.

Plasticity of Dopaminergic Neurons

Parkinsonian animals that underwent surgical trauma (cavitation) alone, without tissue implants display significant recovery of dopaminergic axonal fibers and behavioral improvement (Plunkett et al. 1990). This indicates that the dopaminergic cell system has remarkable plasticity to promote recovery and that inflammatory cells associated with trauma may play a determinant role in eliciting dopaminergic axonal sprouting (Plunkett et al. 1990). Further investigation demonstrated that injury-induced IL-1 expression may be a common pathway for recovery in parkinsonian animals since striatal implants of IL-1 promote behavioral recovery by stimulating compensatory dopaminergic sprouting (Wang et al. 1994a). This finding led us to investigate whether the beneficial effects of IL-1 could be through the regulation of astroglia-derived dopaminergic neurotrophic factors. Among the astroglia-derived dopaminergic neurotrophic factors aFGF, bFGF and GDNF, which have been shown to induce survival and plasticity of dopaminergic neurons when exogenously applied (Date et al. 1990c; Otto and Unsicker 1990; Tomac et al. 1995), a selective upregulation of only bFGF synthesis by IL-1 was observed. Although this study focus primarily on the effects of IL-1β on bFGF synthesis, we found that injection of IL-1α was able to induced a similar induction as well, if not higher. The selective induction of bFGF induction by IL-1 suggests that bFGF could be the putative dopaminergic neurotrophic factor mediating IL-1-induced sprouting of dopaminergic neurons. Since we observed that IL-1 could elicit dopaminergic sprouting by increasing bFGF syntheisis, this prompted us to investigate the endogenous role of IL-1 and trophic factor activities in MPTP-induced dopaminergic sprouting.

The use of MPTP to induce selective degeneration of SN dopaminergic neurons by systemic injections has created an advantageous tool for studying immunological reactions inherent to the brain repair process. Consistent with previous reports, we demonstrated that young mice challenged with MPTP have the capacity for spontaneous

sprouting of dopaminergic nerve terminals whereas middle-aged mice do not (Ricaurte et al. 1986, 1987a,b; Date et al. 1990a). Young mice display a progressive recovery of dopamine uptake levels and TH-immunoreactive fibers in the dorsal striatum between 14 and 30 d post-lesion. The reinnervation of the denervated striatum is found to arise from ingrowth of remaining dopaminergic afferents originating from neurons in the VTA. To examine IL-1 and trophic factor activities associated with MPTP-induced sprouting, we evaluated the expression of IL-1 and astroglia-derived dopaminergic neurotrophic factors during the time course of dopaminergic loss and recovery of axonal terminals in young but not middle-aged mice after MPTP-induced lesion. We found that young mice display a dramatic induction of IL-1α mRNA in the denervated striatum for several weeks which directly correlated with the time period during which the MPTP-induced axonal changes occured. Maximal induction of IL-1α is detected at 8 d in both the dorsal and ventral striatum. While IL-1α induction remain elevated up to 14 d post-lesion in the dorsal striatum, prior to the detectable recovery period, a sustained increase in IL-1α is observed until 30 d post-lesion in the ventral striatum of young mice. In contrast, MPTP-induced increase in IL-1α mRNA is markedly attenuated with age. For IL-1α induction is only seen during the first 8 d exclusively in the dorsal striatum, and not at all in the ventral striatum. Together with evidence that IL-1 stimulates dopaminergic axonal outgrowth when administered (Wang et al. 1994a) and our finding of the MPTP-induced increases in IL-1α mRNA in the denervated striatum of young mice strengthens the possibility that IL-1α may play an important role in spontaneous sprouting responses. Furthermore, the reduced capacity to maintain IL-1α synthesis in middle-aged mice could contribute to the age-associated decline in neuronal plasticity.

Despite the proposed role for IL-1 in eliciting dopaminergic sprouting being mediated by astroglia-derived bFGF, we observed no changes in bFGF mRNA or the astroglia-derived dopaminergic neurotrophic factors aFGF and GDNF in the denervated striatum of young mice following MPTP. These results were confounding in that when IL-1 was

exogenously administered, a dramatic induction of bFGF mRNA is observed; however, when IL-1α is markedly induced in the denervated striatum of following MPTP, we did not observe any changes in bFGF mRNA subsequent to the IL-1α induction.

An important aspect that we found through the course of these studies is the fact that IL-1α induction in response to injury is specific and differs upon different type of injuries and may explain these confounding results. For example, we found that in comparison to a stab injury in which induction of IL-1α is detected within several hours after injury by microglia (Rostworowski et al. 1997), induction of IL-1α is not detected until several days after MPTP lesion and IL-1α is mainly localized to hypertrophied astroglial cells. Therefore, we speculated that when astrocytes are induced to express IL-1 in response to some types of injuries that this affects the subsequent expression of other astroglia-derived factors such as bFGF. To investigate this, we treated glial cultures with LPS, a potent microglia-derived IL-1 inducer, and MPTP. We found that when glial cultures were exposed to LPS, a more rapid induction in IL-1α synthesis is observed compared to a slower onset of IL-1α production after MPTP treatment. Moreover, we found that while activated microglia were the predominant source of IL-1α production following LPS stimulation, microglia as well as astrocytes were responsible for IL-1α production after MPTP. Interestingly, we found that induction of bFGF mRNA coincides with the endogenous increase in IL-1α after LPS stimulation but not after MPTP. This suggests that different injury-induced inflammatory reactions can alter the molecular profile and responsiveness of astrocytes. Moreover, these results also suggest that bFGF is unlikely to attribute to MPTP-induced sprouting response; however, it may be involved in directing other types of axonal outgrowth in the adult brain.

An interesting observation that we found in our studies is that the brain's reaction to injury by cytokine expression is region specific. We found that although MPTP induced IL-1α synthesis in the denervated striatum, activation of IL-1α was not observed in the midbrain in either age group despite glial reaction. The fact that the IL-1α activation was

only found at the terminal fields of dopaminergic neurons following MPTP lesion opens an entirely new perspective for IL-1 in brain function. Previous evidence have also demonstrated region specificity of IL-1α expression to projection areas that are anatomically connected with the lesioned area. Tchelingerian and colleages (1993) demonstrated that after surgical injury to the hippocampus, IL-1α expression is first triggered in the striatum, then in other forebrain regions that are anatomically linked by neuronal projections to and from the lesioned hippocampus implicating the selective cytokine network associated with brain injury.

Pronounced regional differences in the expression of IL-1 molecule and its receptor have also provided evidence to support the notion that IL-1 may act as a target-derived neurotrophic factor. Previous studies have shown distinct distribution of IL-1 receptor binding in the SN, but failed to detect its expression in the striatum (Farrar et al. 1987; Cunningham et al. 1992; Akaneya et al. 1995). Our demonstration of colocalization of IL-1 receptor and TH immunoreactivity within neurons of the SN and VTA further supports the idea that IL-1 may act directly on these neurons to stimulate the MPTP-induced sprouting response.

Molecular Alterations in the Aging Brain

As the brain ages, the ability to promote sprouting after injury is delayed and reduced. Two principal findings from these studies that can account for the age-associated decline in recovery processes could be due to 1) decline in growth factor, cytokine(s) production and 2) inappropriate substrate for axonal extension.

Increasing evidence supports the role of endogenous growth factors in promoting neuronal sprouting in response to injury. For example, increases in endogenous NGF in response to denervated hippocampus have been shown to play an important functional role in collateral sprouting since NGF neutralizing antibody delivery resulted in attenua-

tion of axonal sprouting (Crutcher and Collins 1986; Van der Zee et al. 1992; Conner et al. 1994). It appears that while young rats show increases in NGF expression in response to hippocampal lesions, aged rats fail to induce NGF and such impairments in the expression of NGF may contribute to the reduced axonal plasticity with age (Scott et al. 1994). We have shown in these studies that the ability to induce IL-1α synthesis following MPTP is significantly attenuated with age. More importantly, we found that the reduction in the ability to induce IL-1α synthesis depends on the nature of the injury since a stab injury elicited a prompt and marked increase of IL-1α in the striatum of middle-aged mice similar to that seen in young mice. It has been well-documented that aging animals undergo a decline in immune competence, partly due to alterations in cytokine production and/or changes in the responsiveness of immune cells to cytokines in the periperal system (for review, see Hodes 1995). In these studies, we have demonstrated that similar molecular alterations may also exist in the aging brain which could account for the differential immune reaction following MPTP and ultimately leads to the attenuated sprouting response.

Differences in the ability to promote axonal growth may be due to inhibition by the extracellular environment. It seems that the ability of young mice to induce spontaneous dopaminergic sprouting following MPTP-induced lesion is most likely the result of the dynamic balance of actions between growth-promoting and growth-inhibitory molecules. Although we found that young mice display a transient increase in ECM inhibitory molecule CSPG immunoreactivity in the denervated striatum, it is likely that growth-promoting molecules such as IL-1α and TGF-β1, which are greatly enhanced following MPTP treatment might mask the growth-inhibitory potential of CSPG, thereby triggering axonal sprouting. In contrast, the poor sprouting response exhibited by aging mice is probably due to the imbalance in the relative expression of these molecules. We found that while middle-aged mice displayed an extended induction of CSPG immunoreactivity in the denervated striatum, the ability to maintain IL-1α and TGF-β1 induction markedly declines.

These studies indicate that these age-associated decrements may profoundly affect the response of the aging brain to promote functional recovery.

CONCLUSION

In these studies, we characterized IL-1 and neurotrophic activities associated with plasticity of dopaminergic neurons and factors that can contribute to the age-associated decline in axonal sprouting. IL-1 pleiotrophic actions associated with inflammation and sprouting following injury may depend on several factors: time of induction, cellular source, region specificity and the nature of the injury. IL-1 can elicit direct or indirect effects on dopaminergic neurons by regulating the production of astroglia-derived dopaminergic neurotrophic factor bFGF. The dynamic balance of actions between growth-promoting and growth-inhibitory molecules associated with neuronal injury is critical for facilitating axonal sprouting. Enhanced induction of IL-1 production observed in the denervated striatum of young mice and its absence in middle-aged mice strongly suggests a role for IL-1α in eliciting, propagating or maintaining dopaminergic axonal sprouting processes. The inability to maintain the induction of IL-1 as well as enhanced levels of growth-inhibitory molecule CSPG, could underlie the progressive decline associated with MPTP-induced sprouting of middle-aged mice. Understanding the brain's ability to sprout new connections after injury could help to ameliorate age-related impairments in brain function.

APPENDIX

PUBLICATIONS

CHAPTER 3

Peer Reviewed Report

Ho A, Blum M (1997) Regulation of astroglial-derived dopaminergic neurotrophic factor gene expression by interleukin-1β in the striatum of young and middle-aged mice. *Experimental Neurology* 148:348-359.

Abstract

Ho A, Blum M (1995) Regulation of bFGF and GDNF mRNA by interleukin-1β in striatal astrocyte cultures; potential mechanisms for dopaminergic sprouting in parkinsonian rats. *Society of Neuroscience Abstracts* 21:295.

CHAPTER 4

Peer Reviewed Report

Ho A, Blum M (1998) Induction of interleukin-1 associated with compensatory dopaminergic sprouting in the denervated striatum of young mice: model of aging and neurodegenerative disease. *Journal of Neuroscience* (Accepted).

Abstract

Ho A, Blum M (1996) Characterization of the regulation of dopaminergic trophic factor and cytokine gene expression associated with neurotoxin-induced plasticity of dopamine neurons in young and aging mice. *Society of Neuroscience Abstracts* 22:545.

CHAPTER 5

Abstract

Ho A, Blum M (1997) Characterization of TGF-β1 neurotrophic factor cascade associated with MPTP-induced injury of dopamine neurons in young and middle-aged mice. *Society of Neuroscience Abstracts* 23:2246.

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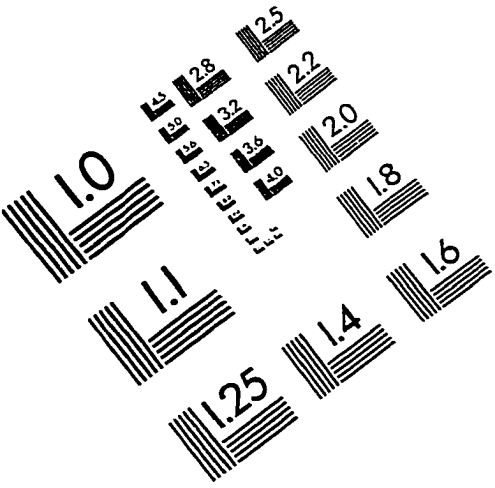
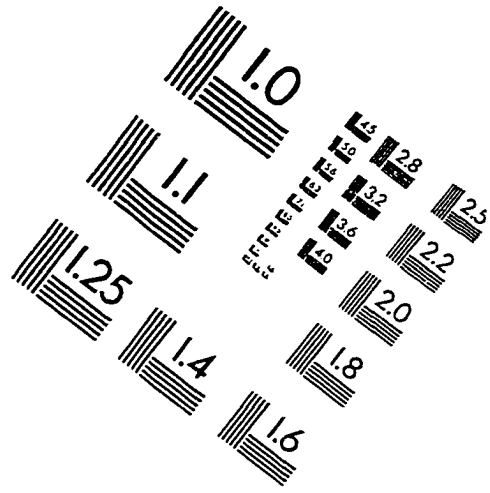
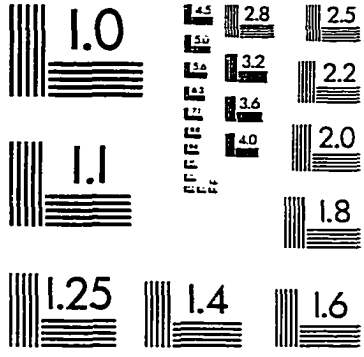
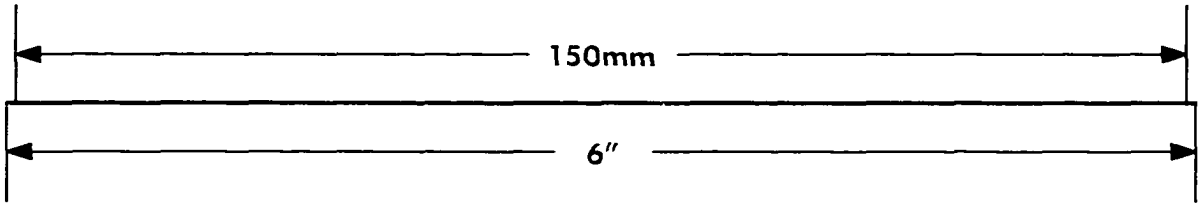
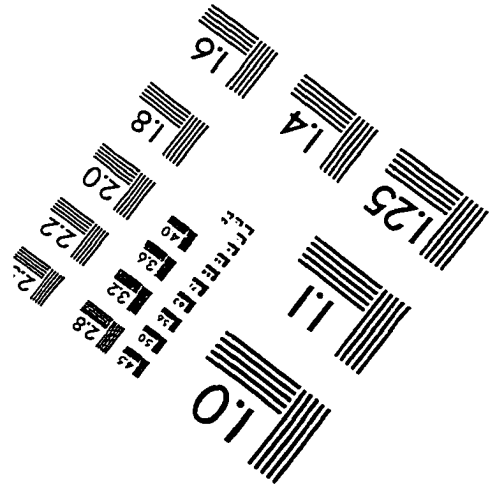
IMAGE EVALUATION TEST TARGET (QA-3)

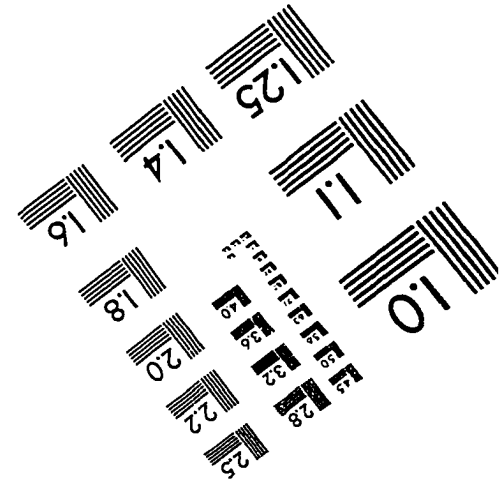












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