Factors Affecting the Extent of Dopamine Cell Loss and Behavioral Sparing after Partial Unilateral Lesions of the Nigrostriatal Dopamine Pathway: the Role of basic Fibroblast Growth Factor

Isabella Anna Moroz

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ABSTRACT

Factors Affecting the Extent of Dopamine Cell Loss and Behavioral Sparing after Partial Unilateral Lesions of the Nigrostriatal Dopamine Pathway:

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Isabella Anna Moroz, Ph.D.
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Behavioral recovery from partial 6-hydroxydopamine lesions of the nigrostriatal dopamine neurons is associated with gradual normalization of basal extracellular levels of dopamine released from the surviving terminals in the striatum. This enhancement of function within the remaining dopamine neurons is reminiscent of the enduring changes in functioning of the dopamine system that accompany behavioral sensitization to psychostimulants. In this thesis, the hypothesis that endogenous expression of basic fibroblast growth factor (FGF-2), a neurotrophic and neuroprotective factor produced by astrocytes and shown to mediate behavioral sensitization to amphetamine, plays a role in behavioral recovery after 6-hydroxydopamine (6-OHDA) lesions of nigrostriatal dopamine neurons was studied.

Initial findings revealed that, despite the increases in FGF-2 expression after lesions, there was a progressive loss of tyrosine hydroxylase immunoreactive (TH-IR) cells in the dopamine cell body regions and no behavioral recovery. Manipulations of gonadal hormones in the neonatal and adult life, known to enhance behavioral sensitization to amphetamine, reduced both the lesion-induced increases in astrocytic FGF-2 expression and losses in TH-IR cells in the dopamine cell body regions, but had

no effect on behavioral recovery. Paradoxically, forced limb-use, shown previously and here to stimulate sparing after 6-OHDA lesions, did not further enhance lesion-induced increases in FGF-2, and did not protect against loss of TH-IR cells, but, in itself, increased FGF-2 expression. Finally, pre-lesion treatment with a sensitizing regimen of amphetamine, known to increase FGF-2 expression in the vicinity of dopamine neurons, led to remarkable sparing of function after subsequent lesions, but did not protect against loss of striatal tissue dopamine.

It is proposed that the increases in endogenous expression of astrocytic FGF-2 seen here after extensive 6-OHDA lesions reflect a magnitude-dependent response of dopamine neurons to degeneration. These increases in FGF-2 may be effective in promoting recovery when combined with behavioral demands, such as forced use, or when induced before the injury. Although the mechanisms underlying these effects remain to be unraveled, it is proposed that the ability of forced limb-use and amphetamine treatment to induce FGF-2, and possibly other neurotrophic factors, may underlie their effects on neuronal growth and morphology. Consequently, their beneficial effects on behavioral sparing may be mediated by structural changes and enhanced synaptic connectivity between the surviving dopamine neurons and their targets.

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DEDICATION

I dedicate this work to my beloved grandmother Anna Romanowska and to the memory of Zbigniew Romanowski.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CONTRIBUTIONS OF AUTHORS

Jane Stewart, my supervisor and the principal investigator, was critically involved in all aspects of all the experiments comprising the present thesis.

CHAPTER II
Demetra Rodaros and *Heshmat Rajabi* provided invaluable technical expertise throughout all studies, particularly with neonatal gonadectomies in Experiment 3 of Chapter II. Heshmat Rajabi conduced HPLC analysis of postmortem tissue in Experiment 1B (Chapter II) and in Experiment 9 (Chapter V).

CHAPTER III
Patricia Boksa provided rats that underwent perinatal anoxia manipulation (Chapter III).

CHAPTER IV
Timothy Schallert shared with us his model of forced limb use. Annie Cohen assisted during surgeries and casting manipulation for Experiment 5 and together with Jennifer Tillerson performed scoring of behavioral tests for Experiment 5 and 6. Kimberley Maxwell carried out casting manipulations and tissue processing of Experiment 7.

CHAPTER V
Susana Pecina independently performed Experiment 9.

CHAPTER I

INTRODUCTION

Growing evidence indicates that the adult brain retains the capacity to be influenced by environmental challenges and insults, such as exposure to psychostimulant drugs and brain injury, or by changes within the organism itself, such as hormonal fluctuations or aging. Neural plasticity is most apparent during ontogeny, the time of dynamic changes characterized by a strict temporal order of molecular and cellular events that guide cell proliferation, growth, and differentiation. In recent years, however, the idea that plasticity in the adult brain is governed by the same signaling mechanisms that operate in the developing brain has been increasingly recognized. Of particular importance to the present thesis is the finding that neurotrophic factors, or proteins that control neuronal growth, differentiation, and synapse formation during brain development are also key mediators of plastic changes in adult neurons.

Basic fibroblast growth factor (FGF-2), a neurotrophic and neuroprotective factor of the midbrain dopamine (DA) system, has been shown to play a crucial role in the development of behavioral sensitization to amphetamine, an experience-dependent plasticity. The characteristic feature of behavioral sensitization is the enduring enhancement in functioning of the DA neurons. Another example of plasticity in the adult midbrain DA system is observed after partial lesions of the nigrostriatal neurons. Behavioral recovery from these lesions is accompanied by normalization of extracellular DA in the striatal terminal regions, suggesting compensatory enhancement in functioning of the surviving DA neurons, similar to that observed after sensitization to amphetamine. The experiments undertaken in this thesis were aimed at exploring the idea that similar

mechanisms mediate the enduring enhancement in functioning of the midbrain DA system seen in recovery from partial lesions and in behavioral sensitization. Specifically, the expression of endogenous FGF-2 was examined after partial lesions of the nigrostriatal DA neurons, in an attempt to demonstrate that lesions enhance the expression of basic fibroblast growth factor and these increases, in turn, mediate behavioral recovery.

In the sections that follow I first describe the behavioral and neurochemical effects of 6-hydroxydopamine lesions of the midbrain DA system and show how they relate to the likelihood of recovery from these lesions. Next, I present evidence supporting the idea that similar mechanisms mediate the enduring enhancement of DA system functioning that accompanies both recovery from partial nigrostriatal lesions and behavioral sensitization. Specifically, I discuss the importance of the role of glutamatergic-dopaminergic interaction within the DA cell body regions, and, I describe changes in neuronal morphology observed in the terminal regions of DA neurons after both phenomena. I then introduce the idea that neurotrophic factors exert profound trophic and neuroprotective effects on the midbrain DA neurons and may thus underlie the enduring enhancements in DA functioning observed after partial lesions and after sensitization. In particular, I provide the rationale behind the idea that endogenous expression of FGF-2 after 6-OHDA lesions may play a role in behavioral recovery following this type of lesion.

6-Hydroxydopamine Lesions of the Midbrain Dopamine Neurons

6-hydroxydopamine (2,4,5-trihydroxyphenethylamine, 6-OHDA) is a transmitter-selective neurotoxin, which destroys catecholaminergic neurons (Thoenen & Tranzer, 1973; Ungerstedt, 1968) and can be used in combination with other pharmacological agents to deplete the tissue of dopamine (DA) without affecting noradrenaline (NA), or vice versa (Schallert &Wilcox, 1985). The ability of 6-OHDA to selectively deplete DA has been utilized in an attempt to model Parkinson's disease (PD), which is characterized by a progressive degeneration of DA neurons originating in the substantia nigra pars compacta (SNc). The 6-OHDA lesion model has been widely used to investigate and develop new anti-Parkinsonian drugs and treatment strategies, including transplantation of neural tissue or exogenous administration of neurotrophic factors. In addition, 6-OHDA serves as an invaluable tool for examining the endogenous capability of the injured brain to defend itself from and compensate for specific neurotoxin-induced neurochemical depletions.

Specificity of 6-OHDA Neurotoxicity and Possible Mechanisms of Action

Stereotaxic injections of 6-OHDA into discrete brain sites were found to produce a loss of central catecholamine-containing terminals (Ungerstedt, 1968). Importantly, these effects were produced with little apparent permanent effects on other neuronal systems in the brain (Agid, Javoy, Glowinski, Bouvet, & Sotelo, 1973; Jacks, De Champlain, & Cordeau, 1972; Kim, 1973). The selectivity of 6-OHDA for the catecholaminergic neurons is explained by the fact that the toxin is a structural analogue of catecholaminergic neurotransmitters, DA and NA. The high-affinity catecholamine

uptake systems located on the plasma membrane of catecholaminergic neurons, which usually transport their transmitters back into the cells, accept and transport 6-OHDA into these neurons, where the substance is then accumulated. Due to its high electroactivity, 6-OHDA readily autooxidizes at physiological pH, leading to production of several cytotoxic species, such as hydrogen peroxide, hydroxyl radical, superoxide anion, and reactive quinones (Cohen & Heikkila, 1974; Sachs & Jonsson, 1975). A number of hypotheses have been put forward to explain the mechanisms of degenerative action of 6-OHDA, but it is generally believed that the accumulation of the toxic metabolites of 6-OHDA oxidation within catecholaminergic neurons destroys the neurons by damaging proteins, membrane lipids and DNA, leading to destruction of the neuronal membrane and cell death in all-or-none manner (Jonsson, 1980; Jonsson, 1983). The selectivity of 6-OHDA can be further increased by coadministration of selective inhibitors of NA or DA reuptake, thus depleting the tissue of DA without affecting NA, or vice versa (Schallert &Wilcox, 1985). Thus, 6-OHDA can be used to produce relatively specific lesions of central DA- and/or NA-containing neurons and hence has become a standard tool in the study of these transmitters. In the present thesis, 6-OHDA was used to selectively damage the midbrain DA neurons. In the following section, a short anatomical description of the midbrain DA system is provided in order to establish a context for discussion of the behavioral and neurochemical effects of such lesions.

The Midbrain DA System – Anatomical Considerations

The midbrain DA system is a diverse group of neurons involved in a wide variety of behaviors, such as movement and sensorimotor integration, reward and motivation, and cognition and higher cortical processing. The organization of this system is complex

and its detailed neuroanatomical description in beyond the scope of this thesis (see reviews by Fallon & Loughlin, 1987; Fallon & Loughlin, 1995; Haber & Fudge, 1997; Hasue & Shammah-Lagnado, 2002). The midbrain DA system has been differentiated into several components, including: mesolimbic, mesocortical, and nigrostriatal system. The mesolimbic system arises from groups of neurons located in the ventral tegmental area (VTA, A10 group) that send projections to nucleus accumbens (NAcc) and other limbic regions such as the olfactory tubercle, lateral septum and amygdala. The mesocortical system arises from VTA cells and projects to various cortical sites including the piriform, entorhinal, prefrontal and anterior cingulate. Finally, the nigrostriatal system originates from cells in the substantia nigra (SN, A9 group) that project mainly to dorsal striatum (or neostriatum). Furthermore, the DA neurons of the SNc extend posteriorly into the retrorubral field (RRF, A8) and innervate the striatal areas including the central extended amygdala and NAcc. The DA fibers ascending from the midbrain cell body sites travel along the nigrostriatal pathway and the medial forebrain bundle (MFB) to reach their terminal targets. Behaviorally and physiologically the effects of 6-OHDA lesions of the midbrain DA neurons have been most often attributed to DA depletions in the dorsal striatum. These depletions can be produced by administration of the toxin at the level of the DA terminals, the axons, or the somata, either bilaterally or unilaterally.

Behavioral Effects of 6-OHDA Lesions of the Midbrain DA System

The bilateral destruction of DA neurons with the resulting substantial depletion of striatal DA is accompanied by the appearance of a behavioral syndrome that is considered an animal analog of PD (Schultz, 1982). In rats, this behavioral syndrome is

characterized by bradykinesia, sensorimotor neglect, aphagia, adipsia, short-step locomotion, postural abnormalities, and cognitive dysfunction (Marshall, Richardson, & Teitelbaum, 1974; Schallert & Whishaw, 1978; Schallert, Whishaw, Ramirez, & Teitelbaum, 1978; Whishaw & Dunnett 1985; Whishaw, O'Connor, & Dunnet, 1986; Whishaw, Robinson, Schallert, De Ryck, & Ramirez, 1978, Ungerstedt 1971; Zigmond & Sticker, 1972; Zis, Fibiger, & Phillips, 1974). The severity of the behavioral deficits induced by bilateral administration of 6-OHDA, particularly the aphagia and adipsia, make it very hard, if not impossible, to study such animals long term, due to a high postoperative risk of lethality and special care requirements, such as intragastric feeding. To combat these problems, a unilateral lesion approach has been developed and represents the most useful and most frequent application of the 6-OHDA lesion technique. The unilateral 6-OHDA lesion model provides the opportunity to compare the behavioral and neurochemical effects of the toxin within the same animal. Numerous behavioral tests of lateralized deficits have been developed to assess asymmetries of posture and behavior in unilaterally lesioned rats that occur as a result of dopaminergic imbalance between the lesioned and the intact hemisphere. In general, the greater the DA depletion on the side of the lesion, the greater the resulting behavioral asymmetries. In fact, the degree of behavioral deficits induced by 6-OHDA infusions has often been taken as an accurate indication of the neurochemical extent of the lesion.

Turning behavior has been used most frequently to describe and quantify behavioral effects of unilateral 6-OHDA lesions. In the pioneering studies, Ungerstedt, (1968) and Ungerstedt and Arbuthnott (1970) reported that rats with unilateral 6-OHDA lesions showed spontaneous turning movements toward the side of the lesion (termed as

ipsiversive). The direction of turning was proposed to reflect the fact that the sensorimotor projections are crossed so that movements are directed away from the dopaminergic dominant hemisphere. More detailed analyses of stepping patterns revealed that turning asymmetries after 6-OHDA lesions were not due to the loss of the ability to move the contralateral limb, but rather, to the loss of the ability to exert force to adjust posture and produce movement (Miklyaeva, Martins, & Whishaw, 1995).

Pharmacological agents, such as amphetamine and apomorphine, have also been used to elicit turning behavior in 6-OHDA lesioned rats. Amphetamine is known to increase synaptic levels of DA by inducing DA release from, and inhibiting DA reuptake into the intact terminals (Seiden, Sabol, & Ricaurte, 1993), causing ipsilateral rotation due to increased dopaminergic activity on the intact side (Ungerstedt, 1971b; Ungerstedt & Arbuthnott, 1970). The postsynaptic DA receptor agonist, apomorphine, induces rotation contralateral to the lesioned side because of denervation-induced DA receptor D_2 supersensitivity (Creese, Burt, & Snyder, 1977; Mishra, Gardner, Katzman, & Makman, 1974; Neve, Kozlowski, & Marshall, 1982; Ungerstedt, 1971c). Furthermore, specific degrees of behavioral asymmetry under a certain drug dose (turns/min) are often taken as indicators of specific degrees of striatal DA depletion.

The other major deficit in unilaterally lesioned 6-OHDA rats is the occurrence of sensory neglect, manifested as a profound decline, or a total loss of behavioral reactivity of these rats to sensory stimuli of various modalities. Deficits in head-orientation have been observed when visual, tactile, or olfactory stimuli were applied to the contralateral, but not the ipsilateral side of the body (Marshall, Turner, & Teitelbaum, 1971). In addition, the unilaterally 6-OHDA lesioned animals preferentially orient to and remove

adhesive stimuli from the nonimpaired forelimb (Schallert et al. 1982; Schallert & Tillerson, 2000) and show preferential scanning of an unfamiliar environment with the ipsilateral side of the body (Fornaguera, Carey, Huston, & Schwarting, 1994; Fornaguera, Schwarting, Boix, & Huston, 1993; Steiner, Bonatz, Huston, & Schwarting, 1988).

Numerous other tests, based on the motor abnormalities seen in PD have been designed to test behavioral impairments in the unilaterally 6-OHDA lesioned rats. For example, akinesia is found in the forelimb contralateral to the lesion on stepping tests (Schallert, Norton, & Jones, 1992) and on paw-reaching and staircase tests (Miklyaeva & Whishaw, 1996; Montoya, Campbell-Hope, Pemberton, & Dunnett, 1990; Schallert & Hall, 1988; Whishaw et al., 1986). 6-OHDA lesioned rats also cannot effectively use the limbs contralateral to the lesion to initiate movements that shift body weight and control posture during vertical exploration in a cylinder test (Schallert & Tillerson, 2000), or to regain stable equilibrium during experimenter imposed weight shifting challenges in a bracing test (Olsson, Nikkhah, Bentlage, & Bjorklund, 1995; Schallert, De Ryck, Whishaw, Ramirez, & Teitelbaum, 1979; Schallert & Tillerson, 2000).

The multitude of behavioral tests designed to accurately assess the deficits produced by destruction of DA neurons reflects the on-going search for tests sensitive to partial depletions of DA. In general, it has been estimated that only animals with residual striatal DA levels of about 20% or less will show consistent behavioral asymmetries, thus discovery of tests that can detect a wide range of DA depletion is highly desirable. Interestingly, a similar critical level of DA depletion has been reported in Parkinson's disease (Bernheimer, Birkmayer, Hornykiewicz, Jellinger, & Seitelberger, 1973;

Hornykiewicz, 1972; Hornykiewicz, 1993), indicating that the 6-OHDA lesion model may be relevant for investigation of deficits relevant to the human disease.

In conclusion, the behavioral tests described in this section have been valuable in assessment of behavioral deficits produced by unilateral infusion of 6-OHDA. In the following section, the neurochemical effects of such lesions will be discussed with an emphasis on depletion in tissue levels of DA and the resulting cellular changes in DA neurons. Furthermore, the indices of 6-OHDA-induced degeneration of DA neurons will be related to the behavioral deficits.

Neurochemical Effects of 6-OHDA Lesions of the Midbrain DA System – Relation to Behavioral Deficits

Quantitative assessment of the extent of the lesion produced by the unilateral administration of 6-OHDA has been typically based on post-mortem analysis of residual tissue levels of DA and its metabolites in the lesioned hemisphere. This measurement is often expressed in terms of dopamine depletion in relation to the contralateral (intact) region of interest, or in relation to the region of interest of unlesioned control animals. Moderate reduction of tissue DA levels in the ipsilateral dorsal striatum were reported as early as one day after the lesion (20%, Neve et al., 1982) and significant reductions were reported 2 days after the lesion (95%, Neve et al., 1982; 77% Nisenbaum, Kitai, Crowley, & Gerfen, 1994; 50% Mishra, Marshall, & Varmuza, 1980). Within 3-5 days after the lesion, the depletion of striatal tissue DA has been reported to reach its maximum (as high as 98%) and remained so for months after lesion (Altar, Marien, & Marshall, 1987; Mishra et al., 1980; Neve et al., 1982). Neither acute nor chronic effects of 6-OHDA

lesions in the striatum contralateral to the lesion have been reported (Altar, O'Neil, & Marshall, 1984; Day, Tham, & Fibiger, 1994; Nisenbaum et al., 1994). In contrast to the lesioned striatum, the tissue levels of DA in the SN or VTA were found to be less severely reduced after 6-OHDA lesions (Barneoud et al., 1995; Hefti, Melamed, & Wurtman, 1980; Saavedra, Setler, & Kebabian, 1978). Specifically, after 6-OHDA lesions of the SN, striatal DA was reduced by 99% whereas SN DA was reduced by 43% (Hossain & Weiner, 1993; Hossain & Weiner, 1995).

In addition to the analysis of tissue DA depletions, degeneration of DA neurons have been demonstrated by means of histofluorescence and immunocytochemistry. Early studies revealed that 6-OHDA infusion into the SN reduced the size of DA cell bodies and was accompanied by change in fluorescent color, indicative of neurodegenerative changes within the DA cell bodies, as early as 24-72 hours after the lesion (Hokfelt & Ungerstedt, 1973; Ungerstedt 1968). Although loss of DA terminals in the striatum was observed as early as one day after intrastriatal 6-OHDA infusion, and 7 to 12 days after intra-SN infusion, in the latter case, reduced number of DA cell bodies was found only after 2 months. Similar results have been obtained with immunocytochemical method of visualizing catecholaminergic neurons and their terminals by labeling the rate-limiting enzyme for catecholaminergic synthesis, tyrosine hydroxylase (TH). Loss of TH-immunoreactive (IR) cell bodies was evident only weeks and months after 6-OHDA infusion, especially if the neurotoxin was injected further away from the DA cell body regions, at the level of the MFB or into the striatal terminals (Gordon, Schreier, Ou, Holcomb, & Morgan, 1997; Ichitani, Okamura, Nakahara, Nagatsu, & Ibata, 1994; Sauer & Oertel, 1994). The general finding that DA depletion in the terminal regions occurs

earlier than the loss of TH-IR cell bodies suggests that the changes in the terminals reflect initially, at least, abnormal cell functioning, and that it is only later that the cells actually die (Schwarting & Huston, 1996).

As with all other types of brain injury, there is a considerable variation in the degree of degeneration induced by unilateral administration of 6-OHDA. This variation depends on several factors, such as the site and dose of 6-OHDA administration. In general, injections of 6-OHDA into the MFB lead to more substantial DA depletion of tissue DA levels in the striatum (Costall, Marsden, Naylor, & Pycock, 1976) and more substantial loss of TH-IR neurons in the SN (Carman, Gage, & Shults, 1991; Perese, Ulman, Viola, Ewing, & Bankiewicz, 1989), than injections into the SN or VTA. The more extensive damage to the DA neurons produced as a result of administration of 6-OHDA at the level of the MFB reflects the fact that such infusions are likely to result in the toxin affecting both the nigrostriatal as well as the mesolimbic DA systems.

In general, behavioral deficits emerge only after the extent of striatal denervation and nigral cell loss exceeds some critical threshold, which may differ depending on the test used. For example, spontaneous ipsiversive turning was reported to correlate well with striatal DA depletions greater than 50 % (Fornaguera, Carey, Dai, Huston, & Schwarting, 1994; Fornaguera, Carey, Huston et al., 1994b, Schwarting & Huston, 1997). Amphetamine-induced rotation has been observed with striatal DA depletions greater than 85 %, whereas apomorphine-induced rotation was evident in animals with virtually complete lesions that produced an average of 99.8 % striatal DA depletion (Barneoud, Descombris, Aubin, & Abrous, 2000). Furthermore, apomorphine- but not amphetamine-induced rotation was correlated with the percentage of striatum innervated by TH-IR

axons and with the number of TH-IR cells remaining in the SNc (Carman et al., 1991; Kirik, Rosenblad, & Bjorklund, 1998). Impairments in skilled paw use (staircase test) and in the ability to make adjusting steps (bracing test) have been shown in rats with striatal DA depletions greater than 80%, striatal TH-IR fiber density reductions greater than 85 %, and reductions in the number of nigral TH-IR neurons in the range of 50-75 % (Chang, Wachtel,Young, & Kang, 1999; Kirik et al., 1998; Lee, Sauer, & Bjorklund, 1996). More sensitive tests, such as the forelimb asymmetry test (the cylinder test) or the adhesion removal test have been found to detect striatal DA depletions as low as 30 % (Schallert & Tillerson, 2000). Lesions placed at the level of the MFB, previously reported to result in the highest striatal DA depletion and the greatest loss of TH-IR cells in the DA cell body regions, also produce the greatest behavioral deficits on tests of drug-induced rotation, skilled reaching, and skilled paw use (Carman et al., 1991; Kirik et al., 1998; Lee et al., 1996).

In conclusion, lesion studies have demonstrated that within 3-5 days of 6-OHDA injection, the DA input to striatal targets reaches its maximum depletion whereas the DA cell bodies only begin to show changes indicative of degeneration. Thus, the initial period after toxin administration reflects dysfunctional DA neurons, which begin to die weeks and months later. Furthermore, the extent of DA neuron damage and behavioral deficits can vary considerably as a function of variables such as lesion placement.

Recovery of Function after 6-OHDA Lesions of the Midbrain DA System

The extent of the unilateral 6-OHDA lesion is critically related to the likelihood of recovery from the lesion-induced behavioral deficits. In particular, striatal DA

depletions greater than 95% have been shown to result in chronic behavioral deficits from which the animals never recover (Marshall, 1985; Robinson, Castaneda, & Whishaw, 1990; Steiner et al., 1988; Stricker & Zigmond, 1976). Extensive but partial depletions (80-95%) of striatal DA lead to severe behavioral deficits, the magnitude of which is positively correlated with the striatal DA depletion in postmortem tissue and cell loss in SNc (Lees, Kydd, & Wright, 1985), but from which most of the animals eventually recover (Marshall, 1974; Marshall et al., 1974; Marshall, 1979; Zigmond & Stricker, 1973). Striatal depletions of less than 80%, however, do not lead to obvious behavioral deficits (Schwarting & Huston, 1997; Stricker & Zigmond, 1976), unless tests sensitive to smaller DA depletions are employed (Schallert & Tillerson, 2000). It has been proposed, therefore, that only 10-20% of the DA input to the striatum is required to maintain relatively normal behavioral function. Similar nonlinear relation between the extent of DA depletion and behavioral deficits is also evident in PD patients, where the disease becomes clinically apparent only when depletion of striatal dopamine levels reaches 80% (Bernheimer et al., 1973; Hornykiewicz, 1972; Hornykiewicz, 1993). This recovery of function after partial 6-OHDA lesions reflects remarkable plasticity of the nigrostratal DA system and has generated considerable interest in the nature of neuroadaptations that could account for these effects.

A number of presynaptic and postsynaptic adaptations within the nigrostriatal DA system have been identified after 6-OHDA lesions. At the presynaptic level, it has been shown that DA neurons spared by the lesions seemed to increase their activity by increasing transmitter synthesis, metabolism, fractional release, impulse-flow, and by decreasing transmitter reuptake (Agid et al., 1973; Altar et al., 1987; Hefti et al., 1980;

Hefti, Enz, & Melamed, 1985; Snyder, Keller, & Zigmond, 1990; Stachowiak, Keller, Stricker, & Zigmond 1987; Zigmond, Acheson, Stachowiak, & Stricker, 1984). At the postsynaptic level, severe 6-OHDA lesions that deplete striatal DA by more than 90 % have been found to lead to long-lasting compensatory increases in the number of the D_2 DA receptors in the striatum, evident within two weeks after lesions (Creese et al., 1977; Mishra et al., 1974; Neve et al., 1982; Ungerstedt, 1971c).

In rats with 85-90 % of striatal DA depletions, maximal increases in presynaptic adaptations have been observed three days after 6-OHDA lesions (Altar et al., 1987), even though behavioral recovery developed gradually and was evident only three weeks later (Kozlowski & Marshall, 1981; Marshall et al., 1979). Thus, in rats with partial 6-OHDA lesions, the presynaptic adaptations could not account for the protracted time course of behavioral recovery. Rather, it was hypothesized that an increase in DA release from the remaining DA terminals in conjunction with the loss of DA reuptake sites could potentially result in much higher extracellular concentrations of DA that would be predicted from the tissue concentrations of DA. Such an ability of 6-OHDA lesioned animals to maintain relatively normal concentrations of extracellular DA was proposed to be more critical for behavioral recovery. These hypotheses began to be tested with in vivo methods of DA measurements, such as microdialysis and voltametry.

Using microdialysis, it was shown that following behavioral recovery from partial unilateral 6-OHDA lesions of the SN, the remaining DA terminals were maintaining normal concentrations of extracellular DA, not different from those measured in neurologically intact rats (Robinson & Whishaw, 1988). Importantly, striatal extracellular DA levels were significantly higher 3-4 weeks after 6-OHDA lesions than 4

days after the lesions, an effect which corresponded in time to behavioral recovery as reflected by a decrease in amphetamine-induced asymmetry of turning behavior (Robinson, Mocsary, Camp, & Whishaw, 1994). Thus, the normalization of extracellular DA was shown to be a relatively gradual process that corresponded to the protracted time course of behavioral recovery. Nonetheless, although the compensatory changes are sufficient to maintain normal extracellular DA concentrations during the resting state, they are not sufficient in demanding situations requiring increased DA release. Deficits can be reinstated if the system is "challenged" by treatment with low doses of antidopaminergic drugs or stress (Robinson & Whishaw, 1988; Snyder, Stricker, & Zigmond, 1985), possibly because such challenges deplete the remaining stores of DA on the side of the lesion.

In summary, the extraordinary ability of only a small fraction of DA terminals to maintain normal extracellular DA concentrations may be responsible for recovery of function after up to 95 % DA inputs to the striatal terminals are depleted. Despite the considerable amount of research on compensatory presynaptic and postsynaptic adaptations and their roles in recovery of function after 6-OHDA lesions of the nigrostriatal neurons, the search continues for the mechanisms underlying this effect. Identification and understanding of such mechanisms has implications for effective treatments of degenerative disease, such as PD, where function may be maintained if methods can be devised to protect even a small fraction of DA inputs to the striatum. In the next section I will present evidence suggesting that the compensatory enhancement in functioning of the midbrain DA system after partial 6-OHDA lesions may be mediated through mechanisms underlying other forms of neural plasticity in the adult DA neurons.

Similarities between Recovery from Partial 6-OHDA Lesions and Sensitization to the Effects of Psychostimulant Drugs

The fact that the clinical manifestation of PD in human patients, and the behavioral deficits associated with 6-OHDA lesions in animals, are not apparent until the depletion of striatal DA content surpasses 80% demonstrates a remarkable example of plasticity within the adult midbrain DA system. In particular, the gradual normalization of basal extracellular levels of DA released from the surviving striatal terminals, which parallels in time the protracted course of behavioral recovery, bears close resemblance to the long-lasting changes in functioning of the adult midbrain DA system that accompany behavioral sensitization to psychostimulant drugs. I will now describe similarities between the enhanced functioning of the DA system that accompanies both phenomena and I will present evidence suggesting that similar mechanisms may underlie their behavioral and neurochemical outcomes.

Enhanced Functioning of the Midbrain DA System In Behavioral Recovery from 6-OHDA Lesions and Sensitization to Psychostimulants

Behavioral sensitization refers to the increased sensitivity to the behavioral activating effects of psychostimulant drugs, such as amphetamine or cocaine, that develops after repeated exposure to these drugs and persists long after termination of drug treatment (Kalivas and Stewart, 1991; Robinson and Becker, 1986). This behavioral enhancement is a compelling example of experience-dependent plasticity associated with enduring changes in the functioning of the midbrain DA system. Acutely, drugs such as amphetamine or cocaine increase the extracellular levels of DA in both the cell body and

terminal regions of the midbrain DA neurons (Kalivas & Stewart, 1991). After repeated exposure to these drugs, however, two indications of increased DA activity remain evident in the striatal regions after termination of drug treatments: higher basal levels of DA metabolites (Akimoto, Hamamura, Kazahaya, Akiyama, & Otsuki, 1990; Patrick, Thompson, Walker, & Patrick, 1991; Robinson, Jurson, Bennett, & Bentgen, 1988; Vezina, 1993), and increased extracellular DA levels in response to a subsequent drug challenge (for reviews see: Kalivas and Stewart, 1991; Robinson and Becker, 1986). Both, the behavioral and neurochemical correlates of behavioral sensitization to stimulant drugs take time to develop and become evident several days to weeks after the termination of drug treatment (Kalivas & Duffy, 1993; Kolta, Shreve, De Souza, & Uretsky, 1985; Paulson & Robinson, 1995). This finding is very similar to what has been observed after partial 6-OHDA lesions, specifically, behavioral recovery and the corresponding normalization of extracellular levels of DA in the striatal regions also take time to develop, appearing only few weeks after the insult. In search of processes mediating the enhancement in functioning of the midbrain DA system that accompanies both phenomena, numerous investigations focused on the study of changes in the dynamics of major neurotransmitter systems and their receptors in the vicinity of DA neurons. Their results provided further evidence in support of the idea that similar mechanisms underlie the behavioral and neurochemical outcomes of recovery from partial 6-OHDA lesions and sensitization to psychostimulant drugs.

Role of Glutamatergic-Dopaminergic Interaction in Sensitization and Behavioral Recovery from 6-OHDA Lesions

An important feature of sensitization to psychostimulant drugs is that the events that lead to enhanced DA functioning are likely initiated by the actions of these drugs in the DA cell body regions. For example, repeated administration of amphetamine into the VTA, but not into the NAcc, is sufficient to induce sensitized behavioral responses and enhanced DA release in the terminal regions of DA neurons in response to subsequent drug challenges (Bjijou, Stinus, Le Moal, & Cador, 1996; Kalivas & Weber, 1988; Vezina, 1993; Vezina, 1996). The DA cell body regions receive substantial glutamatergic innervation; the VTA from the medial prefrontal cortex (Sesack & Pickel, 1992), and the SN from the pendunculopontine nucleus, subthalamus (Smith & Grace, 1992) and cerebral cortex (Carter, 1982; Kornhuber, Kim, Kornhuber, & Kornhuber, 1984). Importantly, glutamate transmission in the areas of the DA cell bodies has been found necessary for the development of sensitization to psychostimulant drugs. For example, both systemic and intra-VTA delivery of NMDA, AMPA, or metabotropic glutamate receptor antagonists concurrently with injections of amphetamine or cocaine, were found to prevent the development of behavioral sensitization (Cador, Bjijou, Cailhol, & Stinus, 1999; Kalivas & Alesdatter, 1993; Karler, Calder, Chaudhry, & Turkanis, 1989; Karler, Calder, & Turkanis, 1991; Kim & Vezina, 1998; Li, Vartanian, White, Xue, & Wolf, 1997; Stewart & Druhan, 1993; Wolf & Jeziorski, 1993) as well as the cellular correlates of behavioral sensitization observed in the VTA (Berhow, Hiroi, & Nestler 1996; Li et al., 1999; Wolf, White, & Hu, 1994). Similarly, daily treatment with glutamate receptor antagonists, MK801 and CPP, for eight days after partial unilateral 6-

OHDA lesions of the SN, blocked the behavioral recovery and the normalization of striatal DA levels on the side of the lesion (Emmi, Rajabi, & Stewart, 1996). These findings support the idea that the compensatory changes in the remaining DA neurons are similar to those responsible for sensitization within the midbrain DA system. In particular, glutamate seems to act during the periods with high potential for neural plasticity, such as immediately after exposure to psychostimulant drugs or immediately after 6-OHDA lesions, to bring about the enduring enhancement in DA functioning that accompanies the corresponding behavioral changes. In the case of 6-OHDA lesions, increased activity in glutamatergic neurons projecting to the SN has been proposed to result from loss of inhibition by the DA neurons (Albin, Young, & Penney, 1989; Greenamyre & O'Brien, 1991; Hollerman & Grace, 1992). In the case of sensitization to psychostimulant drugs, both systemic and intra-VTA injections of amphetamine and systemic injections of cocaine have been found to increase extracellular glutamate in this region.

The drug-enhanced glutamate influx in the VTA has been shown to be mediated by the activation of D_1 DA receptors, residing on terminals (possibly glutamatergic) of afferents to these regions arising from the cortex and striatum (Altar & Hauser, 1987; Dewar, Rompre, Stewart, & Warren, 1997; Sesack & Pickel, 1992). Intra-VTA injections of D_1 receptor agonists increase glutamate release in this region, whereas systemic or intra- VTA injections of D_1 receptor antagonists block the increase in glutamate release in the VTA induced by amphetamine or cocaine (Kalivas & Duffy, 1995, Kalivas & Duffy, 1998; Wolf & Xue 1998, Wolf & Xue, 1999). Importantly, D_1 receptor antagonists injected into the VTA block the development of sensitization to

amphetamine (Cador, Bjijou, & Stinus, 1995; Stewart & Vezina, 1989; Vezina, 1996), and, systemic injections of D_1 receptor antagonists for eight days after partial lesions of SN block behavioral recovery and normalization of extracellular DA in the striatum on the side of the lesion (Emmi, Rajabi, & Stewart 1997). These findings provide further evidence that the compensatory changes in the remaining DA neurons are similar to those responsible for sensitization within the midbrain DA system. Specifically, D_1 receptor stimulation in the SN and VTA leading to an increase in NMDA receptor activation by glutamate in these regions is a key step in the development of neuroadaptations underlying the enduring enhancement in the functioning of the midbrain DA system observed with sensitization and recovery from 6-OHDA lesions. In the case of sensitization, drug-induced increases in extracellular DA levels stimulate D_1 receptors and increase glutamate release within this region. In the case of 6-OHDA lesions, the enhanced glutamatergic tone that arises as a result of the lesion, could act via NMDA receptor activation to stimulate DA release from dendrites thereby increasing extracellular DA in the cell body regions. The DA could then act at the D_1 receptors in the regions and facilitate the release of glutamate, or it could influence the NMDA receptor function directly. The increases in glutamatergic tone could then act to bring about enduring changes in DA functioning that accompanies behavioral sensitization and recovery from partial 6-OHDA lesions.

Neuroadaptations Underlying the Enhanced Functioning of the Midbrain DA System that Accompanies Behavioral Recovery from 6-OHDA Lesions and Behavioral Sensitization to Psychostimulants

As mentioned frequently throughout this chapter, the striking feature of behavioral and neurochemical outcomes that accompany both recovery from 6-OHDA lesions and sensitization to psychostimulant drugs is their gradual development and persistence. Although the glutamatergic-dopaminergic interaction in the cell bodies of the midbrain DA neurons clearly plays an important role in enduring enhancement of DA functioning that accompanies both phenomena, the precise mechanisms through which these neurotransmitter systems bring about the neuroadaptations that could account for the long-lasting changes within the DA system remain to be identified. Accumulating evidence coming from the study of experience-dependent plasticity strongly suggests that truly persistent alterations in behavior are most likely to be mediated by structural modifications in neuronal circuitry and alterations in patterns of synaptic connectivity (Kolb, Forgie, Gibb, Gorny, & Rowntree, 1998; Ivanco & Greenough, 2000). Interestingly, repeated injections of psychostimulants, known to produce robust and persistent behavioral sensitization, leads to structural modifications in the brain similar to those seen in association with other forms of experience-dependent plasticity.

Repeated exposure to sensitizing regimens of amphetamine and cocaine has been found to produce increases in the length of dendrites, the density of dendritic spines, and in the number of branched spines on the medium spiny neurons in the NAcc and on the pyramidal neurons of the prefrontal cortex, as indicated by analysis of Golgi-stained material (Robinson & Kolb, 1997; Robinson & Kolb, 1999). In contrast, in animals

allowed access to wheel running, there was a decrease, rather than an increase in dendritic branching, suggesting that the effects of amphetamine and cocaine on neuronal morphology were not due to their ability to increase motor activity. Such profound changes in neuronal morphology, observed one month after termination of drug treatments, were interpreted to reflect a fundamental reorganization of synaptic inputs onto these neurons, as a consequence of past drug exposure. It is believed that the striatal medium spiny neurons and cortical pyramidal neurons receive excitatory (probably glutamatergic) inputs from the cortex and thalamus, as well as DA inputs from the SNc and VTA (Berger, Gaspar, & Verney, 1991; Freund, Powell, & Smith, 1984; Goldman-Rakic, Leranth, Williams, Mons, & Geffard, 1989; Smith & Bolam, 1990). Thus, enhancement of synaptic efficacy on the striatal and cortical DA output neurons, where DA and glutamate inputs converge, provides the structural basis for the enduring enhancement in the responsiveness of the DA neurons that develops after exposure to psychostimulant drugs, shown in the previous section to depend on the interaction between the DA and glutamate.

In the case of recovery from partial 6-OHDA lesions, it has also been suggested that the gradual normalization of extracellular DA levels could be mediated by morphological changes within the remaining DA neurons and their postsynaptic targets. Unfortunately, the few studies that examined neuronal morphology of postsynaptic DA neurons after 6-OHDA lesions, selectively studied rats with striatal DA depletions greater than 90%, as assessed by rotational asymmetry in response to apomorphine. Given that there is a permanent loss of function following such massive lesions, not surprisingly, 7 to 13 months later, the density of spines was reduced on the medium spiny neurons in

dorsal striatum ipsilateral to the lesions (Ingham, Hood, & Arbuthnott, 1989; Ingham, Hood, van Maldegem, Weenink, & Arbuthnott, 1993). Interestingly, however, small but significant increases in the length of asymmetric synapses were detected in the striatum on the side of the lesion (Ingham et al., 1993). Further, in a subsequent study, a significant increase in the numerical density of a subpopulation of asymmetric synapses with complex, discontinous synapses that appeared perforated has been found (Ingham, Hood, Taggart, & Arbuthnott, 1998). Ipsilaterally to the lesion, this population of perforated asymmetric synapses was surrounded by higher density of particles immunogold-labeled for glutamate, indicating a possible hyperactivity in these synapses. These findings are important since synapses with complex or perforated postsynaptic densities have been shown to have increased efficacy and have been implicated in synaptic remodeling in the hippocampus, where their numbers increase with long-term potentiation (Calverley & Jones, 1990; Geinisman, de Toledo-Morrell, & Morrell, 1991). Furthermore, a similar analysis of the morphological characteristics of the synapses in brains of PD patients revealed significant increases in the length of the postsynaptic synapses and the number of perforated synapses in the neurons of the caudate nucleus, indicating plasticity of the putative corticostriatal synapses in PD (Anglade, Mouatt-Prigent, Agid, & Hirsch, 1996). Thus, even with near complete depletions of striatal DA, there is evidence of structural changes on the major postsynaptic DA neurons in the striatum, suggestive of complex synaptic interactions that have been implicated in other forms of experience-dependent synaptic plasticity.

Massive 6-OHDA lesions of the MFB have also been shown to lead to morphological changes in the NAcc (Meredith, Ypma, & Zahm, 1995). In the NAcc

core, a near total loss of DA was associated with shortened dendrites and spine loss. In the medial part of NAcc shell, where the loss of tyrosine hydroxylase-immunoreactive fibers was never complete, higher dendritic tortuosities (lengthening of dendrites due to complex twisting and curving of parts of some dendritic segments) were observed. Due to the lengthening of the dendrites, the actual number of spines was preserved, even though the spine density (number of spines per unit of the dendrite) was reduced. Thus, it is tempting to hypothesize that the surviving DA terminals may be sufficient to preserve the spines and bring about the increase in dendritic tortuosity in the medial part of NAcc shell. This possibility, however, needs to be addressed in studies of partial, and not massive, 6-OHDA lesions before any conclusions about structural adaptations and behavioral and neurochemical recovery can be reached.

Finally, in addition to the morphological changes in the postsynaptic target neurons of the midbrain DA system after 6-OHDA lesions, one must also consider the morphology of the DA neurons themselves, especially their ability to reinnervate the denervated striatum. Compensatory sprouting of dopaminergic fibers has been in fact proposed to account for the remarkable normalization of extracellular DA levels and the corresponding behavioral recovery after partial 6-OHDA lesions. Evidence of morphological reorganization were obtained after unilateral lesions of SNc, where sprouting of hypertrophic TH-IR fibers entering the ventrolateral part of the striatum was detected on the lesioned side 4 and 7 months, but not 10 days, after lesioning (Blanchard, Anglade, Dziewczapolski, Savasta, Agid, & Raisman-Vozari, 1996). These fibers were more numerous after a post-lesion delay of 7 rather than 4 months, reflecting a progressive process. Ultrastructural examination revealed hypertrophic dopaminergic

fibers and growth-cone-like structures, confirming the existence of re-growth and sprouting of DA fibers. TH-IR varicosities (the sites of synaptic contacts) were observed frequently to make symmetric synapses with dendritic processes or spines. On the heads of the spines in contact with TH-IR varicosities, TH-immunonegative terminals filled with synaptic vesicles formed asymmetric synapses characterized by thick postsynaptic densities. Thus, DA neurons have the capacity for spontaneous axonal re-growth and reinnervation of the partially denervated striatum, but these effects have not been looked at in context of behavioral recovery from 6-OHDA lesions.

To my knowledge, only one investigation quantified the extent of axonal sprouting by SNc neurons in response to varying degrees of 6-OHDA-induced loss of SNc neurons. Although behavioral recovery was not assessed in this study, its likelihood could be inferred from the extent of DA cell loss, based on the relationships between cell loss and behavioral recovery presented in the earlier section. The results revealed that after partial lesions of the SNc, leading to 25-55 % depletion of the total number of SNc neurons, the surviving neurons sprouted extensively within the dorsal striatum (Finkelstein, Stanic, Parish, Tomas, Dickson, & Horne, 2000). The increased terminal arborization was manifested by a substantial increase in collateral branching (increasing branching points) and increased axonal varicosity size (the sites where synapses occur). Furthermore, these increases in the number of branch points and axonal varicosities was proportional to the size of the lesion, as quantified by loss in TH-IR cells or Neutral Red counterstained neurons of the SNc. After depletion of the total number of SNc neurons exceeding 75%, the remaining SNc neurons seemed unable to increase the size of the axonal arbor as estimated by the dopamine transporter (DAT)-IR to visualize striatal

terminals. Thus, after massive lesions, previously reported to lead to a permanent loss of behavioral function, the capacity for regrowth and striatal reinnervation appears to be lost. Exactly how these structural changes on DA neurons would affect the dendritic arborization of the post-synaptic neurons remains to be tested.

In summary, growing evidence suggests that structural modifications in neuronal circuitry and synaptic connectivity, similar to those observed in other forms of experience-dependent synaptic plasticity, underlie the enduring enhancement of function within the DA system that accompanies behavioral sensitization to psychostimulant drugs and recovery from partial 6-OHDA lesions. Although factors mediating these structural modifications remain to be elucidated, molecules normally involved in neural plasticity, such as neurotrophic factors and other precursors to structural adaptations, are likely to play a role. In the next section I will provide evidence that neurotrophic factors profoundly affect the structure and function of the midbrain DA neurons and may thus initiate the morphological and neurochemical changes observed after exposure to psychostimulant drugs and after partial 6-OHDA lesions.

Neurotrophic factors

Neurotrophic factors possess an extraordinarily wide array of activities and can be broadly defined as endogenous soluble proteins regulating neuronal growth and differentiation in the developing brain, as well as survival, maintenance, and morphological plasticity in the adult brain (Hefti, Denton, Knusel, & Lapchak, 1993). There are several major classes of neurotrophic factors that act within the nervous system and that are synthesized by a variety of cell types, including neurons and glial cells.

Some appear to act exclusively in the nervous system, while others act on a number of cell types throughout the body in addition to the nervous system, some seem to regulate specific aspects of development, while others function throughout life. The developmental function of neurotrophic factors has been studied extensively in the peripheral nervous system and led to the formulation of the neurotrophic factor hypothesis. This hypothesis proposed that the selective survival of only a fraction of neurons initially generated during development is due to the competition of the innervating neuronal processes for a limited amount of neurotrophic factors supplied by the target tissue; only the successful competitors survive (Barde, 1989; but see Oppenheim, 1989). In the central nervous system, on the other hand, the actions of neurotrophic factors are less clearly defined. In fact, the concept of neurotrophic factors as specialized, target-derived molecules, each mediating survival and enhancing differentiation of distinct neuronal types had to be modified, as these factors emerged as highly pleiotrophic molecules with a considerable overlap in biological activities (Korsching, 1993). In addition to their classic target-derived actions, they can be also secreted into the extracellular milieu, where they may act in either paracrine fashion on other cells, or autocrine fashion on the cells that secrete them.

The responsiveness of a cell to a particular growth factor is dependent on the expression of specific receptors on the plasma membrane. The receptors for many of the neurotrophic factors are themselves protein kinases, whose enzymatic activity is stimulated upon association with their specific ligand, thereby initiating intracellular signaling events that may lead to gene transcription (Segal & Greenberg, 1996). The expression of specific receptors is highly regulated during development and temporally

assures the sensitivity of cells to neurotrophic factors present in their environment. As mentioned earlier, individual neurons or glial cells are responsive to a number of different growth factors, however, high degree of specificity can be achieved due to the developmental regulation of the spatial and temporal expression pattern for all subtypes of the various neurotrophic factors, their receptors and intracellular signaling components.

Several neurotrophic factors have been found in the adult brain, suggesting that they play important roles on brain function throughout life span of an individual. Initially, their presence in the adult brain was thought to be limited to brain injury or damage, occurrences believed to "recapitulate" a cascade of developmental signaling processes in an attempt to promote neuronal survival and reestablishment of functional connectivity of neural circuits (Mattson & Scheff, 1994; Nieto-Sampedro & Cotman, 1985). However, localization studies employing in situ hybridization or immunocytochemistry techniques in the intact adult brain revealed the presence of the mRNA or functional protein product, respectively, for neurotrophic factors and their receptors in various neuronal and non-neuronal populations. Interestingly, the presence of numerous neurotrophic factors and their receptors has been consistently found in hippocampal and cortical neurons, the areas of key importance for learning and memory, suggesting that these factors may be involved in the regulation of morphological changes that accompany experience-dependent plasticity in the adult brain.

The midbrain DA system also remains highly plastic throughout life, as indicated by the phenomena of behavioral sensitization and recovery of function after partial 6-OHDA lesions of the nigrostriatal neurons. Several neurotrophic factors have been shown

to be expressed by and to influence the functioning of the midbrain DA neurons. The actions of representative factors from the three prominent families encompassing the neurotrophins (prototype member: brain-derived neurotrophic growth factor, BDNF), the transforming growth factor B superfamily (prototype member: glial cell line-derived neurotrophic factor, GDNF) and fibroblast growth factor family (prototype member: basic fibroblast growth factor, FGF-2) on functioning of the developing, adult, and injured DA neurons will be presented in the following sections.

Effects of Neurotrophic Factors on DA Neurons In Vitro

GDNF, BDNF, and FGF-2 have all been shown to exert neurotrophic and neuroprotective effects on DA neurons during early development. Exposure of embryonic mesencephalic cultures to these factors has been found to enhance the survival, morphological differentiation, and high-affinity DA uptake on DA neurons (GDNF: Hou, Lin, & Mytilineou, 1996; Lin, Doherty, Lile, Bektesh, & Collins, 1993; BDNF: Beck, Knusel, & Hefti, 1993; Hyman et al., 1991; Spina, Hyman, Squinto, & Lindsay, 1992; Spina, Squinto, Miller, Lindsay, & Hyman, 1992; Studer et al., 1995; FGF-2: Beck et al., 1993; Ferrari, Minozzi, Toffano, Leon, & Skaper, 1989; Knusel, Michel, Schwaber, & Hefti, 1990; Matsuda, Saito, & Nishiyama, 1990) Some of the morphological changes included increases in soma size, primary neurite length and number, density of neuritic varicosities, and growth cone elaboration, suggesting that these neurotrophic factors play a significant role in the structural plasticity of developing DA neurons.

GNDF, BDNF, and FGF-2 have also been shown to protect cultures of embryonic mesencephalic DA neurons from neurotoxins, such as MPP^+ ion or 6-OHDA. Treatment

of cultures with these factors improved survival of TH-IR cells, prevented further cell death and stimulated recovery of DA uptake after exposure to MPP^+ or 6-OHDA (GNDF: Hou et al., 1996; BDNF: Beck et al., 1992; Hyman et al., 1991; Spina, Hyman, et al., 1992; Spina, Squinto, et al., 1992; FGF-2: Hou, Cohen, & Mytilineou, 1997; Krieglstein, Reuss, Maysinger, & Unsicker, 1998; Otto & Unsicker, 1993; Park & Mytilineou, 1992). Furthermore, GDNF and FGF-2 stimulated extensive neurite outgrowth after toxin-induced structural damage. This regrowth of damaged processes occurred concomitantly with the pronounced increase in DA uptake, which even surpassed the DA uptake observed in untreated controls. Unlike GDNF and BDNF, however, FGF-2 required the presence of astrocytes to exert its neurotrophic and neuroprotective effects (Engele & Bohn, 1991; Hou et al., 1997; Park & Mytlineou, 1992). Thus, in vitro, neurotrophic factors exert profound effects on plasticity within the developing and injured DA neurons, as indicated by the enhancements in morphological and neurochemical parameters of DA functioning.

Effects of Neurotrophic Factors on DA Neurons in Intact Rats

Intracranial administration of GDNF or BDNF (within the nigrostriatal pathway or intracerebroventricularly) has been shown to exert long-lasting and potent stimulatory effects on spontaneous locomotor behavior, suggestive of enhanced activity within the midbrain DA system (GDNF: Hebert & Gerhardt, 1997; Hebert, Van Horne, Hoffer, & Gerhardt, 1996; Hudson et al., 1995; Martin et al.,1996; BDNF: Martin-Iverson & Altar, 1996; Martin-Iverson, Todd, & Altar, 1994). Several studies reported that administration of GDNF or BDNF induced enhancements in DA turnover (Altar et al., 1992; Horger et al., 1998; Hudson et al., 1995; Lapchak, Jiao, Collins, & Miller, 1997; Lucidi-Phillipi et

al., 1995; Martin-Iverson et al., 1994), extracellular DA levels (Herbert & Gerhardt, 1997), and tissue levels of DA and its metabolites (Herbert et al., 1996; Herbert & Genhard, 1997; Martin et al., 1996) within the DA cell body or terminal regions (but see Lapchak, Beck, Araujo, Irwin, Langston, & Hefti, 1993). Furthermore, supranigral infusions of BDNF have been shown to increase the electrical activity of SNc DA neurons in vivo, as illustrated by increases in the number of spontaneously active cells, their average firing rate and the number of action potentials contained within bursts of activity (Shen, Altar, & Chiodo, 1994). The behavioral activating effects of GDNF and BDNF and the concomitant indices of enhanced DA functioning, which in the case of GDNF were observed even after a single injection of this factor, persisted for several weeks after the administration of these factors. Such long-lasting and persistent effects of these factors on functioning of the DA system in vivo are reminiscent of those accompanying behavioral sensitization to psychostimulant drugs, and support the idea that neurotrophic factors may mediate the development of sensitization.

On the morphological level, injection of fibroblasts genetically modified to express BDNF was found to promote sprouting of TH– and neurofilament-IR fibers from the DA neurons in vivo six weeks after implantation into the SN of intact rats (Lucidi-Phillipi et al., 1995). Administration of GDNF into the SN of intact rats, resulted in a robust increase in the density of TH-IR processes in the SN and striatum ipsilateral to the injection observed 24 hours to 7 days later, reflecting either increased TH activity within these neurons or increased sprouting (Hudson et al., 1995; Lapchak et al 1996). Furthermore, two weeks after 6 intrastriatal injections of GDNF, the size of the DA cell bodies in the SNc ipsilateral to the side of the injections increased in a dose dependent

manner and striatal TH-IR profiles reminiscent of growth cones in the developing DA system became significantly more numerous in GDNF treated rats than in the vehicle-treated rats (Shults, Shin, Ernesto, & Martin, 1995). Thus, intracranial administration of neurotrophic factors in intact adult animals exerts long-lasting enhancements on the behavioral, neurochemical, and morphological indices of DA neuron functioning, similar to those observed after sensitization.

Several studies examined whether neurotrophic factors can alter the effects of psychostimulant drugs by microinfusing neurotrophic factors intracranially and assessing behavioral and neurochemical changes to subsequently administered amphetamine or cocaine. Continuous infusion of BDNF into the VTA or SN resulted in a persistent increase (up to 12 months) in the amphetamine-induced rotations contraversive to the site of BDNF injection, consistent with the increased activity of the nigrostriatal DA system on the side of injection (Altar et al., 1992; Altar, Boylan, Fritsche, Jackson, Hyman, & Lindsay, 1994; Martin-Iverson et al., 1994; Shults, Matthews, Altar, Hill, & Langlais 1994). This behavioral effect was accompanied by enhanced striatal DA turnover at the time when peak rotational effects of amphetamine occurred and was blocked by administration of DA antagonists. Intra-VTA and intra-NAcc infusions of BDNF also enhanced the stimulant effects of an acute injection of cocaine and facilitated the development of sensitization to repeated cocaine injections (Horger, Iyasere, Berhow, Messer, Nestler, & Taylor, 1999; but see Pierce, Pierce-Bancroft, & Prasad, 1999). This enhanced response to cocaine in BDNF-treated animals persisted for more than a month after termination of BDNF treatment. Similarly, intra-SN administration of GDNF was accompanied by increased amphetamine-induced locomotor activity (Hebert et al., 1996;

Horger et al., 1998; Hudson et al., 1995), which persisted for at least a week after the injection of the factor. Unilateral overexpression of GDNF in the striatum, via viral-vector mediated delivery, was found to induce a marked turning bias, either spontaneously or after amphetamine injection (Georgievska, Kirik, Rosenblad, Lundberg, & Bjorklund, 2002; Kirik, Rosenblad, Bjorklund, & Mandel, 2000), accompanied by a 2- to 3-fold increase in DA turnover in the ipsilateral striatum 3 weeks after vector delivery. In another study, three weeks after GDNF administration, amphetamine-induced release of extracellular DA was augmented in the striatum and NAcc. These findings suggest that infusions of exogenous neurotrophic factors can further enhance the behavioral and neurochemical responses to psychostimulant administration.

Effects of Exogenously Delivered Neurotrophic Factors on Recovery from 6-OHDA Lesions in Rats

The effects of intracranial delivery of GDNF have been the most extensively studied, by far, in the nigrostriatal DA degeneration models. This is due to the fact that GDNF has been cloned and purified based on its ability to stimulate survival and differentiation of DA neurons and because it has been shown to have the highest neurotrophic potency on these neurons (Lin et al., 1993). Several studies revealed beneficial effects of GDNF administration within the DA pathway, via intracranial injections, or via viral vector delivery, administered at various time points before or even after lesioning. Administration of GDNF from 3 weeks to 6 hours before lesioning was shown to protect against loss of cells in the DA cell body regions and/or against loss of DA innervation of the striatum (Choi-Lundberg et al., 1997; Choi-Lundberg et al., 1998; Connor et al., 1999; Georgievska et al., 2002; Kearns & Gash, 1995; Kirik et al., 2000;

Kirik, Rosenblad, & Bjorklund, 2000; Kozlowski, Connor, Tillerson, Schallert, & Bohn, 2000; Mandel, Spratt, Snyder, & Leff, 1997; Natsume et al., 2001). Late delivery of GDNF (1-4 weeks after lesioning) led to recovery of TH-IR cells in SN and sprouting of remaining DA fibers in the striatum (Bowenkamp et al., 1996; Kozlowski et al., 2000; Wang et al., 2002; Winkler, Sauer, Lee, & Bjorklund, 1996). Behavioral improvements have been observed after both the pre-lesion and post-lesion administration of GDNF. In addition, pretreatment with GDNF before 6-OHDA lesions led to enhanced DA neuron functioning as evidenced by significant increases in basal and stimulated DA release measured using in vivo microdialysis (Opacka-Juffry, Ashworth, Hume, Martin, Brooks, & Blunt, 1995; Gerin, 2002).

The neuroprotective effects of BDNF and FGF-2 after nigrostriatal lesions have not been studied as extensively as those of GDNF. Chronic intra-SN infusions of BDNF were found to decrease the amphetamine-induced turning after 6-OHDA lesions of the striatum, and to increase striatal levels of DA metabolites (Altar, Boylan, Fritsche, Jones, et al., 1994). Grafting of BDNF secreting astrocytes significantly attenuated amphetamine-induced rotation after 6-OHDA lesions of the SN (Yoshimoto et al., 1995), but, it did not protect against the loss of TH-IR in the striatum on the side of the lesion. Intrastriatal implantation of BDNF- (Levivier, Przedborski, Bencsics, & Kang, 1995) or FGF-2-producing fibroblasts (Shults, Ray, Tsuboi, & Gage 2000) prior to intrastriatal 6-OHDA lesions led to preservation of TH-IR nigral DA neurons and striatal DA innervation and, in the case of FGF-2, resulted in a strikingly attenuated amphetamine- and apomorphine-induced rotation. In the MPTP lesion model, exogenous delivery of BDNF (Frim et al., 1994) or FGF-2 (Chadi et al., 1993; Date et al., 1993; Otto &

Unsicker, 1990) has also been reported to markedly increase TH-IR cell survival in the SNc, TH-IR striatal innervation, and the levels of DA and TH activity. Nonetheless, in the case of BDNF, lack of neuroprotection also has been reported; BDNF-producing fibroblasts did not protect against axotomy induced degeneration of DA neurons via MFB transections (Knusel et al., 1992; Lapchak et al., 1993) and against intra-MFB 6-OHDA infusions (Lucidi-Phillipi et al., 1995).

In summary, the multitude of findings described above consistently show that neurotrophic factors, such as GDNF, BDNF, and FGF-2 exert potent neurotrophic and neuroprotective effects on the functioning of DA neurons in vitro and in vivo. The ability of these factors to enhance neurochemical and morphological plasticity within developing, adult, and injured DA neurons and to produce concomitant changes in behavior, strongly implies that their actions may underlie the long-lasting behavioral and neurochemical outcomes accompanying recovery from partial 6-OHDA lesions and sensitization to psychostimulant drugs. The role of endogenous expression of neurotrophic factors in these phenomena remains to be elucidated. In the study of behavioral sensitization, endogenous expression of FGF-2, in particular, has been shown to play a critical role in the development of long-lasting changes induced by repeated administration of amphetamine. These findings will be presented in the next section leading up to a proposal that endogenous expression of FGF-2 may also play a role in recovery from partial unilateral 6-OHDA lesions of the nigrostriatal neurons.

A Rationale for Proposing a Role for Endogenously Induced Expression of FGF-2 in Recovery from 6-OHDA Lesions

Studies examining the developmental course of FGF-2 expression consistently reveal that both the protein and the message are barely detectable at the time of birth but increase in specific neuronal populations and astrocytes until adult levels are reached at postnatal day 28 (Eckenstein, Andersson, Kuzis, & Woodward, 1994; Kuzis, Reed, Cherry, Woodward, & Eckenstein, 1995; Riva & Mocchetti, 1991). Thus, the most likely role of FGF-2 is restricted to regulating maturation, maintenance, and repair within the mature nervous system. As is the case for many neurotrophic factors, the high affinity FGF-2 receptor, FGFR1, is a tyrosine kinase receptor that is dimerized upon ligand binding leading to activation of signaling cascades (Schlessinger & Ullrich, 1992). In vitro, FGFR1 inhibitor has been shown to potently and selectively antagonize the neurotrophic actions of FGF-2 (Skaper, Kee, Facci, Macdonald, Doherty, & Walsh, 2000). In the adult brain, the expression of mRNA and protein product for FGF-2 and FGFR1 is widespread throughout neuronal and non-neuronal populations, with astrocytes containing the highest levels within the nucleus. Exceptionally high levels of mRNA and protein for the high affinity receptor FGFR1 have been found in the cell body and terminal regions of DA neurons (Cintra et al., 1991; Gonzalez, Berry, Maher, Logan, & Baird, 1995; Wanaka, Johnson, & Milbrandt, 1990). Cytoplasmic FGF-2-IR has been demonstrated within the majority of nigral DA neurons (Bean et al., 1991; Cintra et al., 1991), whereas nuclear astrocytic FGF-2-IR has been found within both SNC and SNR and within striatum (Cintra et al., 1991).

The results of the recent series of experiments indicated that endogenous astrocytic FGF-2 plays an important role in the development of behavioral sensitization to amphetamine. As few as three injections of amphetamine have been found to induce increased expression of the FGF-2, in astrocytes in the DA cell body regions, VTA and SNc, evident for up to one month after the last injection of the drug (Flores, Rodaros, & Stewart, 1998). Furthermore, a two-week escalating-dose regimen of amphetamine was shown to induce increases in FGF-2 expression in the DA striatal terminal regions, as well as in VTA and SNc (Flores and Stewart, 2000b). This finding, in particular, supports the idea that FGF-2 may be involved in the persistent structural modifications on the major DA output neurons in the nucleus accumbens (NAcc) and prefrontal cortex reported after similar treatment with amphetamine (Robinson and Kolb, 1997; Robinson and Kolb, 1999).

More importantly, however, it was found that the number of FGF-2-IR astrocytes in the VTA and SNc was strongly and positively correlated with the magnitude of behavioral sensitization, and that infusions of a neutralizing antibody to FGF-2 into the VTA prior to amphetamine administration prevented the development of sensitization (Flores, Samaha, & Stewart, 2000). Since behavioral sensitization to amphetamine and increased expression of endogenous FGF-2 were both prevented when amphetamine injections were preceded by injections of NMDA receptor antagonists, FGF-2 has been proposed to function as a possible mediator of the effects of glutamate on the development of sensitization. As mentioned earlier, glutamate has been also found to mediate recovery from partial 6-OHDA lesions. Thus, endogenous expression of neurotrophic factors, particularly FGF-2, may critically contribute to initiation of the

long-lasting behavioral and neurochemical changes after repeated administration of amphetamine and after recovery from nigrostriatal DA lesions.

The evidence presented in this chapter supports that idea that similar mechanisms may underlie the enhanced functioning of the midbrain DA system that accompanies recovery after partial 6-OHDA lesions and sensitization to psychostimulant drugs. Since endogenous expression of FGF-2 is critically involved in the long-lasting behavioral and neurochemical changes induced by repeated administration of amphetamine, it is reasonable to hypothesize that endogenous FGF-2 is likely to be involved in recovery from 6-OHDA lesions. In fact, increases in astrocytic FGF-2 mRNA and protein product have been observed after unilateral 6-OHDA lesions of SN, but no information was given about behavioral outcomes of these lesions (Chadi, Cao, Petterson, & Fuxe, 1994).

In the present thesis, the expression of endogenous astrocytic FGF-2 was examined in relation to behavioral and cellular outcomes of intra-MFB infusions of 6-OHDA. Experiments presented in Chapter 2 and 3 begin with a study of the time course of FGF-2 expression after 6-OHDA lesions, followed by an examination of the effects of manipulations known to affect sensitization to psychostimulant drugs on 6-OHDA-induced FGF-2 expression. In Chapter 4, the effect of forced limb-use, a behavioral manipulation known to facilitate recovery after extensive lesions of nigrostriatal DA pathway, was studied on 6-OHDA–induced expression of FGF-2. Finally, in Chapter 5, the effects of pre-lesion treatment with sensitizing regimens of amphetamine, known to increase endogenous expression of FGF-2 in the vicinity of DA neurons, were examined on behavioral and neurochemical outcomes after subsequent 6-OHDA lesions.

CHAPTER II

EFFECTS OF SEX AND HORMONAL STATUS ON ASTROCYTIC FGF-2- AND TH-IMMUNOREACTIVITY AFTER MEDIAL FOREBRAIN BUNDLE 6-HYDROXYDOPAMINE LESIONS OF THE MIDBRAIN DOPAMINE NEURONS

I. A. Moroz, H. Rajabi, D. Rodaros and J. Stewart

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Abstract

We examined astrocytic basic fibroblast growth factor immunoreactivity (FGF-2-IR) and tyrosine hydroxylase immunoreactivity (TH-IR) in the cell body region of midbrain dopaminergic neurons after unilateral infusions of the neurotoxin 6-hydroxydopamine into the medial forebrain bundle in male and female rats. In addition, to determine whether neonatal exposure to gonadal hormones has consequences on the expression of astrocytic FGF-2 and cell loss in response to injury in adulthood, we studied the effects of these lesions in adult male and female rats that had been exposed or not to testosterone in the neonatal period. In both males and females there was a progressive loss of TH-expressing cells that peaked five weeks after the lesions. Females showed less loss of TH-expressing cells than males, but this effect was not estrogen dependent. Lesions led to an increase in expression of astrocytic FGF-2 that was greater in males than in females. Finally, it was found that, regardless of genetic sex, rats exposed to testosterone neonatally showed greater astrocytic FGF-2 expression after lesions than those not exposed, and that among those not exposed to testosterone, estrogen treatment had a modest protective effect.

Analysis of behavior and striatal dopamine content showed that the percent of striatal dopamine depletion 14 days after the lesion correlated with the amount of behavioral asymmetry displayed by animals on all tests conducted after lesioning. In groups killed 2 and 5 weeks after the lesion, the amount of behavioral asymmetry correlated with the percent loss of TH-IR cells and with the percent increase in FGF-2-IR cells in the midbrain. These relationships were not evident in groups killed 3 and 7 days

after the lesion, possibly because the changes in the number of FGF-2- and TH-IR cells were not fully manifested.

The present findings show that hormonal events early in life can alter the response of midbrain dopamine neurons to insult and injury in adult life and suggest that the slow degeneration of these neurons may release signals triggering a sustained activation of adjacent astrocytes which, in turn, may lead to induction of astrocytic FGF-2.

Introduction

The nigrostriatal and mesolimbic dopaminergic systems, with dopamine (DA) cell bodies in the substantia nigra compacta (SNc) and ventral tegmental area (VTA) projecting to the dorsal and ventral striatum, respectively, play significant roles in sensorimotor integration, movement, and motivation. Unilateral application of 6-hydroxydopamine (6-OHDA), a selective catecholaminergic neurotoxin, has been used frequently to study the capacity of these neurons to recover from or to compensate for damage or insult. As with all other types of brain injury, there is a considerable variation in the degree of degeneration induced by unilateral administration of 6-OHDA. This variation depends on several factors, including the site and dose of 6-OHDA administration (Kirik et al., 1998; Przedborski et al., 1995), but in addition may depend on other factors such as hormonal status (Dluzen, 1997), age (Marshall, Drew, & Neve, 1983), and post-lesion experiences (Tillerson et al., 2001).

Behavioral recovery from partial unilateral lesions is accompanied by gradual normalization of basal levels of extracellular DA in the striatum measured using microdialysis (Altar et al., 1987; Castaneda, Whishaw, & Robinson, 1990; Robinson & Whishaw, 1988). This normalization is taken as evidence for compensatory changes in the functioning of the remaining DA neurons. These changes are gradual and include increases in the synthesis, metabolism, and release of DA per impulse in remaining terminals, as well as a decrease in uptake sites in the area (Altar et al., 1987; Robinson et al., 1994; Snyder et al., 1990). Compensatory changes have also been observed within the midbrain DA system after repeated injections of the stimulant drug amphetamine,

known to cause massive increases in extracellular DA in midbrain cell body and terminal regions. Such changes are observed several days, even weeks, after termination of amphetamine treatment and include enhanced extracellular DA levels in response to amphetamine challenge (Kalivas & Stewart, 1991; Robinson & Becker, 1986). Interestingly, it has been found that manipulations that prevent the development of sensitization to amphetamine, such as the administration of NMDA receptor antagonists or D1/D5 DA receptor antagonists, also interfere with behavioral recovery from partial 6-OHDA lesions and normalization of basal levels of DA in the striatum (Emmi et al., 1996; Emmi et al., 1997).

Another common feature that studies of the effects of such neurotoxic lesions and the stimulant drugs have revealed is a sex difference in response to these manipulations. For example, there are sex differences in the susceptibility of the nigrostriatal system to neurotoxic agents; female mice show less striatal DA depletion in response to nigrostriatal toxins such as MPTP (Brooks, Jarvis, & Wagner, 1989; Freyaldenhoven, Cadet, & Ali, 1996; Miller, Ali, O'callaghan, & Laws, 1998) and methamphetamine (Wagner, Tekiran, & Cheo, 1993; Yu & Wagner, 1994). There are also robust sex differences in the behavioral response to psychostimulants; female rats show greater behavioral responsiveness to both acute and repeated injections of amphetamine than male rats (Becker, Robinson, & Lorenz, 1982; Camp & Robinson, 1988; Forgie & Stewart, 1993; Forgie & Stewart, 1994) and greater amphetamine-stimulated DA release in striatum both in vitro and in vivo (Becker & Ramirez, 1981; Becker, 1990; Becker & Rudick, 1999). These sex differences may be attributable to estrogen which has been shown to exert modulatory effects on functioning of the nigrostriatal and mesolimbic DA

systems, affecting the synthesis (Pasqualini, Olivier, Guibert, Frain, & Leviel, 1995), metabolism (Di Paolo, Rouillard, & Bedard, 1985), and release (McDermott, Liu, & Dluzen, 1994) of DA.

Estrogen may function as a neuroprotectant of the nigrostriatal system. Estrogen administration before treatment with the selective dopaminergic neurotoxin MPTP has been reported to significantly attenuate the amount of striatal DA depletion produced by MPTP in both gonadectomized female and male mice (Dluzen, McDermott, & Liu, 1996a; Dluzen, McDermott, & Liu, 1996b) and in intact male mice (Callier, Morissette, Grandbois, & Di Paolo, 2000; Grandbois, Morissette, Callier, & Di Paolo, 2000). This capacity of estrogen to preserve striatal DA concentrations was also observed in the 6-hydroxydopamine lesioned rat (Dluzen, 1997) and the methamphetamine treated CD1 mouse (Doherty, 2000).

The gradual nature of the changes induced by repeated administration of stimulant drugs and those accompanying behavioral recovery from partial 6-OHDA lesions suggests the operation of neurotrophic factors, known to play a critical role in the survival, maintenance, and morphological plasticity of adult neurons. Interestingly, increased expression of neurotrophic and neuroprotective factor basic fibroblast growth factor (bFGF or FGF-2) in the VTA and SNc has been shown after repeated administration of amphetamine (Flores et al., 1998) and after 6-OHDA lesions (Chadi et al., 1994). Furthermore, estrogen loss has been reported to increase the expression of FGF-2 in the VTA and in cortical projection regions of DA cells after ovariectomy in adult rats (Flores, Salmaso, Cain, Rodaros, & Stewart, 1999), suggesting that estrogen loss itself is capable of inducing injury-like reactions in DA neurons. Thus, the enhanced

expression of FGF-2 seen in the DA cell body regions may be part of the cascade of intracellular and intercellular neuroprotective events that lead to long-lasting changes in the functioning of the DA neurons.

The present experiments were carried out to examine astrocytic FGF-2 expression within the nigrostriatal DA system after injections of the neurotoxin 6-OHDA, in male and female rats and to determine whether neonatal exposure to testosterone would alter the effects of the adult lesions on the expression of astrocytic FGF-2 in response to injury in adulthood.

Experimental Procedures

Subjects

Adult male and female Wistar rats (Charles River, St. Constant, Quebec) served as subjects in Experiment 1 and 2, and were mated to produce subjects for Experiment 3. All animals were housed individually in stainless steel cages and maintained in a temperature- and humidity-controlled environment under a 12 hr light/dark cycle. Food and water were available *ad libitum*. Animals obtained as adults were handled and habituated to the laboratory before surgery. All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the Concordia University Animal Care Committee.

Materials

6-OHDA (6-hydroxydopamine hydrochloride, Sigma) was dissolved in 0.9 % saline containing 0.05 % ascorbic acid (Aldrich Chemical Company, Inc.) immediately

before being injected. Desmethylimipramine (RBI Biochemicals) was dissolved in nanopure water and Pargyline (Sigma) was dissolved in 0.9 % saline. Estradiol benzoate (EB, Sigma) and testosterone proprionate (TP, Sigma) were dissolved in peanut oil. EB was injected subcutaneously at a dose of 5 μg/0.1 ml per animal and TP was injected subcutaneously at a dose 200 μg/0.1 ml per animal. Primary antibodies generated in mice and rabbits were used at the following concentrations: mouse monoclonal anti-FGF-2 (1:500; Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal anti-TH (1:5000; Chemicon), mouse monoclonal anti-GFAP (1:1000, Sigma), and mouse monoclonal anti-OX42 or CD11b/c (1:1000; Cedar Lane, Hornsby, Ontario).

Lesions

Rats were anesthetized with sodium pentobarbital (males: 30 mg/kg i.p.; females: 21 mg/kg) followed by atropine sulfate (0.5 mg/ml, 0.2 ml/rat, s.c.). Methoxyflurane (Metofane) was used to supplement the anesthesia throughout the surgery. Animals were given desmethylimipramine (15 mg/kg, i.p.), a norepinephrine reuptake inhibitor (to protect the noradrenergic cells from 6-OHDA), and pargyline (40 mg/ml s.c.), an MAO-inhibitor, 30 min before infusion of 6-OHDA. 6-OHDA (2 μl of 8 μg/4 μl solution) was infused unilaterally using a Hamilton microsyringe (0.2 μl/min) over a period of 10 min into the medial forebrain bundle (MFB). Stereotaxic coordinates, with flat skull, were: 2.9 mm posterior, 1.7 mm lateral to bregma, and 7.6 mm ventral to dura for males, and 2.9 mm posterior, 1.9 mm lateral to bregma, and 7.4 mm ventral to dura for females. The injector was slowly removed 5-10 min after the end of the infusion. All animals were injected with an injectable antibiotic Penlong XL (0.2 ml/rat i.m.) after surgery.

Behavioral testing

To verify lesions, animals were tested for lateralized behavioral deficits, specifically, ipsiversive turning (turning toward the side of the lesion) without restraint (i.e., harnesses utilized in rotometers) and without the use of drugs. Behavioral tests were conducted in wooden boxes (58 x 58 x 48 cm) painted flat black and open at the top to allow the camera suspended from the ceiling to record the test. Animals were tested 3, 7, 14 days, and 5 wks after lesioning (depending on the survival time of the group). Test duration was 10 min. The number of compact (within the diameter of approximately 20 cm) 360 degree turns and 180 degree half-turns ipsilateral and contralateral to the side of the lesion were recorded and summed across the 10 min sessions. To assess the degree of behavioral asymmetry, the number of ipsilateral turns was presented as a percent of the total number of turns displayed by an animal (ipsi/[ipsi + contra] x 100%).

Post mortem tissue analysis (Experiment 1B)

Animals were killed by decapitation and their brains were rapidly removed, placed in isopentane, cooled on dry ice, and frozen overnight at –80 degrees C. The following day the brains were sliced on a cryostat into 200 μm sections. Punches were taken from the dorsal striatum (1, 2 mm) and nucleus accumbens (2, 1 mm) of the lesioned and non-lesioned hemisphere, from two sections about 1.2 and 1.5 mm posterior to bregma, and from the SNc (3, 0.5 mm) and VTA (1, 0.5 mm) of the lesioned and non-lesioned hemisphere, from two sections about 5.2 and 5.5 posterior to bregma (Palkovits & Brownstein, 1988). Punches were suspended in phosphate buffer (PB) and frozen overnight. The following day samples were thawed and centrifuged at 4000 rpm for 15

min. Pellets were suspended in 0.1 M NaCl and analyzed for protein content. The supernatant was removed and assayed for DA, dihydroxyphenylacetic acid (DOPAC), and homovanilic acid (HVA) using HPLC-EC. The supernatant was injected into a 15 cm C_{18} column (5 μm particle size, Scientific Products and Equipment, Ont.) The mobile phase consisted of 30 mM citric acid, 60 mM sodium phosphate monobasic, 0.10 mM EDTA, 14 % acetonitrile, and 0.08 mM sodium dodecyl sulphate, pH 3.35. The mobile phase was pumped through the system at 1.2 ml/min using a Waters 515 HPLC pump. Compounds were detected and quantified with and ESA coulochem detector (model 5100A) equipped with analytical cell (model 5011; E1 = + 0.35 V, E2 = - 0.3 V, ESA, Inc.) The concentrations were estimated from peak height by comparison with injection of known amounts of pure standards (Sigma) and expressed as μg/mg of protein.

Immunohistochemistry

Animals were deeply anesthetized with sodium pentobarbital (120 mg/kg) and perfused transcardially with 200 ml of cold PBS, followed by 100 ml of a cold solution of paraformaldehyde (w/v) and 15% picric acid (v/v) in phosphate buffer (PB; pH 6.9). Brains were removed and stored overnight in the fixative solution at 4 degrees C. Coronal 50 μm sections were cut on a vibratome and stored overnight in PB at 4 degrees C. Before slicing, a small mark was cut in each brain to allow discrimination of the lesioned from the intact hemisphere in each section. Sections were then double labeled for FGF-2 and tyrosine hydroxylase (TH) using immunohistochemistry. Double labeling was obtained by processing the sections first for FGF-2 immunoreactivity and then for TH immunoreactivity. Free-floating tissue sections were incubated for 24 hrs at 4

degrees C with the mouse anti-FGF-2 antibody diluted to 1:500 with 0.3% Triton X-100 (Sigma) in PB and 1% Normal Horse Serum (NHS; Vector Laboratories, Burlingame, CA). After incubation in the primary antibody, sections were rinsed three times for 5 min in cold PB and incubated for 1 hr at room temperature (RT) in a solution of rat adsorbed biotinylated anti-mouse antibody (Vector) diluted to 1:200 with PB and 1% NHS. After three 5 min washes in cold PB, sections were incubated in an avidin-horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector) for 30 min at RT, and rinsed again three times for 5 min in cold PB. Next, sections were incubated for 10 min at room temperature and under constant agitation in a solution of 0.05% 3,3'-diaminobenzidine (Sigma) in PB. Then, without washing, the sections were transferred to a 3,3'-diaminobenzidine/PB solution, pH 7.8 with 0.01% H_2O_2 to catalyze the reaction and with 8% $NiCl_2$ to darken the reaction product. This incubation was terminated 8 min later by washing the sections three times for 10 min in cold PB.

For TH immunoreactivity, the tissue sections previously processed for FGF-2 immunoreactivity were preincubated in 0.3% Triton X-100 PB and 1% Normal Goat Serum (NGS) for 1 hr at room temperature. They were then incubated for 24 hrs at 4 degrees C with the rabbit anti-TH polyclonal antibody diluted to 1:5000 (Chemicon) in PB and 1% NGS. Secondary antibody (rat adsorbed biotinylated anti-rabbit antibody) and the ABC reagent were applied as described above. No $NiCl_2$ was added to the 3,3'-diaminobenzidine-PB-H_2O_2 solution in order to obtain an orange-brown reaction product clearly distinguishable form the black FGF-2 reaction product. This incubation was terminated 5 min later by washing the sections three times for 10 min in cold PB. Tissue from all groups was included in each batch of immunohistochemical processing.

Sections were then mounted on gelatin-coated slides, dried for at least 24 hours, hydrated in distilled water (1 min) and gradually dehydrated in 70%, 95%, and 100% ethanol. Slides then were cleared in Hemo-De and were coverslipped with Permount (Fisher Scientific).

Alternate sections from the mid-brain of 2 rats killed 7 days after a unilateral 6-OHDA lesion were collected and double-labeled for FGF-2 and GFAP and for FGF-2 and OX42 to determine whether FGF-2 was expressed by astrocytes or microglia, respectively. Double labeling was performed by processing the sections first for FGF-2 immunohistochemistry and then for either GFAP or OX42 immunohistochemistry. GFAP and OX42 immunolabeling was performed by using the ABC method. No $NiCl_2$ was added to the 3,3'-diaminobenzidine-PB-H_2O_2 solution in order to obtain an orange-brown reaction product clearly distinguishable form the black FGF-2 reaction product.

Image Analysis

Immunostained sections were analyzed under a Leica microscope (Leitz DMRB). The number of astrocytic FGF-2- and TH-immunoreactive (IR) cells per squared millimeter was estimated from digitized images of sample areas within SNc, VTA, and SNr, nucleus accumbens shell and core, and dorsomedial striatum, using computerized image-analysis system (NIH Image 1.6). Structure boundaries were defined according to the Paxinos and Watson stereotaxic atlas (Paxinos & Watson, 1997).

FGF-2 IR cells were counted in the DA cell body and terminal regions as described previously (Flores et al., 1998). Briefly, three images were taken in each hemisphere from VTA and SNr and 4 from SNc at 2 different levels from bregma: -5.2

and -5.3. For the DA terminal regions, four images were taken from each region at 3 different levels from bregma: 0.6mm, 1.2 mm, and 1.7 mm, in each hemisphere. TH-IR cells were counted in a minimum of 8 images taken from SNc and 4 from VTA at two different levels from bregma: -5.2 and -5.3mm, in each hemisphere. Only sections in which the medial and lateral parts of the substantia nigra were clearly separated by the medial terminal nucleus of the accessory optic tract were selected (Lee et al.,1996; Sauer & Oertel, 1994). No attempt was made to estimate the total TH-IR neurons number in three dimensions. The number of TH-IR cells calculated from a few selected levels from bregma has been previously shown to be an accurate representation of 6-OHDA induced degeneration of the SNc and VTA neurons (Carman et al., 1991; Gordon et al., 1997). The images were assigned code names and the FGF-2 and TH-IR cells were counted by an individual blind to the code assignment. The cell counts from the areas sampled in each hemisphere of each brain region were summed and divided by the total area examined.

Design and Procedures

Experiment 1A. Time course of FGF-2 and TH expression in males. Four groups of male rats, weighing 350-450 g at the time of surgery, were killed 3d (n = 17), 7d (n = 14), 14d (n = 10), and 5 wks (n = 10) after 6-OHDA lesioning and their brains were processed for FGF-2 and TH immunoreactivity. Sham operated rats from another experiment were used to provide baseline numbers of FGF-2 and TH-IR cells. These rats underwent identical stereotaxic surgical procedure up to, but not including, lowering of the infusion syringe tip (dura was pierced). These rats received desipramine but not pargyline pretreatment. Six sham-lesioned animals were killed 10 days and 7 were killed

28 days after the sham surgery. There were no differences between the sham-operated side and control side in the numbers of FGF-2- and TH-IR cells. Thus the mean of the two hemispheres was used to provide scores for these rats.

Experiment 1B. Evaluation of behavioral asymmetry and striatal DA content in males. Rats with unilateral 6-OHDA lesions (n = 14) were tested for spontaneous rotational asymmetry on 3 occasions: 3d, 7d, and 14 days after lesioning. After completion of the last behavioral test, the animals were killed and punches from striatum, nucleus accumbens, SNc and VTA were analyzed for DA and its metabolites using high-performance liquid chromatography with electrochemical detection (HPLC-EC).

Experiment 2. Time course of FGF-2 and TH expression in females. Eight groups of 90-day old females (n = 7-9/group) were ovariectomized under Metofane anesthesia by bilateral dorsal incisions. Animals were then randomly assigned to receive an injection of EB or Oil every 4 days for 4 weeks, a replacement regimen used by (Forgie et al., 1993) and shown to potentiate the locomotor-stimulating effects of amphetamine in ovariectomized rats. After four weeks, 24 h after an EB or Oil injection, the animals were lesioned. EB treated animals weighed 240-330 g and Oil treated animals weighed 310-400 g at the time of surgery. The EB/Oil replacement regimen was continued for the remaining survival time of the groups; animals were killed 24 h after the last injection and their brains were processed for FGF-2 and TH-immunoreactivity.

Experiment 3. The effect of neonatal hormonal manipulations on FGF-2 and TH-expression after 6-OHDA lesions in adulthood. Neonatal manipulations. Pups born to adult male and female rats obtained from Charles River and mated in the laboratory were

used. Successful mating was assessed by noting the presence of sperm in daily vaginal smears. On the day on which sperm was noted (embryonic Day 0, E0) the female was removed from the mating cage and housed individually. Beginning on E20, the breeding cages were checked every few hours for the presence of pups. Immediately after giving birth, postnatal Day 0 (PN0), the mother was removed from the breeding cage and the litter was sexed. Male pups were either gonadectomized (GDX) under hypothermic anesthesia (M/GDX – no-T) or subjected to the anesthetic procedure alone (M - T). Female pups were injected with TP (F/TP - T) or Oil (F no-T) on PN0 and PN1. The site of injection was sealed with Collodion (Fisher Scientific). Dams were given litters containing either TP-treated females and males or GDX males and Oil-treated females. This was done to avoid the possibility of exposing Oil-treated females and GDX males to TP. The litters remained undisturbed except for weekly cage cleaning until weaning. Animals were group housed until they were about 60-65 days old.

Adult hormonal manipulations. When rats were about 80-90 days of age, they were gonadectomized (except for GDX/M) under Metofane anesthesia. Animals from each of the four groups M (T), GDX/M (no-T), F/TP (T), F (no-T) were randomly assigned to receive EB or Oil every 4 days for 4 weeks (total of 8 groups, n = 5-9/group). Rats were lesioned 24 h after an EB or Oil injection, following which they continued to receive EB or Oil every four days for another 5 weeks. Animals were killed 24 h after the last injection and their brains were processed for FGF-2 and TH immunoreactivity. Body weights of animals at the time of surgery varied depending on hormonal treatment. In general males weighed more than females, T animals weighed more than no-T animals, and Oil-treated weighed more than those treated with EB in adulthood (no-T/EB females:

255-345 g, no-T/Oil females: 350-410 g, T/EB females: 308-400 g, T/Oil females: 370-420 g, no-T/EB males: 375-420g, no-T/Oil males: 400-480 g, T/EB males: 400-520 g; T/Oil males: 445-590 g).

Statistical Analyses

All analyses were done on raw data, using estimated numbers of FGF-2- and TH-IR cells per square millimeter. For Experiment 1, mixed factor ANOVAs were carried out for each brain region and each antibody (FGF-2 and TH) with *Time* (3d, 7d, 14d, and 5 wks) as a between factor and *Side* (Lesion, No Lesion) as a repeated factor. For Experiment 2, repeated measure ANOVAs were carried out for each brain region and each antibody with *Time* (3d, 7d, 14d, and 5 wks) and *Treatment* (EB and Oil) as between factors and *Side* (Lesion, No Lesion) as a repeated factor. In Experiment 3, two groups were exposed to testosterone (T) in the neonatal period, one male (M) and one female (F/TP), and two groups that were not exposed to T (no-T) in the neonatal period, one male (M/GDX) and one female (F). As adults, rats from each group were treated with either EB or Oil. ANOVAs were carried out for each brain region and each antibody with *Sex* (M, F) x *Neonatal Treatment* (T, no-T) and *Adult Treatment* (EB, Oil) as between factors and *Side* (Lesion, No Lesion) as a repeated factor. Appropriate post hoc tests ($p < .05$) were used to test for significant differences between means. Linear correlation analyses were performed by calculating Pearson's r coefficients. Very occasionally sections from an area were poorly stained and for that reason had to be discarded, making for slightly different degrees of freedom for the F-values for FGF-2-IR and TH-IR from the same group of animals.

Results

FGF-2 expression

The anti-FGF-2 antibody was previously shown by means of double-label immunohistochemistry to detect FGF-2 immunoreactivity in astrocytes (Flores et al., 1998). Consistent with those observations, Figure 1 shows that in the present study, FGF-2 immunoreactivity in all areas examined was confined to GFAP-immunoreactive astrocytes, and not to cells labeled with the monocyte/macrophage/microglial marker OX42.

Experiment 1A. Time course of FGF-2 and TH expression in males

FGF-2 in SNc, VTA, SNr: Regardless of time after lesion, there were more FGF-2–IR cells on the lesion than on the no-lesion side: SNc, $F(1, 47) = 105.87$, $p < .0001$; VTA, $F(1, 47) = 126.05$, $p < .0001$; and SNr, $F(1, 47) = 45.26$, $p < .0001$. In both the SNc and VTA, there were significant Side by Time interactions $F(3, 47) = 6.04$, $p < .01$, and $F(3, 47) = 10.40$, $p < .0001$ respectively; although there were more FGF-2 – IR cells on the lesion side than on the no-lesion side, this difference increased over days up to 14 days after lesion (see Figure 2, top panel). Paired t-tests conducted to analyze simple main effects revealed that the side difference was statistically significant 7, 14 days, and 5 weeks after the lesion in both the SNc and VTA ($p < .05$, Bonferroni corrected). The number of FGF-2-IR cells on the no-lesion side at all time points (see Figure 2, top panel) was similar to that expressed by sham lesioned animals (SNc: 419.74 ($\pm$ 32.80), VTA: 382.05 ($\pm$ 25.90).

Figure 1. Digitized images of SNc sections from the lesioned hemisphere of an animal killed 7 days after the 6-OHDA infusion into the MFB. A: Section labeled with both GFAP and FGF-2. The black arrows point to astrocytes that were double labeled with both FGF-2 and GFAP. B: Section labeled with both OX-42 and FGF-2. FGF-2-IR (white arrows) was not found within the OX-42-positive cells (dashed arrows). Oil immersion, scale bar = 10 μm.

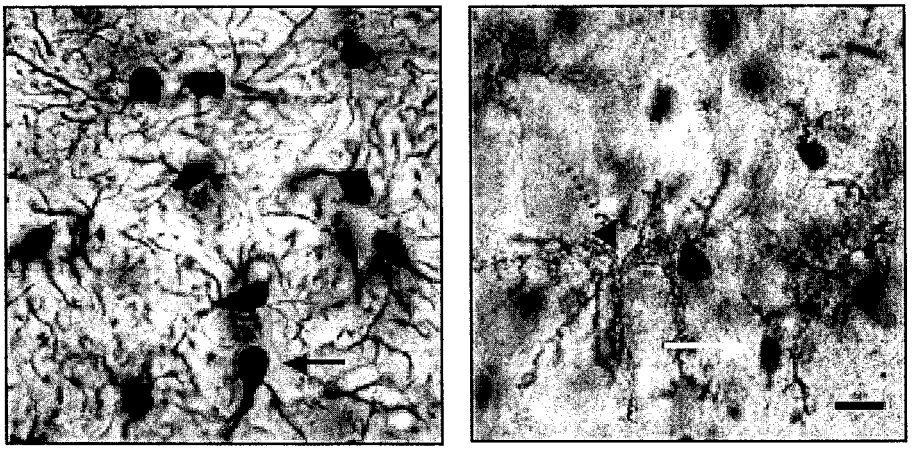
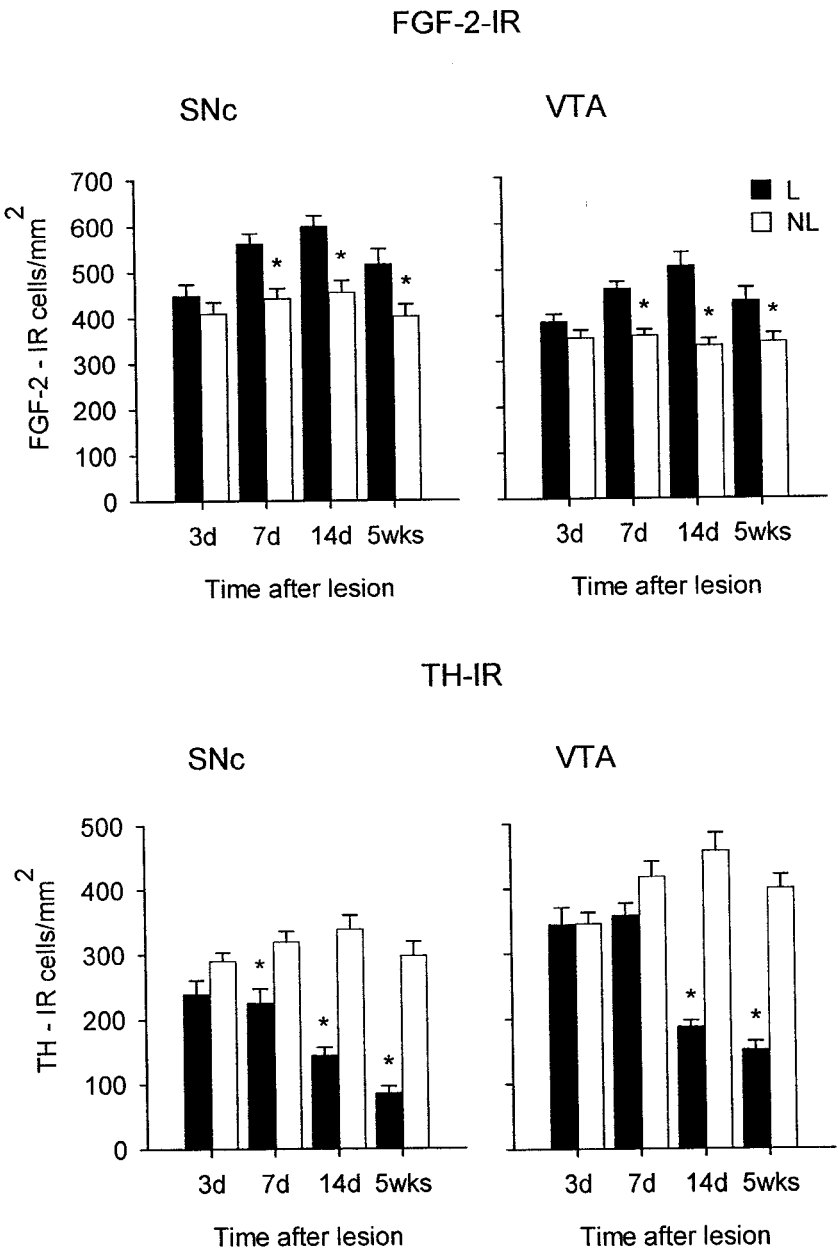


Figure 2. Time course of changes in FGF-2-IR (top panel) and TH-IR (bottom panel) in 6-OHDA-lesioned male rats: Mean (± SEM) number of FGF-2- and TH-IR cells per square millimeter in the SNc and VTA in groups (n = 10-14 / group) killed 3, 7, 14 days and 5 weeks after 6-OHDA lesion of the MFB. Asterisks indicate significant differences between lesioned (L) and non-lesioned (NL) sides as assessed by analyses for simple main effects.


FGF-2-IR
SNc
VTA
FGF-2 - IR cells/mm 2
700
600
500
400
300
200
100
0
L
NL
3d 7d 14d 5wks
Time after lesion
TH-IR
SNc
VTA
TH - IR cells/mm 2
500
400
300
200
100
0
3d 7d 14d 5wks
Time after lesion

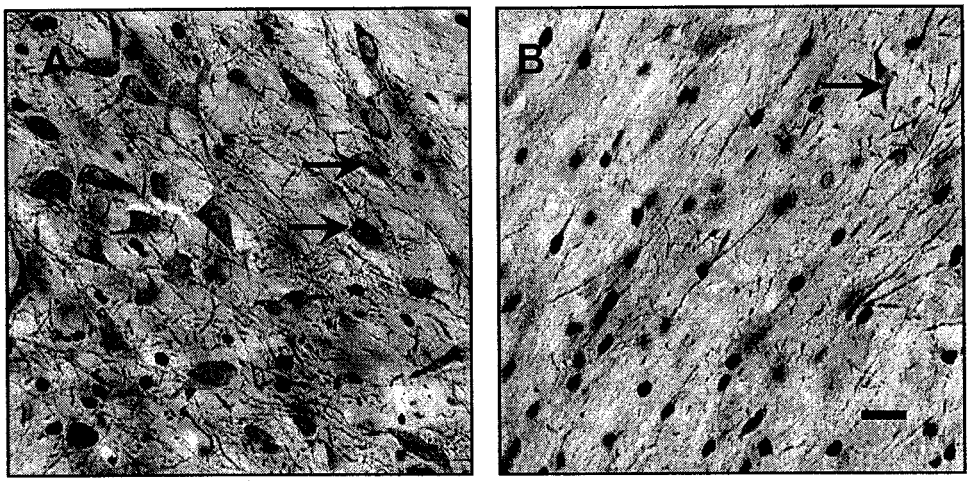
TH in SNc, VTA: It can be seen in Figure 2, lower panel, that in both the SNc and VTA, there were fewer TH positive cells on the lesion side and that this effect increased with the passage of time. These observations are confirmed by the significant main effects of Side (SNc, $F(1,43) = 108.95$, $p < .0001$; VTA, $F(1, 43) = 205.80$, $p < .0001$), Time (SNc, $F(3, 43) = 5.33$, $p < .01$; VTA, $F(3, 43) = 5.58$, $p < .01$) and the significant Side by Time interactions (SNc, $F(3, 43) = 9.30$, $p < .0001$; VTA, $F(3, 43) = 45.70$, $p < .0001$). Paired t-tests conducted to analyze simple main effects revealed that the side difference was statistically significant 7, 14 days, and 5 weeks after the lesion in the SNc, and 14 days and 5 weeks after lesion in the VTA ($p < .05$, Bonferroni corrected). Figure 3 shows digitized images from representative sections of the SNc double-stained for FGF-2- and TH-IR taken from the lesion and no-lesion side of a male rat killed 14 days after the lesion. It can be seen that TH-IR cells are greatly reduced in number on the lesion side whereas FGF-2-IR cells are greatly increased. The number of TH-IR cells on the no-lesion side at all time points (Figure 2, lower panel) was similar to that expressed by sham-lesioned animals (SNc: 277.12 ($\pm$ 24.4), VTA: 363.03 ($\pm$ 18.5).

FGF-2 in nucleus accumbens (shell and core) and dorsomedial striatum: There were no significant effects of lesion on FGF-2-IR in any of the terminal regions, at any time point.

Relationship between behavioral asymmetry, FGF-2-IR and TH

In the SNc, the percent increase in FGF-2-IR cells on the side of the lesion found in groups killed at 2 and 5 weeks was correlated with the percent ipsilateral turning displayed 3 ($r = .45$, $p < .05$) and 14 days after the lesion ($r = .57$, $p < .05$). In the

Figure 3. Digitized images showing darkly labeled FGF-2-IR cells (black dots) and TH-IR cells (arrows) taken from the central part of the SNc from a representative male rat that was lesioned with 6-OHDA in the MFB and killed 14 days after the lesion. A: no-lesion side. B: lesion side. As indicated in the text, FGF-2-IR was not found within TH-IR cells. Scale bar = 20 μm.

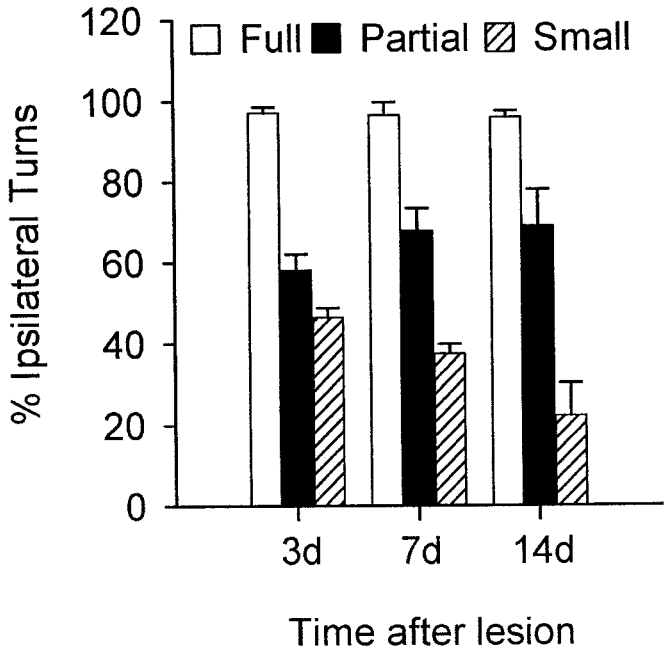

A
B

same animals, the percent TH-IR loss in the SNc on the side of the lesion was also correlated with percent ipsilateral turning 3 days after lesioning ($r = .51$, $p < .05$). No significant correlations between behavioral asymmetry and the number of IR cells were found for groups killed 3 and 7 days after the lesion. In the VTA, none of the correlations between either the percent increase in FGF-2-IR cells or the percent decrease in TH-IR cells and the percent ipsilateral turning was significant.

Experiment 1B. Relation between behavioral asymmetry and striatal DA content in males

Based on the percent of striatal DA depletion in the lesioned hemisphere 14 days after the lesion, rats were assigned to three groups: Full - more than 90% loss, Partial - 70-90% loss, and Small - less than 50% loss. A repeated measures ANOVA was carried out for behavioral asymmetry with Group as the between and Test Day as the within factor. It can be seen in Figure 4 that the Full lesion group displayed almost 100% ipsilateral turning on all 3 tests given 3, 7, and 14 days after the lesion, whereas there was an increase in the percent of ipsilateral turns across test days in the Partial lesion group from 58 to 68%, and a decrease in the percent of ipsilateral turns in the Small lesion group from 46 to 22%. These observations were confirmed by the significant main effect of Group ($F(2, 11) = 57.14$, $p<.0001$) and a significant Group by Test Day interaction ($F(4, 22) = 5.46$, $p < .01$. Highly significant positive correlations were found between the percent of striatal DA loss in the lesioned hemisphere and the percent of ipsilateral turns for each test: Day 3, $r = .74$, $p < .01$; Day 7, $r = 85$, $p < .001$; Day 14, $r = .93$, $p < .0001$.

Figure 4. Behavioral asymmetry: Mean (± SEM) percent of spontaneous ipsilateral turns in the novel environment (ipsi/[ipsi + contra] x 100%) displayed by animals with Full, Partial or Small lesions (see text for details) on tests conducted 3, 7, and 14 days after 6-OHDA lesions of the MFB..


120
100
80
60
40
20
0
% Ipsilateral Turns
Full
Partial
Small
3d
7d
14d
Time after lesion

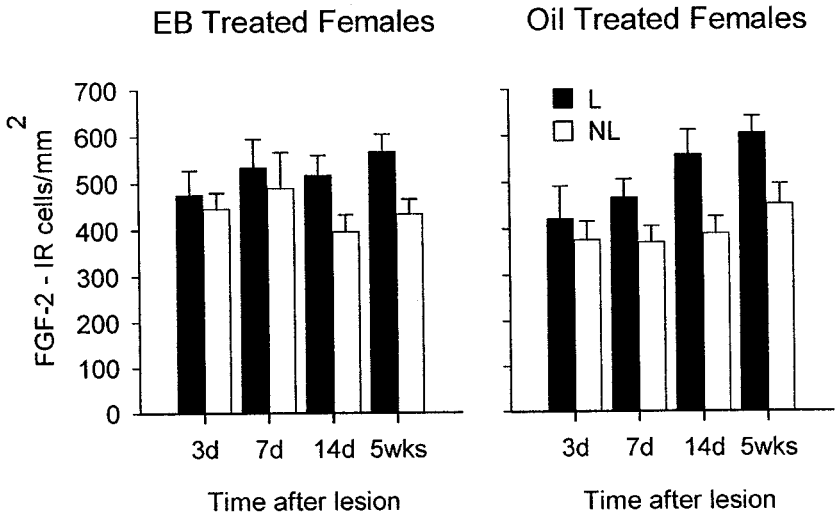
The DA depletion in the nucleus accumbens, SNc and VTA at 14 days was not as pronounced as in the striatum. In the SNc, the percent of DA depletion was, however, significantly correlated with the percent of striatal DA depletion ($r = .81$, $p < .05$) and with the percent of ipsilateral turns on each test day: Day 3, $r = .69$, $p < .05$; Day 7, $r = .82$, $p < .05$; Day 14, $r = .77$, $p < .05$. In the VTA, the percent DA depletion was moderately correlated with the percent of DA depletion in the nucleus accumbens ($r = .54$; $p = .07$), but was not correlated with behavioral asymmetry on any test day.

Experiment 2. Time course of FGF-2 and TH expression in females

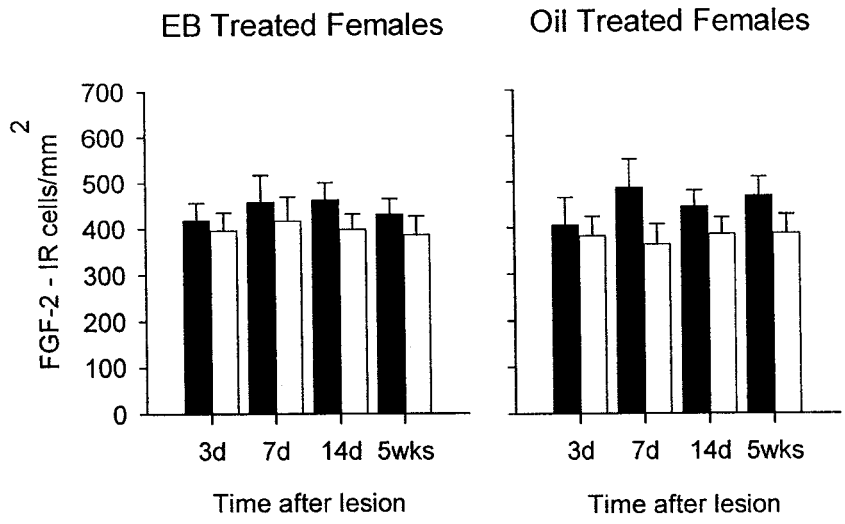
FGF-2 in SNc, VTA, SNr: As in males, there was a greater number of FGF-2-IR cells on the lesion side in all regions (SNc, $F(1, 52) = 56.45$, $p < .0001$; VTA, $F(1, 52) = 43.15$, $p < .0001$; SNr, $F(1, 51) = 28.65$, $p < .0001$). In SNc and SNr, there were significant Side by Time interaction effects (SNc, $F(3, 52) = 4.24$, $p < .05$; SNr, $F(3, 51) = 4.10$, $p < .05$) reflecting the fact that on the lesion side the number of FGF-2-IR cells increased with time after lesioning. Figure 5 shows the number of FGF-2-IR cells in the SNc and VTA in the groups treated with EB and Oil separately. Although the overall ANOVA did not reveal a significant interaction effect for Side by Time by Hormone Treatment, it can be seen that in both regions the differences between sides tended to be greater in the Oil-treated females at each time point.

Figure 5. Time course of changes in FGF-2-IR in 6-OHDA-lesioned female rats: Mean (± SEM) number of FGF-2- IR cells per square millimeter in the SNc (top panel) and VTA (bottom panel) in groups of ovariectomized females treated with EB (left) or Oil vehicle (right) killed 3, 7, 14 days and 5 weeks after 6-OHDA lesion of the MFB.

SNc


EB Treated Females
Oil Treated Females
FGF-2 - IR cells/mm 2
700
600
500
400
300
200
100
0
L
NL
3d
7d
14d
5wks
Time after lesion

VTA


EB Treated Females
Oil Treated Females
FGF-2 - IR cells/mm 2
700
600
500
400
300
200
100
0
3d
7d
14d
5wks
Time after lesion

TH in SNc, VTA: Figure 6 shows that, as in males, there were fewer TH-IR cells on the lesioned side in both areas and the number decreased over time (Side: SNc, $F(1, 50) = 75.43$, $p < .0001$; VTA, $F(1, 51) = 232.28$, $p < .0001$; Time: (SNc, $F(3, 50) = 6.1$, $p < .005$; VTA, $F(3, 51) = 6.03$, $p < .005$; Time by Side interactions: SNc $F(3, 50 = 8.99$, $p < .0001$; VTA, $F(3, 51) = 10.69$, $p < .0001$). There was no effect of hormonal treatment on the number of TH-IR cells in either SNc, $F(1, 50) = .054$, or in the VTA, $F(1, 51) = 2.6$, $p > .05$. Paired t-tests conducted to analyze simple main effects revealed that the side difference was statistically significant 7, 14 days, and 5 weeks after the lesion in the SNc, and at all time points in the VTA ($p < .05$, Bonferroni corrected).

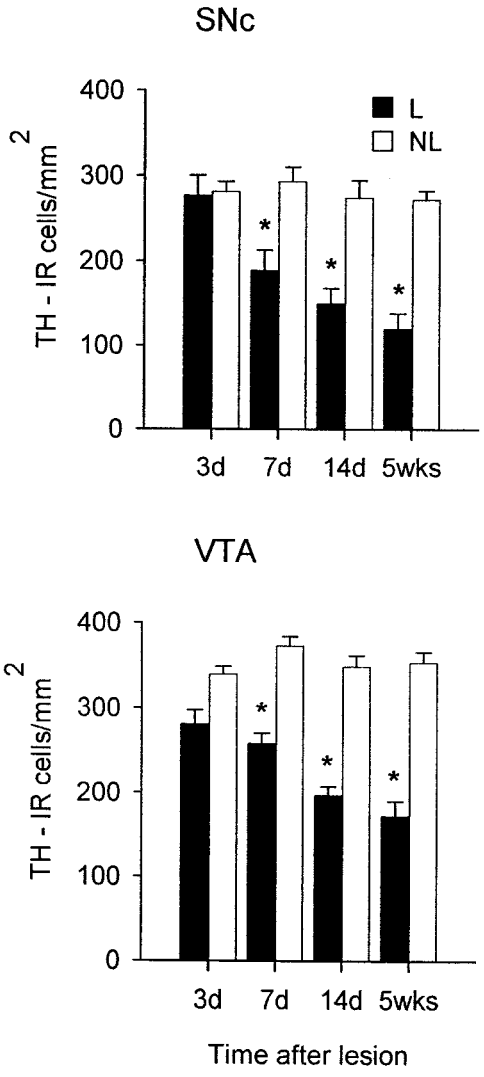
FGF-2 in nucleus accumbens (shell and core) and dorsomedial striatum: There were no consistent differences or significant effects of the lesion on FGF-2-IR in any of the terminal regions, at any time point.

Relationship between behavioral asymmetry, FGF-2-IR and TH

In the SNc, the percent increase in FGF-2-IR cells on the side of the lesion found in groups killed at 2 and 5 weeks was moderately but significantly correlated with the percent ipsilateral turning displayed 3 ($r = .50$, $p < .05$) and 14 days after the lesion ($r = .35$, $p = .05$). In the same animals, the percent TH-IR loss in the SNc and VTA on the side of the lesion was also moderately correlated with percent ipsilateral turning 3 (SNc, $r = .53$, $p< .05$; VTA, $r = .68$, $p < .05$) and 14 days after the lesion 3 (SNc, $r = .52$, $p< .05$; VTA, $r = .67$, $p < .05$). No significant correlations between behavioral asymmetry and the number of IR cells were found for groups killed 3 and 7 days after the lesion.

Figure 6. Time course of changes in TH-IR in 6-OHDA-lesioned female rats: Mean (± SEM) number of FGF-2- IR cells per square millimeter in the SNc (top panel) and VTA (bottom panel) in groups of female rats treated with EB and Oil vehicle killed 3, 7, 14 days and 5 weeks after 6-OHDA lesion of the MFB. Asterisks indicate significant differences between lesioned (L) and non-lesioned (NL) sides as assessed by analyses for simple main effects.

TH-IR


SNc
400
300
200
100
0
TH - IR cells/mm 2
L
NL
*
*
*
3d
7d
14d
5wks
VTA
400
300
200
100
0
TH - IR cells/mm 2
*
*
*
3d
7d
14d
5wks
Time after lesion

Experiment 3. The effect of neonatal hormonal manipulations on FGF-2 and TH expression five weeks after 6-OHDA lesions in adulthood

FGF-2 in SNc, VTA: Figure 7 shows that the effect of lesion on FGF-2-IR was consistently greater in both regions in animals exposed to testosterone neonatally. The ANOVAs yielded significant main effects for Neonatal Treatment: SNc, $F(1, 42) = 10.29$, $p < 01$; VTA, $F(1, 43) = 18.34$, $p < .001$ and Side: SNc, $F(1, 42) = 156.50$, $p < .0001$); VTA, $F(1, 43) = 124.78$, $p < .0001$. The Neonatal Treatment by Side interaction was statistically significant only for the SNc: $F(1,42) = 17.28$, $p < .001$, and approached significance in the VTA: $F(1,43) = 3.81$, $p = .058$. Note that the effect of sex, per se, was not significant in either region (SNc, $F(1.42) = .42$, $p > .05$; VTA, $F(1, 43) = 2.06$, $p > .05$); the Side effect on the number of FGF-2-IR cells was greater in animals exposed to testosterone neonatally regardless of genetic sex.

FGF-2 in SNr: The ANOVA carried for FGF-IR cells in the SNr yielded no significant effects except for Side, $F(1, 43) = 13.66$, $p < .001$. As in the other areas, lesions led to an increase in FGF-2-IR.

FGF-2 in nucleus accumbens (shell and core) and dorsomedial striatum: There were no consistent differences or significant effects of lesions on FGF-2-IR in any of the terminal regions.

TH in SNc, VTA: Figure 8 shows that the number of TH-IR cells was consistently lower on the lesion side in both regions (SNc, $F(1, 43) = 83.37$, $p < .0001$; VTA, $F(1, 43) = 267.78$, $p < .0001$). In the SNc, there was a significant effect of Neonatal Treatment, $F(1, 43) = 7.01$, $p < .05$; no-T groups had more TH-IR cells than T groups. Inspection of

Figure 7. FGF-2-IR in midbrain of adult rats exposed to testosterone neonatally or not exposed to testosterone neonatally, 5 weeks after 6-OHDA lesions of the MFB. Two groups were exposed to testosterone (T) in the neonatal period, one male (M) and one female (F/TP), and two groups that were not exposed to T (no-T) in the neonatal period, one male (M/GDX) and one female (F). As adults animals from each group were treated with EB or Oil. Values are expressed as mean ($\pm$ SEM) number of FGF-2- IR cells per square millimeter in the SNc (top panel) and VTA (bottom panel).

FGF-2-IR

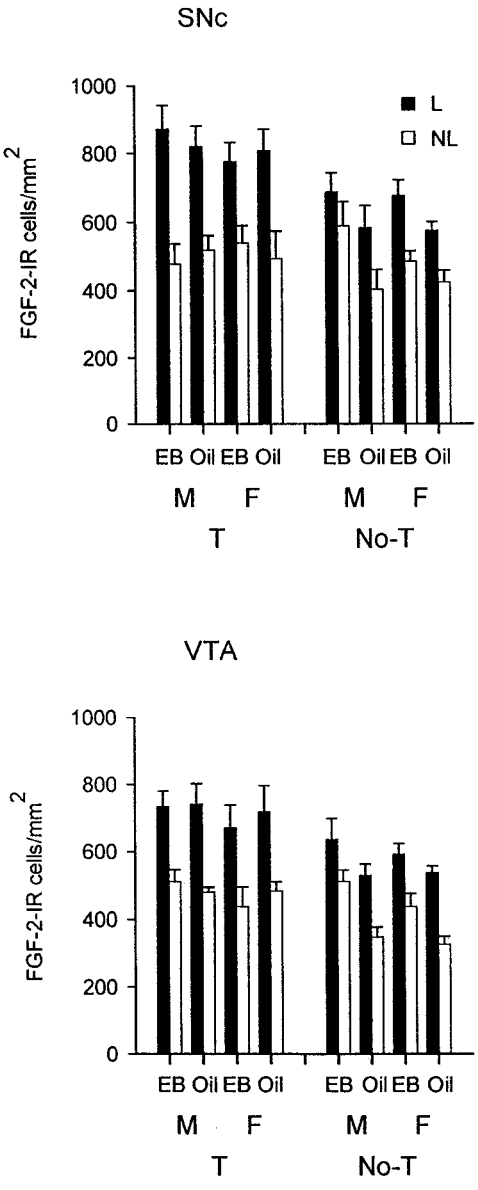
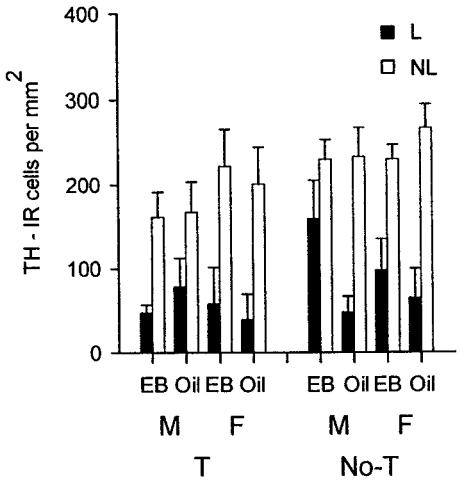

SNc
1000
800
600
400
200
0
FGF-2-IR cells/mm2
L
NL
EB Oil EB Oil EB Oil EB Oil
M F M F
T No-T
VTA
1000
800
600
400
200
0
FGF-2-IR cells/mm2
EB Oil EB Oil EB Oil EB Oil
M F M F
T No-T

Figure 8. TH-IR in midbrain of adult rats exposed to testosterone neonatally or not exposed to testosterone neonatally, 5 weeks after 6-OHDA lesions of the MFB. Two groups were exposed to testosterone (T) in the neonatal period, one male (M) and one female (F/TP), and two groups that were not exposed to T (no-T) in the neonatal period, one male (M/GDX) and one female (F). As adults animals from each group were treated with EB or Oil. Values are expressed as mean (± SEM) number of TH- IR cells per square millimeter in the SNc (top panel) and VTA (bottom panel).

TH-IR

SNc


400
300
200
100
0
TH - IR cells per mm2
L
NL
EB Oil EB Oil
EB Oil EB Oil
M F
M F
T
No-T

VTA

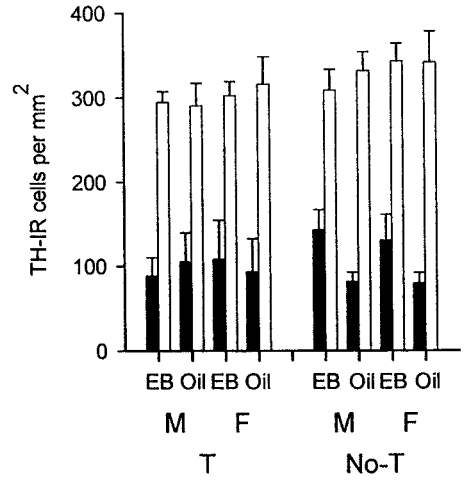

400
300
200
100
0
TH-IR cells per mm2
EB Oil EB Oil
EB Oil EB Oil
M F
M F
T
No-T

Figure 8 indicates that among no-T groups, animals treated with EB as adults appear to have a greater number of TH-IR cells remaining in the SNc on the side of the lesion. ANOVAs carried out on the data from the T groups and the no-T groups separately, revealed that the effect of EB treatment was only significant in the no-T groups, $F(1, 27) = 4.90$, $p < .05$. The same analysis carried out on the data from the VTA revealed a similar finding ($F(1, 27) = 4.20$, $p < .05$). It appears from close inspection of Figure 8 that the number of TH-IR cells in the no-lesion SNc was less in rats exposed to testosterone neonatally. A separate ANOVA carried out on the number of TH-IR cells in the no-lesion SNc revealed a significant effect of Neonatal Treatment, $F(1, 43) = 7.60$, $p < .05$.

Relationship between behavioral asymmetry, FGF-2-IR and TH

The percent increase in FGF-2-IR cells in the SNc and VTA on the side of the lesion found in groups killed 5 weeks after the lesion was moderately and significantly correlated with the percent ipsilateral turning displayed 3 (SNc, $r = .37$, $p < .05$; VTA, $r = .41$, $p < .05$), 7 (SNc, $r = .33$, $p < .05$; VTA, $r = .45$, $p < .05$), 14 days (SNc, $r = .34$, $p < .05$; VTA, $r = .48$, $p < .05$) and 5 weeks after the lesion (SNc, $r = .28$, $p < .05$; VTA, $r = .37$, $p < .05$). In the same animals, the percent TH-IR loss in the SNc and VTA on the side of the lesion was highly correlated with percent ipsilateral turning 3 (SNc, $r = .73$, $p < .0001$; VTA, $r = .61$, $p < .0001$), 7 (SNc, $r = .75$, $p < .0001$; VTA, $r = .72$, $p < .0001$), 14 days (SNc, $r = .81$, $p < .0001$; VTA, $r = .75$, $p < .0001$) and 5 weeks after the lesion (SNc, $r = .76$, $p < .0001$; VTA, $r = .60$, $p < .0001$).

Discussion

The primary purpose of the present experiments was to examine astrocytic FGF-2 immunoreactivity within the nigrostriatal DA system after injections of neurotoxin 6-hydroxydopamine into the MFB in male and female rats, and to determine whether neonatal exposure to gonadal hormones has consequences on the expression of astrocytic FGF-2 in response to injury in adulthood. The principal findings from these experiments are outlined below.

In males, 6-OHDA lesions led to increases in the number of FGF-2-IR cells in the SNc and VTA on the lesioned side. These increases were evident 7 days after the lesion and peaked at 14 days. Similarly, in females, 6-OHDA lesions led to increases in the number of FGF-2-IR cells in SNc and VTA on the lesioned side. Furthermore, in both regions the increases on the lesioned side relative to the non-lesioned side tended to be greater in Oil-treated ovariectomized females compared to those treated with EB. The increases in FGF-2 –IR in females were highest 5 weeks after the lesion. In both males and females the number of TH-IR cells decreased in the SNc and VTA on the lesioned side. This decrease was gradual and was greatest 5 weeks after the lesion.

In rats exposed to testosterone neonatally, the lesion-induced increase in the number of FGF-2-IR cells observed 5 weeks after the lesion was greater than in rats not exposed to testosterone neonatally in both SNc and VTA, regardless of the genetic sex. Among rats not exposed to testosterone neonatally, those treated with EB as adults showed less loss of TH-IR cells in the SNc after lesion.

Analysis of behavior and striatal DA content (Experiment 1B) showed that the percent of striatal DA depletion 14 days after the lesion correlated with the amount of behavioral asymmetry displayed by animals on all tests conducted after lesioning, at 3, 7 and 14 days. Examination of the relationships between behavioral asymmetry and TH-IR cells and FGF-2-IR cells in SNc and VTA revealed that, in groups killed 2 and 5 weeks after the lesion, the amount of behavioral asymmetry correlated with the percent loss of TH-IR cells and with the percent increase in FGF-2-IR cells. In other words, the greater the behavioral asymmetry, the greater the TH-IR cell loss, and the greater the number of FGF-IR astrocytes on the lesioned side. These relationships were not evident in groups killed 3 and 7 days after the lesion, possibly because the changes in the number of FGF-2- and TH-IR cells were not fully manifested.

6-OHDA lesion induced changes in cells expressing FGF-2 and TH

Increases in the astrocytic FGF-2-IR have been demonstrated after various types of brain injury, and specifically, after infusions of 6-OHDA immediately anterior to the DA cell bodies in the SNc (Chadi et al., 1994). Interestingly, in the present experiments, when 6-OHDA was infused into the MFB, the astrocytic expression of FGF-2 was greatest in the cell body region of the DA neurons in SNc and VTA and, most importantly, this enhanced FGF-2 expression increased as the number of TH-expressing cells decreased over the 5-week period. The fact that the loss of TH-expressing cells in SNc and VTA after 6-OHDA lesions of the MFB occurs gradually stands in contrast to what has been reported for rapid loss of DA content in terminal regions in striatum after similar lesions (Costall et al., 1976; Neve et al., 1982; Nisenbaum et al., 1994; Mishra et al., 1980). The pattern of results seen in the cell body regions in the present experiments

is like that seen by Ichitani et al. (1994), who reported that following 6-OHDA infusions into the striatum a significant reduction in the number of TH-IR cells in the SNc was observed at 14 days and 10 months, but not at 1 and 7 days after the lesion. Similarly, Sauer and Oertel (1994) reported that such infusions caused a progressive loss of TH-expressing SNc DA cells starting between one and two weeks after the lesion and continuing at 8 to 16 weeks. A time-dependent loss of TH-IR neurons in the SNc after 6-OHDA infusions in the MFB has also been reported (Gordon et al., 1997), where neuron number was significantly reduced at 14 days relative to the earlier time points. The fact that astrocytic FGF-2 expression increases as cells are lost suggests that the increased expression reflects the recruitment of a neuroprotective mechanism, albeit a relatively ineffective one.

As mentioned in the introduction, these experiments were prompted by the similarities between the compensatory changes in DA neurons after partial 6-OHDA lesions and the development of long-term changes in DA neurons after repeated exposure to amphetamine. Following partial 6-OHDA lesions of the nigrostriatal pathway, there is normalization of basal levels of extracellular DA accompanied by behavioral recovery (Altar et al., 1987; Robinson & Whishaw, 1988; Robinson et al., 1994) and it is known that both normalization of DA levels and behavioral recovery can be blocked by NMDA receptor antagonists given over 7 days immediately following the infusions of 6-OHDA (Emmi et al., 1996). Interestingly, NMDA antagonists given during the period of repeated exposure to amphetamine block the development of behavioral sensitization to amphetamine (Cador et al., 1999; Karler et al., 1989; Kim & Vezina, 1998; Stewart & Druhan, 1993; Wolf & Jeziorski, 1993) and, of relevance to the present results, block the

sustained increases in FGF-2 expression seen in the DA cell body regions following repeated exposure to amphetamine (Flores et al., 1998). Thus, it is possible that both the lesion- and amphetamine-induced increases in FGF-2 expression are mediated by glutamate during these periods with high potential for neuronal plasticity, after lesion or after drug exposure. Somewhat paradoxically, the increases in glutamate observed in these periods of vulnerability may induce neurotrophic factors that play both a neuroprotective and neurotrophic function with respect to the DA neurons. It would be interesting, therefore, in future studies to determine whether NMDA receptor antagonists would block the lesion-induced increase in FGF-2.

The neurotrophic actions of FGF-2 on DA cells *in vitro* have been shown to be mediated by astrocytes (Engele & Bohn, 1991; Hou et al., 1997). *In vivo*, an increase in astroglial activation, as measured by glial fibrillary acidic protein (GFAP) expression, has been demonstrated after striatal (Rodriguez, Gomide, & Chadi, 2001), MFB (Stromberg et al., 1986), and SNc (Sheng, Shirabe, Nishiyama, & Schwartz, 1993) 6-OHDA lesions most often in the striatum, but also in the cell body regions of DA neurons. The increase in GFAP-IR in the striatum in these studies was detected as early as 1 day after the lesion, whereas the increase in SNc was evident only after 3 days. Furthermore, after 6-OHDA lesions of the MFB, the greatest GFAP-IR in the SNc was observed 16 and 32 days postlesion (Stromberg et al., 1986). These increases in reactive astrocytes correspond with the timing of the increase in astrocytic FGF-2 observed in the present experiments. Similarly, Rodriguez et al. (2001) described an intense astroglial reaction in the SNc with about 50% loss of TH-IR neurons, 22 days after unilateral striatal infusions of 6-OHDA. These observations together with the present findings suggest that 6-OHDA-induced

degeneration of DA neurons may release signals triggering a sustained activation of adjacent astrocytes which, in turn, may lead to induction of astrocytic FGF-2. The possibility exists, however, that increased FGF-2 expression increases astrocytic activity. For example, an injection of FGF-2 into the intact cerebral cortex of adult rats has been shown to induce a glial reaction characterized by a higher number of GFAP-IR cells per surface unit and increase in the size and branching of the astroglial processes (Eclancher, Kehrli, Labourdette, & Sensenbrenner, 1996). These increases were comparable in magnitude to those induced by an electrolytic lesion. Injection of FGF-2 into the site of electrolytic lesion was found to accelerate the increase in GFAP-IR, thus implicating FGF-2 expression in injury repair in the adult brain.

The finding that the number of astrocytes expressing FGF-2 in the present experiment was increased in DA cell body regions, but not in terminal regions, raises the question of whether there is something special about the astrocytes within the area of degenerating DA cell bodies. Interestingly, Nomura, Yabe, Rosenthal, Krzan, & Schwartz (2000) showed that the highly polysiatylated neural cell adhesion molecule NCAM (PSA-NCAM) was expressed by astrocytes only in the SNc after 6-OHDA lesions made rostrally to the SNc. PSA-NCAM is expressed during development where it plays a role in neurite outgrowth and cell migration, but is also expressed in discrete structures of the adult brain where neurogenesis continues to occur. Nomura et al. (2000) also showed that PSA-NCAM colocalized with a marker of dividing cells, suggesting that only the dividing astrocytes in the SNc re-expressed NCAM. It would be interesting to examine whether the FGF-2-IR astrocytes are also the ones expressing PSA-NCAM.

In the study by Rodriguez et al. (2001) mentioned above, it was found that 22 days after 6-OHDA infusions into the striatum there was microglial, as well as astroglial activation in the SNc, coincident with the TH-IR loss in the SNc. It has been shown that activated microglia synthesize interleukins that stimulate the activation of astrocytes (Giulian, Vaca, & Corpuz, 1993) which, in turn, produce increases in substances with neurotrophic properties, such as FGF-2 observed in the present experiment. Thus, the 6-OHDA-induced retrograde degeneration of nigrostriatal neurons might lead to astroglial and microglial activation, which could in turn trigger trophic factor synthesis by activating nigrostriatal glial cells in surviving DA neurons.

Effects of gonadal hormones on lesion-induced changes in cells expressing FGF-2 and TH

In light of the recent findings demonstrating neuroprotective effects of estrogen within the nigrostriatal system (Callier et al., 2000; Dluzen et al., 1996a; Dluzen et al., 1996b; Dluzen, 1997; Grandbois et al., 2000) we studied how EB given to adult ovariectomized females (Experiment 2) affected the expression of astrocytic FGF-2 and the number of TH-IR cells in the SNc and VTA in response to injury. We found that compared to males, the lesion-induced increase in FGF-2-IR was modest, but more pronounced in ovariectomized females treated with Oil than in those given EB replacement. In this experiment however, no differences were found in the number of TH-IR cells or in behavior between the Oil- and EB-treated females. It is interesting, however, that even though EB per se did not have an effect on the lesion-induced loss of TH-IR cells, compared to males, all females had about 10-15 % more TH-IR cells remaining on the lesioned side, suggesting that females were affected less by the toxin

than males. This is in agreement with reports of reduced susceptibility of the nigrostriatal system to neurotoxic agents such as MPTP in female mice (Brooks et al., 1989; Freyaldenhoven et al., 1996; Miller et al., 1998) and provides the first demonstration of sex differences in the susceptibility of the nigrostriatal system to 6-OHDA induced degeneration as assessed by TH-IR.

In Experiment 3, it was found that in adults neonatally exposed to testosterone, the expression of FGF-2 after lesion was greater than in those not exposed, regardless of genetic sex. Thus, it appears that early exposure to testosterone for reasons that are unknown at present, increases the astrocytic FGF-2 response in the DA cell body regions after 6-OHDA lesions. Furthermore, in rats exposed to testosterone neonatally, there was no effect of adult treatment with EB on the number of TH-IR cells remaining in the SNc and VTA, whereas in those not exposed to testosterone, EB appeared to provide some protection. The finding that rats exposed to testosterone neonatally are less responsive to estrogens as adults is in accord with the general idea that neonatal exposure to testosterone decreases the sensitivity of the adult brain to the effects of estrogens (Forgie et al., 1993; Juraska, Kopcik, Washburne, & Perry, 1988; Milner & Loy, 1982; Stewart & Kolb, 1994).

Interestingly, neonatal exposure to testosterone appeared to reduce the number of TH-IR cells in the SNc. Although, to our knowledge, the effects of sex steroids on the number of DA cells in the SNc have not been studied previously, the locus coeruleus (LC), the largest group of norepinephrine containing neurons in the brain, has been reported to be a sexually dimorphic structure. The volume and cell number of LC is greater in female than in male rats (Guillamon et al. 1988; Luque et al., 1992) and both

measures are reduced when testosterone is administered to females neonatally (Guillamon et al., 1988).

In general, the present findings are not in agreement with previous reports of neuroprotective actions of estrogen as assessed by striatal DA depletion where EB was shown to prevent depletion of striatal DA concentrations in mice treated with MPTP (Callier et al., 2000; Disshon & Dluzen, 2000; Dluzen et al., 1996a; Dluzen et al., 1996b; Grandbois et al., 2000) and in rats treated with 6-OHDA (Dluzen, 1997). On the basis of these data and those showing that estrogen reduces DA uptake in striatal tissue (Arvin, Fedorkova, Disshon, Dluzen, & Leipheimer, 2000; Disshon, Boja, & Dluzen, 1998; Disshon et al., 2000; Gainetdinov, Fumagalli, Jones, & Caron, 1997; Miller, Gainetdinov, Levey, & Caron, 1999; Thompson, 1999), it has been proposed that the protective effects of estrogen might be due to its ability to inhibit DA transporter activity, thus preventing the transport of neurotoxins into the DA neurons. The only evidence for a neuroprotective effect of estrogen in the present experiments, however, comes from the finding in Experiment 3 that in rats not exposed to testosterone neonatally, there was reduced loss of TH-IR cells in those treated with EB as adults. It would be interesting, therefore, to investigate whether neonatal exposure to testosterone is responsible for the differential responsiveness of the DA transporter to estrogen in male and female rats.

In summary, male rats show a greater astrocytic FGF-2-IR in response to 6-OHDA-induced loss of TH-IR cell bodies in the SNc and VTA. In both males and females, however, there was a progressive loss of TH-IR in the cell body regions of the DA neurons that was greatest 5 weeks after the lesions. In general, female rats showed a reduced loss of TH-IR cells, but this effect was not estrogen dependent. In Experiment 3,

done to assess the interaction between neonatal testosterone exposure adult estrogen treatment on the response to neurotoxic lesions, it was found that all rats exposed to testosterone showed a greater FGF-2 response to lesions than those not exposed, and that among those not exposed, estrogen treatment had a modest protective effect. The lack of an estrogen effect in Experiment 2 may have been due to the fact that the rats in that experiment were exposed to circulating gonadal hormones throughout life. Interestingly, the effects of estrogen seen in Experiment 3 were greatest in neonatally gonadectomized males that had not been exposed to any circulating gonadal hormones until the EB treatment as adults.

In conclusion, we examined astrocytic FGF-2- and TH-IR in the cell body region of midbrain dopaminergic neurons after unilateral infusions of the neurotoxin 6-OHDA into the MFB in male and female rats. In addition, to determine whether neonatal exposure to gonadal hormones has consequences on the expression of astrocytic FGF-2 and cell loss in response to injury in adulthood, we studied the effects of these lesions in adult male and female rats that had been exposed or not to testosterone in the neonatal period. In both males and females there was a progressive loss of TH-IR cells that peaked five weeks after the lesions. Females showed less loss of TH-IR cells than males, but this effect was not estrogen dependent. Lesions led to an increase in expression of astrocytic FGF-2 that was greater in males than in females. Rats exposed to testosterone in neonatal life showed greater astrocytic FGF-2 expression after lesions than those not exposed, and among those not exposed to testosterone, estrogen treatment had a modest protective effect. The present findings show that hormonal events early in life can have

long-lasting consequences for the response of midbrain dopamine neurons to insult or injury in adult life.

Acknowledgements

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CHAPTER III

EXPERIMENT 4: EFFECTS OF 6-OHDA LESIONS OF THE NIGROSTRIATAL DOPAMINE PATHWAY IN RATS EXPOSED TO PERINATAL ANOXIA

Introduction

Accumulating evidence suggests that obstetric complications may alter functioning of the midbrain dopamine (DA) system later in life. For example, epidemiological studies have documented an increased incidence of a variety of obstetrical complications, and in particular, birth by Caesarian section (C-section) and fetal hypoxia, among those who later develop schizophrenia (Geddes & Lawrie, 1995; Jones, Rantakallio, Hartikainen, Isohanni, & Sipila, 1998; McNeil, 1995), a disorder believed to reflect a pathological enhancement of midbrain DA function (Abi-Dargham et al., 1998; Breier et al., 1997; Lieberman, Sheitman, & Kinon, 1997). Thus far, however, the relationship between obstetric complications and the vulnerability of the midbrain DA system to neurodegenerative diseases, such as Parkinson's disease, which is characterized by a progressive degeneration of DA neurons projecting from the substantia nigra to the striatum, has not been examined. In the present study, therefore, we examined the effects of perinatal anoxia on the response of the adult DA system to an insult caused by 6-hydroxydopamine-induced (6-OHDA) degeneration of the nigrostriatal DA neurons.

To experimentally study the long-term consequences of oxygen deprivation, a rat model of anoxia during C- section birth has been developed (Bjelke, Andersson, Ogreen, & Bolme, 1991). In this model, the intact uterus containing pups is removed from the pregnant rat at term via an abdominal incision, isolated from its blood supply and placed

in a warm saline bath for several minutes, mimicking an acute global anoxic event before delivery of the pups. Studies using rat models of C-section birth and of intrauterine anoxia revealed that these manipulations lead to enhanced functioning of the DA system in adulthood, particularly in response to environmental challenges, such as exposure to psychostimulant drugs or to stress. Compared to vaginally born rats, adult rats born by C-section, with or without an added period of anoxia, show augmented locomotor activity following amphetamine challenge (El-Khodor & Boksa, 1998) and elevated dopamine release in the NAcc in response to repeated stress (Brake, Noel, Boksa, & Gratton, 1997). Furthermore, in adult rats born by C-section with an episode of anoxia, repeated exposure to stress leads to sensitized behavioral responses to both stress and amphetamine (Brake, Boksa, & Gratton, 1997; El-Khodor & Boksa, 2000).

The ability of birth complications to lead to persistent behavioral and neurochemical alterations indicative of sensitized functioning of the midbrain DA system is likely to be mediated by operation of neurotrophic factors, known to play a critical role in the survival, maintenance, and morphological plasticity of adult neurons (Hefti et al., 1993). In a recent study, perinatal anoxia was found to produce enduring changes in astrocytic expression of the neuroprotective and neurotrophic factors, basic fibroblast growth factor (FGF-2) in the DA cell body regions. Specifically, FGF-2 expression in the VTA and SNc increased in response to repeated exposure to mild stressor only in adult rats that had been exposed to anoxia at birth, suggesting that altered FGF-2 recruitment may be involved in the enduring behavioral and neurochemical consequences of birth complications (Flores, Stewart, Salmaso, Zhang, & Boksa, 2002).

The purpose of the present study was to explore the effects of birth complications on the behavioral and cellular responses to injury of the DA system in adulthood. Specifically, we examined the effects of birth by C-section, with or without an added episode of anoxia, on the behavioral asymmetries and on changes in expression of FGF-2- and TH-immunoreactivity (IR) after unilateral 6-OHDA lesions of the nigrostriatal neurons in adult rats.

Materials and Methods

Drugs and antibodies used in this experiment and procedures for immunocytochemistry and image analysis are identical to those described in Chapter II. In addition, sham-operated rats that underwent identical stereotaxic surgical procedures and drug pretreatment up to, but not including, the lowering of the infusion syringe tip (dura was pierced) were used.

Subjects and Experimental Design

All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the Concordia University and the McGill University Animal Care Committees.

Timed pregnant Spraque Dawley rat dams (Charles River, Quebec, Canada) at 22 days of gestation (the expected day of delivery) were used to generate pups born under three birth conditions: C-section with 15 min of intrauterine anoxia (C-section + anoxia), C-section alone (C-section), and vaginal birth (vaginal). Pups in each birth group were born from at least 4 different dams. To avoid possible prematurity, C-sections were only

begun after one of the groups of timed pregnant dams, mated at the same time, had given birth vaginally. Furthermore, only pups from litters with all members weighing more than 5 g at delivery were used. Only male pups were retained for the study.

Male Spraque-Dawley rats born under the three birth conditions outlined above were grown to adulthood. At approximately three months of age, animals from different birth groups underwent either 6-OHDA lesion or sham-lesion surgery. Tests of behavioral asymmetries were conducted 5 and 14 days after the surgery. Fourteen days after surgeries the rats were killed and their brains were processed for FGF-2- and TH-IR.

C-Section and Acute Intrauterine Anoxia

Rat pups were subjected to acute global anoxia during C-section birth using procedures previously described by Bjelke et al. (1991) and El-Khodor and Boksa (1997, 1998). Briefly, at 22 days of gestation, pregnant dams were decapitated (to avoid the confound of anesthetic use) and hysterectomized. The uterus was quickly isolated from its blood supply and surrounding connective tissue (10-15 sec) immersed into a 37 degree C saline bath for 15 min to induce anoxic episode (C-section + anoxia group). The pups were then delivered and stimulated by gentle tapping until breathing became even (30-40 sec). No other means of artificial resuscitation were employed. The umbilical cord was ligated and the animals were placed on a heating pad for 1-2 hrs until given to their surrogate mothers. Survival after 15 min episode of intrauterine anoxia was 90-95%. Recent studies in which brain lactate, a marker of CNS hypoxia, and brain adenosine triphosphate (ATP) were quantified during the first 24 hrs of life, indicated that this

model produces consistent and reproducible "moderate" hypoxic episode in the pups (Berger, Vaillancourt, & Boksa, 2000; El-Khodor and Boksa, 1997)

A second group of animals was delivered via C-section with no period of added anoxia in the saline bath (C-section group). Time between decapitation of the dam and delivery of the last pup in a litter for the C-section group was less than 1.5 min and survival was 100% in the C-section group. Pups born vaginally served as a control group (vaginal group). Pups born vaginally were removed from their dams at 1-12 hrs after birth and were placed on a heating pad for 1-2 hrs before being placed with the surrogate dams. Pups from all three groups were cross-fostered by surrogate dams in mixed litters (12 pups/dam) to minimize differential rearing effects. Animals were weaned at 21-23 days of age and grown to adulthood (3 months).

Behavioral testing and limb-use observation

Use of each forelimb for upright support and for landing on when descending from a rearing position was analyzed both pre- and post-operatively by videotaping animals in the transparent cylinder for 3 min. Occurrences of forelimb use were scored during slow-motion playbacks from the videotapes (Schallert & Tillerson, 2000). Briefly, occurrences of forelimb use for wall exploration and landing were determined separately and each was expressed in terms of (1) the percent ipsilateral limb use [(ipsi/ipsi+contra+both) x 100] and (2) the percent contralateral limb use [(contra/ipsi+contra+both) x 100]. The percent contralateral limb use was then subtracted from the percent ipsilateral limb use for both the wall behavior and for landing. These

two scores (wall and landing) were averaged to obtain a single overall limb use asymmetry score.

Rats were also tested for spontaneous ipsiversive turning (turning toward the side of the lesion) in a novel environment, as previously described (Moroz, Rajabi, Rodaros, & Stewart, 2002). Briefly, the number of compact (within the diameter of approximately 20 cm) 360 degree turns and 180 degree half-turns ipsilateral and contralateral to the side of the lesion were recorded and summed across 5 min testing sessions. The number of ipsilateral turns was presented as a percent of the total number of turns displayed by an animal (ipsi/[ipsi + contra] x 100%).

Statistical Analyses

Due to low levels of locomotor activity displayed after surgeries, behavioral observations obtained on tests conducted 5 and 14 days after surgeries were combined to produce single scores (one for limb-use and one for turning). For limb-use asymmetry test, 4 animals that performed less than 5 landings and less than 10 wall movements during the combined testing sessions were not included in the analysis. For turning asymmetry, 3 animals that did not display any turns on the second day of testing were not included in the analysis. Behavioral observations were subjected to a 3 X 2 *Birth Type* (vaginal, C-section, C-section + anoxia) by *Surgery* (sham-lesion, lesion) ANOVA.

Analyses of immunocytochemistry results were done on raw data, using estimated numbers of FGF-2- and TH-IR cells per square millimeter. Cell counts from one rat were excluded from the analyses due to inactivity on both tests of behavioral asymmetries. Mixed factor ANOVAs were carried out for each brain region and each antibody (FGF-2

and TH) with *Birth Type* (vaginal, C-section, C-section + anoxia) and *Surgery* (sham-lesion, lesion) as between factors and *Side* (lesioned/sham-lesioned, non-lesioned) as a repeated factor. Tests for simple main effects and Fisher's PLSD ($p < .05$) were used to determine significant differences between means.

Results

Behavioral Asymmetry

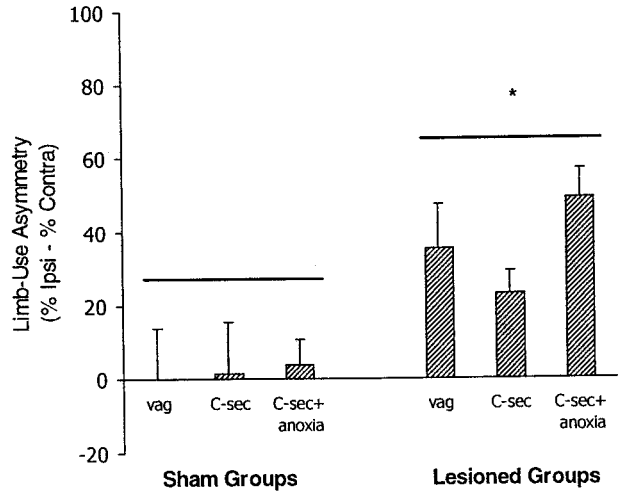
It can be seen in Figure 9 that rats with unilateral 6-OHDA lesions displayed a high degree of asymmetry of limb-use (Figure 9 A) and turning (Figure 9 B), as compared to sham-lesioned groups (limb-use: $F(1, 29) = 15.74$, $p < .001$; turning: $F(1, 30) = 32.96$, $p < .001$). There were no significant effects of *Birth Type* or *Day*, or any significant interactions. It can be readily seen in Figure 9 that the C-section + anoxia group was affected the most on both tests. Separate ANOVAs carried out on the data from lesioned groups only revealed that the C-section + anoxia group differed from C-section group on the limb-use asymmetry test ($p < .05$) and on turning ($p = .06$).

FGF-2 in SNc, VTA, SNr:

It can be seen in Figure 10 that all groups of rats with unilateral 6-OHDA lesions had increased numbers of FGF-2-IR cells on the side of the lesion in both the SNc and VTA. Mixed factor ANOVAs revealed a significant effect of *Side* (SNc, $F(1, 32) = 22.00$, $p < .001$), VTA ($F(1, 32) = 16.74$, $p < .001$, SNr, $F(1, 32) = 13.18$, $p < .001$) and significant *Side* by *Surgery* interactions (SNc, $F(1, 32) = 14.99$, $p < .001$, VTA, $F(1, 32) = 14.44$, $p < .001$), which were clearly due to the side effect in the lesioned groups.

Figure 9. Mean ($\pm$ SEM) asymmetry of (A) limb-use and (B) spontaneous turning, tested 5 and 14 days after 6-OHDA lesions of the MFB. Behavioral observations for days 5 and 14 were combined to produce single scores; one for limb-use and one for turning (see text). The lesioned groups differed significantly from the sham-lesioned groups (* $p < .05$).

A.


100
80
60
40
20
0
-20
Limb-Use Asymmetry
(% Ipsi - % Contra)
*
vag
C-sec
C-sec+
anoxia
vag
C-sec
C-sec+
anoxia
Sham Groups
Lesioned Groups

B.

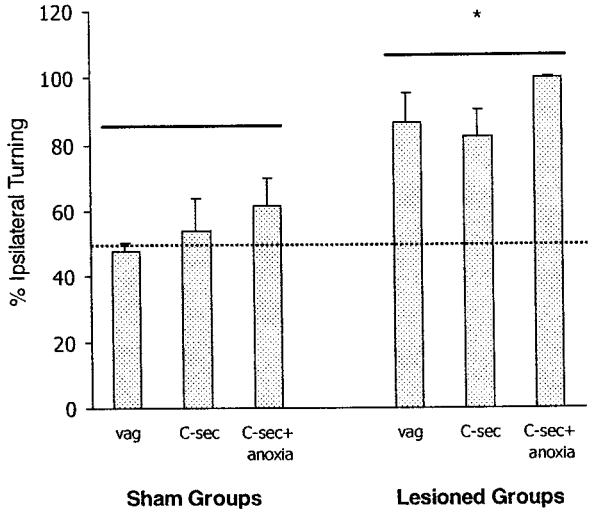
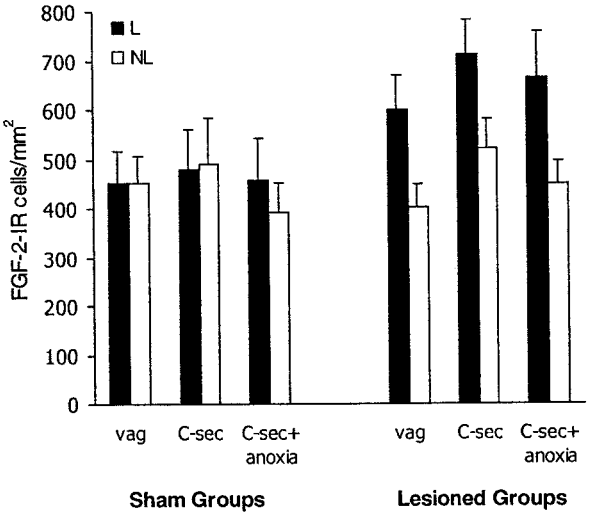
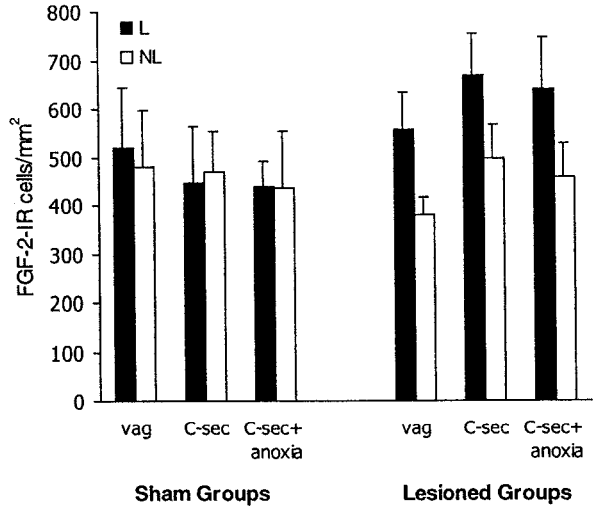

120
100
80
60
40
20
0
% Ipsilateral Turning
*
vag
C-sec
C-sec+
anoxia
vag
C-sec
C-sec+
anoxia
Sham Groups
Lesioned Groups

Figure 10. FGF-2-IR in SNc (top panel) and VTA (bottom panel) 14 days after 6-OHDA lesion of the MFB or sham-lesion surgery for groups shown in Figure 9. * indicate significant differences between the lesioned (L) and non-lesioned (NL) sides as assessed by analyses of simple main effects.

SNc


800
700
600
500
400
300
200
100
0
L
NL
FGF-2-IR cells/mm2
vag
C-sec
C-sec+ anoxia
vag
C-sec
C-sec+ anoxia
Sham Groups
Lesioned Groups

VTA


800
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500
400
300
200
100
0
L
NL
FGF-2-IR cells/mm2
vag
C-sec
C-sec+ anoxia
vag
C-sec
C-sec+ anoxia
Sham Groups
Lesioned Groups

There were no significant effects of *Birth Type, Birth Type* by *Surgery,* or *Birth Type* by *Side* interactions. Thus, lesions increased the numbers of FGF-2-expressing cells and the type of birth had no effect on this lesion-induced effect.

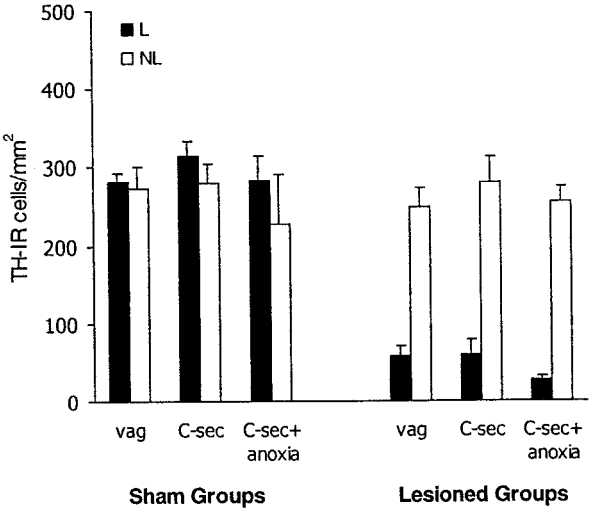
FGF-2 in nucleus accumbens (shell and core) and dorsomedial striatum: There were no significant effects of *Surgery, Side,* or *Birth Type* in any region.

TH in SNc and VTA:

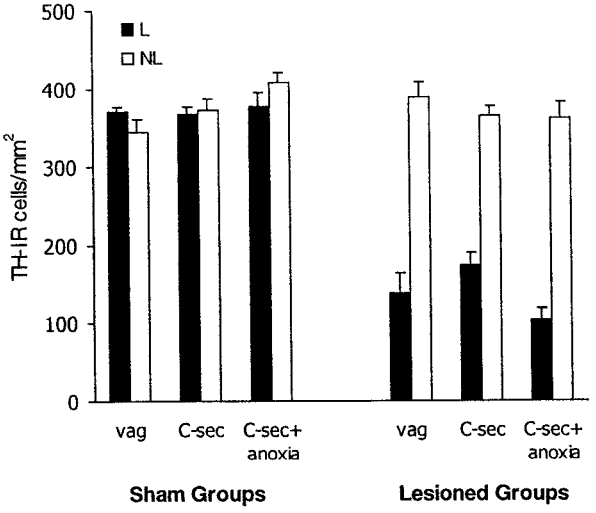
It can be seen in Figure 11 that in all lesioned groups there was a reduction in the number of TH-IR cells on the side of the lesion. Mixed factor ANOVAs revealed significant effects of *Surgery* (SNc, $F(1, 32) = 47.95$, $p < .0001$; VTA, $F(1, 32) = 94.40$, $p < .0001$), *Side* (SNc, $F(1, 32) = 42.12$, $p < .0001$; VTA, $F(1, 32) = 100.88$, $p < .0001$), and significant *Side* by *Surgery* interactions (SNc, $F(1, 32) = 78.61$, $p < .0001$; VTA, $F(1, 32) = 95.99$, $p < .0001$), which were clearly due to the side effect in the lesioned groups. It appears from inspection of Figure 3 that animals born by a C-section with anoxia (C-section + anoxia), had lower numbers of TH-IR cells remaining in the SNc and VTA as compared to the other lesioned groups. One-way ANOVAs carried out on the number of TH-IR cells remaining on the side of the lesion revealed a significant effect of *Birth Type* only in the VTA ($F(2, 23) = 3.99$, $p < .05$). Comparisons of the means revealed that the group born by a C-section with anoxia differed from the group born by a C-section alone (C-section) in the VTA ($p < .05$) and in the SNc ($p = .09$).

Figure 11. TH-IR in SNc (top panel) and VTA (bottom panel) 14 days after 6-OHDA lesion of the MFB lesion or sham-lesion surgery for groups shown in Figure 9. * indicate significant differences between the lesioned (L) and non-lesioned (NL) sides as assessed by analyses of simple main effects.

SNc


500
400
300
200
100
0
L
NL
TH-IR cells/mm2
vag
C-sec
C-sec+ anoxia
vag
C-sec
C-sec+ anoxia
Sham Groups
Lesioned Groups

VTA


500
400
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L
NL
TH-IR cells/mm2
vag
C-sec
C-sec+ anoxia
vag
C-sec
C-sec+ anoxia
Sham Groups
Lesioned Groups

Relationship between behavioral asymmetry, FGF-2-IR and TH

In both SNc and VTA, the percent increase in FGF-2-IR cells was correlated with the percent ipsilateral turning (SNc, $r = .46$, $p < .05$; VTA, $r = .43$, $p < .05$); the greater the behavioral asymmetry of turning, the greater the increases in FGF-2-IR on the side of the lesion. Although in the same direction, the correlations between the percent increase in FGF-2-IR cells and limb-use asymmetry did not reach statistical significance. The percent TH-IR loss in the SNc and VTA was correlated with the percent ipsilateral turning (SNc, $r = .81$, $p < .0001$; VTA, $r = .83$, $p < .0001$) and with the limb-use asymmetry (SNc, $r = .62$, $p < .0001$; VTA, $r = .62$, $p < .0001$); the greater the behavioral asymmetries of turning and limb-use, the greater the TH-IR loss on the side of the lesion. Furthermore, the percent increase in FGF-2-IR cells correlated with the percent loss of TH-IR cells (SNc, $r = .54$, $p < .001$; VTA, $r = .58$; $p < .0001$); the greater the losses in TH-IR cells, the greater the increases in FGF-2-IR cells on the side of the lesion.

Discussion

The purpose of the present study was to examine the effects of birth complications on behavioral responses and the expression of FGF-2 and TH after unilateral 6-OHDA lesions of the nigrostriatal neurons in adult rats. In agreement with our previous findings (Moroz et al., in press), 6-OHDA lesions led to increases in the number of FGF-2-IR astrocytes in the SNc and VTA on the lesioned side. These lesion-induced increases in FGF-2-IR cells were not affected by birth complications, suggesting that the astrocytic response to nigrostriatal DA neuron degeneration is not compromised nor augmented in rats born by a C-section, with or without an added episode of anoxia.

As expected, 6-OHDA lesions of the MFB led to dramatic decreases in the number of TH-IR cells in the SNc and VTA on the side of the lesion. These lesion-induced decreases in TH-IR cells tended to be more severe in the group born by C-section with an added episode of anoxia (C-section + anoxia). Tests of asymmetry of forelimb use and turning behavior revealed that the "C-section + anoxia" group also tended to be more devastated in comparison to the "C-section" group. On the test of turning, for example, the "C-section + anoxia" group displayed 99.7 % asymmetry.

The lack of an effect of birth complications on 6-OHDA-induced increases in astrocytic expression of FGF-2 is surprising in light of findings that perinatal anoxia renders FGF-2 responses in the vicinity of DA neurons more sensitive to the effects of challenges in later life. In particular, rats exposed to anoxia at birth have lower numbers of FGF-2 expressing astrocytes in the vicinity of DA neurons at 2 weeks and 3 ½ months after birth, but show enhanced FGF-2 expression after repeated exposure to stress in adulthood (Flores et al., 2002). It has been suggested that these enduring effects of perinatal anoxia on basal and stress-induced FGF-2 expression near DA neurons mediate the enhanced behavioral and neurochemical responses to stress observed in these rats in adulthood (Brake, Boksa, et al., 1997; Brake, Noel, et al., 1997; El-Khodor & Boksa, 1998; El-Khodor & Boksa, 2000). The lack of an effect of perinatal anoxia on lesion-induced FGF-2 expression in the current study may simply reflect differences in the severity of the manipulations used. Stress is typically described as a challenge to the midbrain DA system, whereas unilateral intra-MFB lesion poses a major insult to this system, which may be inducing a maximal response of astrocytes surrounding the degenerating neurons. It would be important therefore, to examine the effects of perinatal

anoxia on the FGF-2 response to milder lesions of the nigrostriatal DA system in the adult rat. Interestingly, the previously observed reduction in the basal numbers of astrocytes expressing FGF-2 in rats exposed to anoxia at birth was not seen in the present study in rats that underwent a sham surgery. It is tempting to hypothesize, therefore, that in the "C-section + anoxia" group, the sham-lesion surgery may have served as a stressful challenge to the midbrain DA system, and increased FGF-2 expression in this group to levels seen in rats born vaginally or by a C-section.

It is possible that the previously reported decreases in the basal number of FGF-2-IR cells in rats exposed to perinatal anoxia reflect an altered development of the DA system that could, in turn, underlie the enhanced vulnerability to neurotoxic insults later in life. The pattern of developmental expression of FGF-2 and its mRNA in the postnatal brain suggests that this factor is involved in postnatal neuronal maturation (Eckenstein et al., 1994; Kuzis et al., 1995; Riva & Mocchetti, 1991). Hence, the lower basal levels of FGF-2 in the vicinity of DA neurons observed in rats exposed to perinatal anoxia have been suggested to reflect a delay in developmental process in these regions (Flores et al., 2002). Such a developmental delay could occur as a result of the acute effects of perinatal anoxia on the DA system, specifically the augmentation in the concentration of DA and its metabolites observed within 20-40 minutes after birth (Ungethum et al., 1996), that could serve as an insult to this system. DA, just like 6-OHDA, is a highly reactive molecule that forms several potentially toxic molecules, such as reactive oxygen species, hydrogen peroxide and superoxide and DA quinone (Cohen, 1984). Oxidation of DA has been shown to occur *in vivo* and led to neurotoxicity (Hastings, Lewis, & Zigmond, 1996; Rabinovic, Lewis, & Hastings, 2000). Such an early insult to the

developing DA system in animals born with anoxia may have serious implications for the development and functioning of the midbrain DA system in adulthood. In the case of cortical lesions, it has been shown that the closer the injury occurs to the time of birth, the more disruptive its consequences on the behavior and neuronal morphology in adulthood (Kolb & Cioe 2000; Kolb, Cioe, & Whishaw, 2000a; Kolb, Cioe, & Whishaw, 2000b). Similarly, numerous behavioral deficits and increases in the responsivity to dopamine receptor agonists in adulthood are seen following neonatal 6-OHDA lesions of the midbrain DA system, with the type and degree of behavioral alteration dependent upon the age at which the lesioning occurs and the extent of lesions (Neal-Beliveau & Joyce, 1999).

Interestingly, some of the behavioral responses seen in adult rats exposed to perinatal anoxia are similar to those observed in adult rats that sustained extensive neonatal intrastriatal 6-OHDA lesions on the day of birth (P0) or postnatal day 1 (P1). In response to amphetamine, animals exposed to anoxia at birth show increased locomotion, duration of sniffing, and duration and frequency of rearing (El-Khodor & Boksa, 1998), whereas, in response to D1 receptor agonists, increases in locomotor activity, rearing and paw treading are observed in rats given 6-OHDA on P0/P1 (Neal-Beliveau & Joyce, 1998). It has been proposed that 6-OHDA lesions made on P0/P1 interfere with the ontogeny of DA receptor development, particularly the early-developing D_1 receptor system. Interestingly, losses of D_1 receptor with no change in D_2 receptor have been reported after both perinatal anoxia (Chen et al., 1997, but see El-Khodor & Boksa, 2001) and after neonatal 6-OHDA lesions (Neal-Beliveau & Joyce, 1992; Neal-Beliveau & Joyce, 1993; Thomas, Neal-Beliveau, & Joyce, 1998). Curiously, however, increases in

D_1 receptor sensitivity were seen in the NAcc after perinatal anoxia (Chen et al., 1997), and in certain parts of the dorsal striatum after neonatal 6-OHDA lesions (Simson et al., 1992). Thus, in spite of the reduction in D_1 receptor number, the increased agonist affinity for D_1 receptors may result in an increase in DA-agonist stimulated motor behaviors observed in adult animals exposed to perinatal anoxia or to neonatal 6-OHDA lesions.

Furthermore, neonatal 6-OHDA lesions have been reported to lead to compensatory region-specific elevation in TH mRNA levels observed in the dorsal medial and ventral medial striatum and in the VTA of adult animals (Frohna, Neal-Beliveau,& Joyce, 1993; Frohna, Neal-Beliveau, & Joyce, 1997). Interestingly, region specific changes in the numbers of TH-IR neurons and levels of TH mRNA have been also observed in animals subjected to perinatal hypoxia (Andersson et al., 1995; Bjelke et al., 1991; Gross et al., 2000). Thus, it is possible that the behavioral, neurochemical, and cellular consequences of perinatal anoxia develop as compensatory responses to the insult sustained by the developing DA system. As such, the enhancements in functioning of the DA system observed in adult in rats born with anoxia may reflect a pathological development leading to the increased vulnerability of this system to neurotoxic insults later in life. This hypothesis is supported by the present findings showing that in response to 6-OHDA lesion in adulthood, rats exposed to anoxia at birth show signs of enhanced vulnerability, manifested in pronounced behavioral asymmetries and substantial losses of TH-IR cell bodies in the SNc and VTA.

In summary, the present study is the first to provide evidence of enhanced vulnerability to insults to the midbrain DA system in adult rats exposed to perinatal

anoxia. In comparison to rats born vaginally or by C-section, exposure to an episode of anoxia resulted in more devastating behavioral asymmetries and pronounced TH-IR cell loss in the DA cell body regions after 6-OHDA lesions in adulthood. The lesion-induced increases in the number of astrocytes expressing FGF-2 in the vicinity of DA neurons were not affected by perinatal anoxia. The previously reported decreases in the basal numbers of FGF-2 expressing cells in rats exposed to perinatal anoxia, may underlie the enhanced vulnerability of these rats to insults to the adult midbrain DA system reported in the present study.

CHAPTER IV

EFFECTS OF FORCED LIMB-USE ON BEHAVIORAL OUTCOME AND FGF-2- AND TH-IMMUNOREACTIVITY AFTER PARTIAL UNILATERAL 6-OHDA LESIONS OF NIGROSTRIATAL DOPAMINE NEURONS

I. Moroz[1]; A.D. Cohen[2]; J.L.Tillerson[3]; K. Maxwell[1]; T. Schallert[3,4], J. Stewart[1*];

1. Center for Studies in Behavioral Neurobiology, Concordia Univ., Montreal, PQ, Canada;
2. Department of Neurology, Univ. Pittsburgh, Pittsburgh, PA, USA,
3. Dept of Psychology and Institute for Neuroscience, Univ. Texas, Austin, TX, USA,
4. Department of Neurosurgery, Univ. Michigan, Ann Arbor, MI, USA

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Abstract

We examined the impact of forced use of the impaired forelimb 10 and 28 days after unilateral 6-hydroxydopamine (6-OHDA) lesions of the medial forebrain bundle on astrocytic basic fibroblast growth factor (FGF-2) and tyrosine hydroxylase (TH) immunoreactivity in adult male rats. When lesioned, rats were fitted with casts forcing them to use the impaired (Ipsi-Cast) or the non-impaired limb (Contra-Cast) for 7 days or were left uncasted. There were two sham-lesioned groups for each time point, casted or uncasted. On all tests of limb use, sham-lesioned and Ipsi-Cast lesioned rats showed no asymmetry, whereas Contra-Cast and No-Cast lesioned rats did. The number of TH-immunoreactive (IR) cells in the ipsilateral SNc and VTA was reduced to about 50 % at Day 10, and to about 30 % at Day 28 after exposure to 6-OHDA. At Day 10, there was significant reduction in the number of TH-IR cells in uncasted animals (40%) and in Contra-casted animals (46%), but the reduction in ipsi-casted animals (64%) was not significant. By Day 28, TH-IR declined significantly to 20%, 42% and 40% in uncasted, contra-casted and ipsi-casted rats, respectively. FGF-2-IR astrocytes were increased in SNc and VTA ipsilateral to the lesion 10 and 28 days after lesioning, but this effect was not enhanced by forced use. Forced use alone increased the number of FGF-2-IR cells in SNc and VTA in both hemispheres 3 days after cast removal. In intact rats casted for 7 days and killed immediately or 7, 14, and 28 days after cast removal, the number of FGF-2-IR cells in SNc and VTA was increased in both hemispheres immediately after cast removal, but not at later time points. Thus, forced limb-use leads to increases in the numbers of astrocytes expressing FGF-2 in SNc and VTA and may contribute to behavioral recovery.

Introduction

Behavioral rehabilitation therapies have been reported to ameliorate motor impairments associated with neural degeneration accompanying stroke and Parkinson's disease (de Goede, Keus, Kwakkel, & Wagenaar, 2001; Liepert et al., 1998). Parkinson's disease is characterized by a progressive motor impairment caused by the gradual degeneration of the nigrostriatal dopamine (DA) neurons projecting from the substantia nigra to the striatum. Various forms of motor therapy, including conventional physiotherapy, treadmill training with body weight support, walking with controlled stride length or to a rhythmic auditory stimulation, cued and conscious movement control, balance and strength/resistance training, upper body karate training, have all been reported to significantly improve motor performance and simple daily activities of the Parkinson's disease patients (Baatile, Langbein, Weaver, Maloney, & Jost, 2000; de Goede et al., 2001; Hurvitz, 1989; McIntosh, Brown, Rice, & Thaut, 1997; Miyai et al., 2000; Morris, Iansek, Matyas, & Summers, 1996; Nieuwboer et al., 2001; Reuter, Engelhardt, Stecker, & Baas, 1999; Scandalis, Bosak, Berliner, Helman, & Wells, 2001; Toole, Hirsch, Forkink, Lehman, & Maitland, 2000; Viliani et al., 1999).

In a rat model of Parkinson's disease, a unilateral infusion of the neurotoxin 6-OHDA is given to selectively and partially destroy nigrostriatal DA cells, leading to a variety of behavioral asymmetries, including a preferential use of the forelimb ipsilateral to the affected hemisphere. It has been shown recently that a targeted motor therapy involving forced use of the impaired forelimb for seven days following the lesion not only prevents the development of forelimb use asymmetry but, in addition, leads to reduced loss of striatal DA and TH, the vesicular monoamine transporter (VMAT) and

the dopamine transporter (DAT) (Tillerson et al., 2001; Tillerson et al., 2002). Although the mechanisms through which behavioral rehabilitation exerts these beneficial effects remain obscure, neurotrophic factors are likely to play a role inasmuch as their actions have been implicated in various forms of plasticity in the adult brain.

Neurotrophic factors are proteins important for neuronal differentiation, growth and survival (Hefti et al., 1993). Basic fibroblast growth factor (bFGF or FGF-2) is one of the most studied molecules within the family of neurotrophic growth factors. FGF-2 is widely expressed during embryonic development and, more importantly, remains expressed in the adult brain (Eckenstein, 1994; Eckenstein et al., 1994). FGF-2 has been found *in vitro* to promote the survival and differentiation of several classes of neurons, including the midbrain DA neurons (Bouvier & Mytilineou, 1995; Hou et al., 1997; Reuss & Unsicker, 2000). Exogenous administration of FGF-2 into mouse striatum has been found to partially counteract the neurotoxin MPTP-induced loss of TH-IR nerve terminals and TH-IR neurons in the substantia nigra, and restored the MPTP-induced reduction of the locomotor activity (Chadi et al., 1993; Date et al., 1993; Otto & Unsicker, 1990). In the rat, intrastriatal implantation of genetically altered fibroblasts that produce FGF-2 have been found to protect against 6-OHDA-induced loss of striatal TH-IR fibers, TH-IR neurons in the substantia nigra, and behavioral asymmetries (Shults et al., 2000). Furthermore, astrocytic expression of FGF-2 was found to increase in the DA cell body regions after unilateral infusions of 6-OHDA (Chadi et al., 1994; Moroz, Rajabi, et al. 2002), possibly as an attempt to promote repair processes in the areas affected by the toxin.

Recent evidence indicates that the expression of neurotrophic factors in the adult brain is also enhanced after physical activity, suggesting that the beneficial aspects of exercise may act directly on the molecular machinery of the brain itself. Voluntary wheel running has been reported to increase FGF-2 mRNA in the hippocampus (Gomez-Pinilla, Dao, & So, 1997) as well as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) mRNA in the hippocampus and cerebral cortex (Neeper, Gomez-Pinilla, Choi, & Cotman, 1996) of adult male rats. Training on a Morris water maze task, a spatial memory task involving swimming, was found to increase both FGF-2 protein and its mRNA in the hippocampus and cerebellum. Interestingly, an active control group matched with the learning group on the amount of time spent swimming, but in which the spatial learning component of the task was minimized, also showed an increase in FGF-2 mRNA (Gomez-Pinilla, So, & Kesslak, 1998). Furthermore, this increase in FGF-2 mRNA was found to be intensity-dependent; the higher the intensity of the physical activity the higher the increase in FGF-2 mRNA. Thus, the ability of physical activity to stimulate the production of endogenous FGF-2 and other neurotrophic factors makes it plausible that the actions of neurotrophic factors may underlie the beneficial effects of physical therapy.

Here we examined the impact of forced use of the impaired forelimb after unilateral 6-OHDA nigrostriatal lesions on FGF-2 expression at two time points: 10 days and 28 days after lesioning. In addition, temporal expression of FGF-2 was examined in intact animals forced to rely on the use of one limb for 7 days.

Methods

Subjects

Adult male Wistar rats (Charles River, St. Constant, Quebec) weighing between 350-400 g at the time of experimental manipulations were used. All animals were housed individually in stainless steel cages and maintained in a temperature- and humidity-controlled environment under a 12 hr light/dark cycle with free access to food and water. Animals were handled and habituated to the laboratory before the experiments began. All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the Concordia University Animal Care Committee.

Materials

6-OHDA (6-hydroxydopamine hydrochloride, Sigma) was dissolved in 0.9 % saline containing 0.05 % ascorbic acid (Aldrich Chemical Company, Inc.) immediately before being injected. Desmethylimipramine (RBI Biochemicals) was dissolved in nanopure water and Pargyline (Sigma) was dissolved in 0.9 % saline. FGF-2 immunoreactivity was detected by using a mouse monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) at a concentration of 1:500, previously shown by means of double-label immunocytochemistry to detect FGF-2 immunoreactivity in astrocytes following injections of amphetamine (Flores et al., 1998; Flores & Stewart, 2000b). Similarly, after 6-OHDA lesions of the MFB, using this antibody, FGF-2 was expressed in astrocytes, but not in microglia (Moroz, Rajabi, et al., 2002). Rabbit polyclonal antibodies were used to detect TH-immunoreactivity (1:5000; Chemicon).

Surgical Procedures

Rats were anesthetized with sodium pentobarbital (30 mg/kg i.p.) followed by atropine sulfate (0.5 mg/ml, 0.2 ml/rat, s.c.). Methoxyflurane (Metofane) (Experiment 5) and Isoflurane (Experiment 6 and 7) were used to supplement the anesthesia throughout the surgery. Animals were given desmethylimipramine (15 mg/kg, i.p.), a norepinephrine reuptake inhibitor (to protect the noradrenergic cells from 6-OHDA), 30 min before infusion of 6-OHDA. 6-OHDA (4 µl of 10 µg/4 µl solution) was infused unilaterally using a Hamilton microsyringe (0.4 µl/min) over a period of 10 min into the medial forebrain bundle (MFB). Stereotaxic coordinates, with flat skull, were: 2.9 mm posterior, 1.7 mm lateral to bregma, and 7.6 mm ventral to dura. The injector was slowly removed 5 min after the end of the infusion. Sham-operated animals underwent identical stereotaxic surgical procedures and drug pretreatment up to, but not including, the lowering of the infusion syringe tip (dura was pierced). All animals were injected with an injectable antibiotic Penlong XL (0.2 ml/rat i.m.) after surgery.

Forelimb immobilization (casting) procedure

Rats in Experiments 5 and 6 were casted immediately following the surgical procedures while they were still anesthetized but recovered to the point of being able to orient their head to whisker stimulation. Rats in Experiment 7 were anesthetized with Isofluorane before casting. Casts were composed of plaster of Paris and were designed to immobilize one forelimb. They were formed around the upper torso into one-holed vests, which permitted unrestricted movement of one forelimb, but limited movements of the

restricted forelimb to a pocket of space between the torso and the inner (cotton) lining of the vest (Jones & Schallert, 1994).

Behavioral testing and limb-use observation

Use of each forelimb for upright support and for landing on when descending from a rearing position was analyzed both pre- and post-operatively by videotaping animals in the transparent cylinder for 3 min. Occurrences of forelimb use were scored during slow-motion playbacks from the videotapes (Schallert & Tillerson, 2000). Briefly, wall exploration and landing scores were determined separately and each was expressed in terms of (1) the percent ipsilateral limb use [(ipsi/ipsi+contra+both) x 100] and (2) the percent contralateral limb use [(contra/ipsi+contra+both) x 100]. The percent contralateral limb use was then subtracted from the percent ipsilateral limb use for both the wall behavior and for landing. These two scores (wall and landing) were averaged to obtain a single overall limb use asymmetry score.

Immunocytochemistry

Animals were deeply anesthetized with sodium pentobarbital (120 mg/kg) and perfused transcardially with 200 ml of cold PBS, followed by 100 ml of a cold solution of paraformaldehyde (w/v) and 15% picric acid (v/v) in phosphate buffer (PB; pH 6.9). Brains were removed and stored overnight in the fixative solution at 4 degrees C. Coronal 50 μm sections were cut on a vibratome and stored overnight in PB at 4 degrees C. Before slicing, a small mark was cut in each brain to allow discrimination of the hemispheres in each section. Sections were then double labeled for FGF-2 and TH using immunocytochemistry as described previously (Moroz et al., 2002b). Double labeling

was obtained by processing the sections first for FGF-2 immunoreactivity and then for TH immunoreactivity. Briefly, free-floating tissue sections were incubated for 24 hrs at 4 degrees C with the mouse anti-FGF-2 antibody diluted to 1:500 with 0.3% Triton X-100 (Sigma) in PB and 1% Normal Horse Serum (Vector Laboratories, Burlingame, CA). After incubation in the primary antibody, sections were rinsed three times for 5 min in cold PB and incubated for 1 hr at room temperature (RT) in a solution of rat adsorbed biotinylated anti-mouse antibody (Vector) diluted to 1:200 with PB and 1% Normal Horse Serum. After three 5 min washes in cold PB, sections were incubated in an avidin-horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector) for 30 min at RT, and rinsed again three times for 5 min in cold PB. Next, sections were incubated for 10 min at room temperature and under constant agitation in a solution of 0.05% 3,3'-diaminobenzidine (Sigma) in PB. Then, without washing, the sections were transferred to a 3,3'-diaminobenzidine/PB solution, pH 7.8 with 0.01% H_2O_2 to catalyze the reaction and with 8% $NiCl_2$ to darken the reaction product. This incubation was terminated 8 min later by washing the sections three times for 10 min in cold PB.

For TH immunoreactivity, the tissue sections previously processed for FGF-2 immunoreactivity were preincubated in 0.3% Triton X-100 PB and 1% Normal Goat Serum (NGS) for 1 hr at room temperature. They were then incubated for 24 hrs at 4 degrees C with the rabbit anti-TH polyclonal antibody diluted to 1:5000 (Chemicon) in PB and 1% NGS. Secondary antibody (rat adsorbed biotynylated anti-rabbit antibody) and the ABC reagent were applied as described above. No $NiCl_2$ was added to the 3,3'-diaminobenzidine-PB-H_2O_2 solution in order to obtain an orange-brown reaction product clearly distinguishable form the black FGF-2 reaction product. This incubation was

terminated 5 min later by washing the sections three times for 10 min in cold PB. Tissue from all groups was included in each batch of immunocytochemical processing.

Sections were then mounted on gelatin-coated slides, dried for at least 24 hours, hydrated in distilled water (1 min) and gradually dehydrated in 70%, 95%, and 100% ethanol. Slides from Experiment 7 were counterstained with 0.1 % cresyl violet to demonstrate anatomical landmarks. Slides then were cleared in Hemo-De and were coverslipped with Permount (Fisher Scientific).

Image Analysis

Immunostained sections were analyzed under a Leica microscope (Leitz DMRB). The number of astrocytic FGF-2- and TH-immunoreactive (IR) cells per squared millimeter was estimated from digitized images of sample areas within SNc, VTA, and SNr, nucleus accumbens shell and core, dorsomedial striatum using computerized image-analysis system (NIH Image 1.6). Structure boundaries were defined according to the Paxinos and Watson stereotaxic atlas (Paxinos & Watson, 1997).

FGF-2-IR cells were counted in the DA cell body and terminal regions as described previously (Moroz et al., 2002b). Briefly, three images were taken in each hemisphere from VTA and SNr and 4 from SNc at 2 different levels from bregma: -5.2 and -5.3. For the DA terminal regions, four images were taken from each region at 3 different levels from bregma: 0.6mm, 1.2 mm, and 1.7 mm, in each hemisphere. TH-IR cells were counted in a minimum of 8 images taken from SNc and 4 from VTA at two different levels from bregma: -5.2 and -5.3mm, in each hemisphere. Only sections in which the medial and lateral parts of the substantia nigra were clearly separated by the

medial terminal nucleus of the accessory optic tract were selected (Lee et al., 1996; Sauer & Oertel, 1994). No attempt was made to estimate the total TH-IR neurons number in three dimensions. The number of TH-IR cells calculated from a few selected levels from bregma has been previously shown to be an accurate representation of 6-OHDA induced degeneration of the SNc and VTA neurons (Carman et al., 1991; Gordon et al., 1997). The images were assigned code names and the FGF-2- and TH-IR cells were counted by an individual blind to the code assignment. The cell counts from the areas sampled in each hemisphere of each brain region were summed and divided by the total area examined.

Design and Procedures

Experiment 5 and 6. FGF-2 and TH-IR after 6-OHDA and forced limb-use manipulations

For each experiment, rats were randomly assigned to one of three lesioned groups or two sham-lesioned groups (n = 6-8 rats/group). The three lesioned groups included: 1) Ipsi-Cast group – fitted with casts immobilizing the "good" limb, or the limb ipsilateral to the lesioned hemisphere, 2) Contra-Cast group, fitted with casts immobilizing the "bad" limb, or the limb contrataleral to the lesioned hemisphere, and 3) No-Cast group, not fitted with casts. The two sham-lesioned groups included a casted group (Cast) – fitted with casts immobilizing a forelimb ipsilateral to the sham-lesioned hemisphere and a group with no casts (No-Cast). In Experiment 5, behavior was assessed before and 9 days after the lesions; FGF-2 and TH-IR was examined 10 days after the lesion. In Experiment 6, behavior was assessed before and 14, 21, and 28 days after the lesion; FGF-2 and TH-IR were examined 28 days after the lesion.

Experiment 7. Time course of FGF-2 expression after forced limb-use in intact rats

Rats were assigned to have either their left or right forelimb immobilized in a cast for 7 days. Groups of rats were killed immediately (n = 8), 7 days (n = 8), 14 days (n = 8) and 28 days (n = 8) after the cast was removed. A control group of 6 rats were handled but not casted.

Statistical Analyses

Behavioral observations of limb use asymmetry were subjected to a one-way ANOVA (Experiment 5) and a mixed factor ANOVA (Experiment 6) with *Group* (lesion + Ipsi-Cast, lesion + Contra-Cast, lesion + No-Cast, sham-lesion + Cast, sham-lesion + No-Cast) as the between factor and *Time* after lesion (14, 21, and 28 days) as the within factor. Analyses of immunocytochemistry results were done on raw data, using estimated numbers of FGF-2- and TH-IR cells per square millimeter. For Experiments 5 and 6, the data from the three lesioned groups (Ipsi-Cast, Contra-Cast, and No-Cast) and the two sham-lesioned groups (Cast, No-Cast) were analyzed separately using mixed factor ANOVAs carried out for each brain region and each antibody (FGF-2 and TH) with *Cast* (Ipsi, Contra, or No) as the between factor and *Side* (lesioned/sham-lesioned, non-lesioned) as the within factor. For Experiment 7, due to the differences in stain quality obtained in 2 separate immunocytochemistry runs, the raw counts of FGF-2-IR cells were presented as percent increase or decrease relative to those of the non-casted controls taken as 100%. Mixed factor ANOVAs were then carried out for each brain region with *Time* after cast removal (immediately, 7, 14, and 28 days) as the between factor and *Side* (ipsilateral, contralateral to the casted forelimb) as the within factor. Tests for simple

main effects and Fisher's PLSD ($p < .05$) were used to determine significant differences between means.

Results

Experiment 5: Effects of forced limb-use on behavioral asymmetry and FGF-2- and TH-IR 10 days after 6-OHDA lesion (3 days after cast removal)

Limb-use asymmetry test

Figure 12 shows that forced use of the impaired limb for seven days after lesioning prevented the preferential use of the "good forelimb" for vertical exploration that was evident in the No-Cast and Contra-Cast groups. The ANOVA revealed that the effect of *Group* was significant ($F(4, 28) = 6.63$, $p < .001$) on this test given 9 days after the lesion. Comparisons of the means revealed that the Ipsi-Cast group did not differ from either of the sham-lesioned groups, but Ipsi-Cast and sham group means differed from those of the No-Cast and Contra-Cast 6-OHDA groups. There were no differences between groups in limb use before the surgery ($F(4, 28) = 1.33$, $p > .05$).

FGF-2 in SNc, VTA, SNr:

It can be seen in Figure 13 that all groups of animals with unilateral 6-OHDA lesions had increased numbers of FGF-2-IR cells on the *Side* of the lesion in both the SNc ($F(1, 19) = 10.33$, $p < .01$) and VTA ($F(1, 19) = 17.18$, $p < .001$). There was no effect of lesion on FGF-2 expression in SNr ($F(1, 19) = .73$, $p > .05$) and no *Cast* or *Side* by *Cast* interaction in any of the regions. Thus, lesions increased the numbers of FGF-2-

Figure 12. Asymmetry of limb-use for vertical and lateral weight shifting movements in the cylinder test nine days after 6-OHDA lesion (Experiment 5). Lesioned rats were either left uncasted (No-Cast) or were forced to use their impaired (Ipsi-Cast) or non-impaired limb (Contra-Cast) for 7 days after lesioning. Sham-lesioned groups were either left uncasted (No-Cast) or were forced to use the limb ipsilateral (Ipsi-Cast) to the sham-operated hemisphere. Forced use of the impaired forelimb (Ipsi-Cast) after lesioning prevented asymmetry. The Ipsi-Cast group did not differ from either of the sham-lesioned groups, but all differed significantly (*) from the No-Cast and Contra-Cast groups.

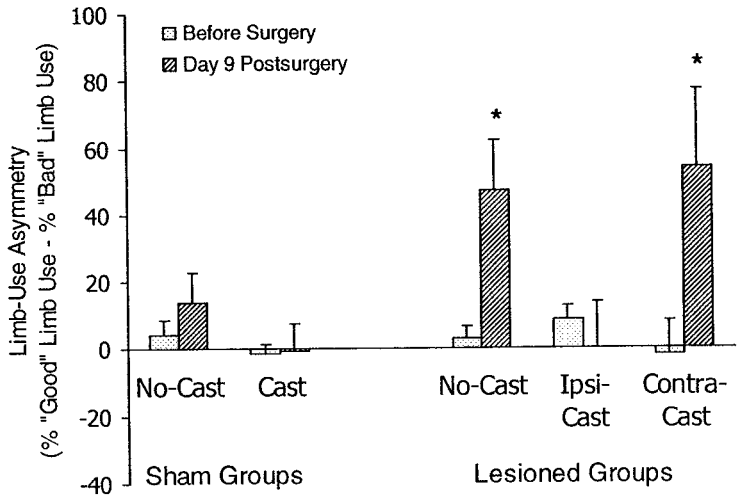
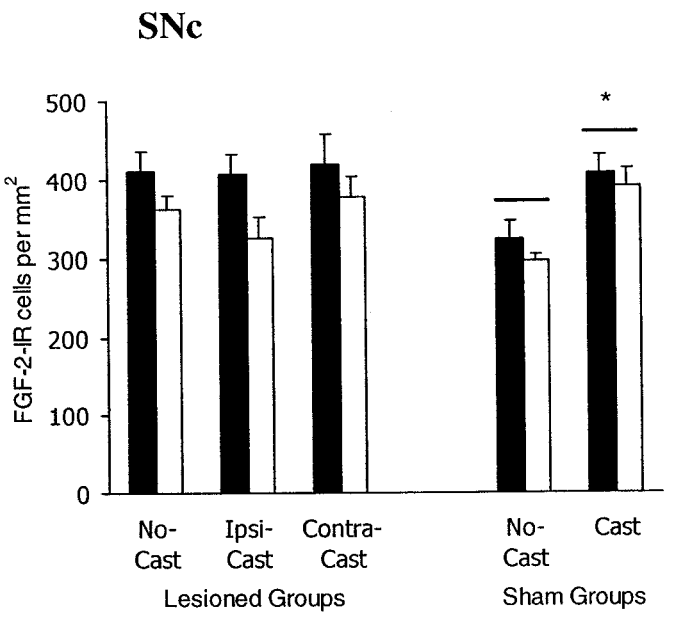
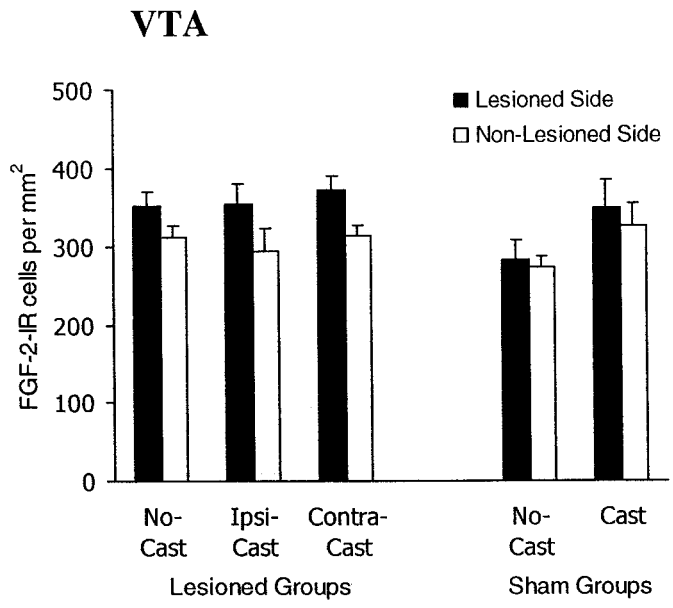

Before Surgery
Day 9 Postsurgery
Limb-Use Asymmetry
(% "Good" Limb Use - % "Bad" Limb Use)
100
80
60
40
20
0
-20
-40
*
*
No-Cast
Cast
No-Cast
Ipsi-
Cast
Contra-
Cast
Sham Groups
Lesioned Groups

Figure 13. FGF-2-IR in SNc (top panel) and VTA (bottom panel) 10 days after 6-OHDA lesion or sham-lesion surgery for groups shown in Figure 12. Both 6-OHDA lesions and forced limb-use, in itself, increased the numbers of astrocytes expressing FGF-2 in SNc and VTA, but the effects of each manipulation on FGF-2-IR were not additive. * indicates a significant effect of *Cast* in the Sham-lesioned groups.


SNc
500
400
300
200
100
0
FGF-2-IR cells per mm2
*
No-Cast
Ipsi-Cast
Contra-Cast
No-Cast
Cast
Lesioned Groups
Sham Groups


VTA
500
400
300
200
100
0
FGF-2-IR cells per mm2
Lesioned Side
Non-Lesioned Side
No-Cast
Ipsi-Cast
Contra-Cast
No-Cast
Cast
Lesioned Groups
Sham Groups

expressing cells and forced limb-use had no additional effect on this lesion-induced effect.

In the sham-lesioned groups, as seen in Figure 13, casted animals had higher levels of FGF-2-IR cells than non-casted animals in both hemispheres. This effect of *Cast* was significant only in the SNc ($F(1, 10) = 10.23$, $p < .01$). There was no effect of *Side* or *Side* by *Cast* interaction in any of the regions.

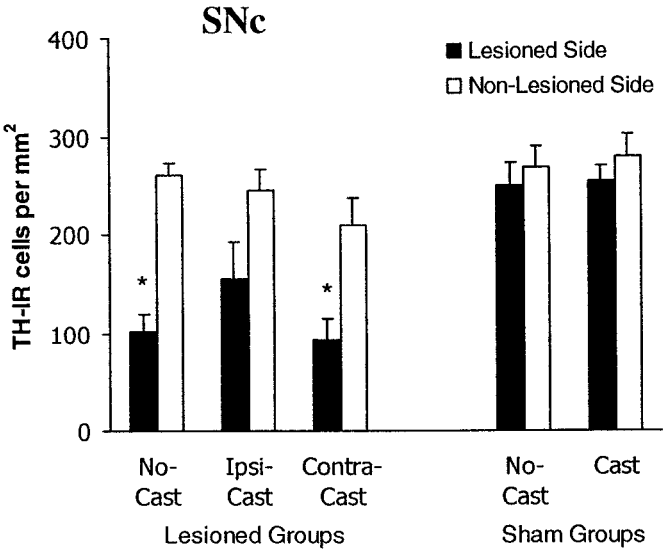
FGF-2 in nucleus accumbens (shell and core) and dorsomedial striatum:

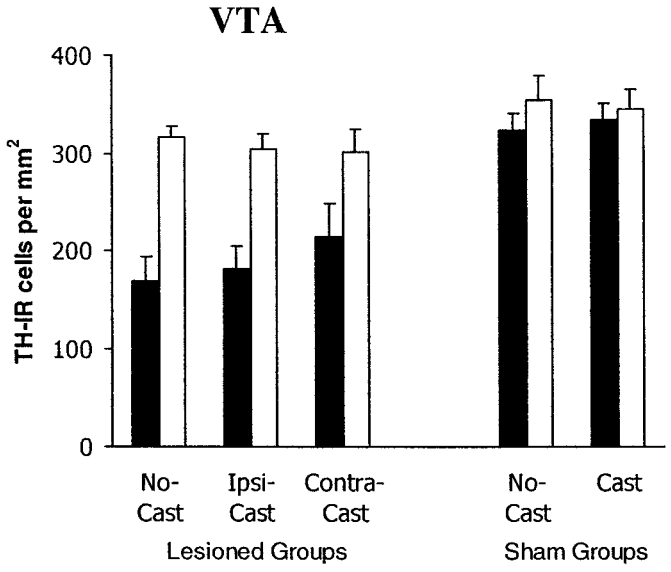
The groups of rats with unilateral 6-OHDA lesions had increased numbers of FGF-2-IR cells on the side of the lesion in the terminal regions examined (NA shell, $F(1, 19) = 6.00$, $p < .05$; NA core, $F(1, 19) = 4.15$, $p = .05$; dorsomedial striatum, $F(1, 19) = 4.67$, $p < .05$). In the sham-lesioned groups there were no significant effects of *Side* or *Cast* on FGF-2-IR in any region.

TH in SNc and VTA:

It can be seen in Figure 14 that in the lesioned groups there was a significant effect of *Side* in both SNc ($F(1, 19) = 49.618$, $p < .0001$) and VTA ($F(1, 19) = 65.24$, $p < .0001$) reflecting the reduction in the number of TH-IR cells on the side of the lesion. As was the case for FGF-2, the ANOVAs revealed no effect of *Cast* or significant *Cast* by *Side* interactions. It appears from inspection of Figure 14 (top panel) that animals forced to use their impaired forelimb (Ipsi-Cast) had a greater number of TH-IR cells remaining in the SNc on the side of the lesion. Paired t-tests were used to assess the *Side* difference in each group. It was found that whereas there were significant differences between the

Figure 14. TH-IR in SNc (top panel) and VTA (bottom panel) 10 days after 6-OHDA lesion or sham-lesion surgery for groups shown in Figure 12. Forced use of the impaired forelimb after lesioning (Ipsi-Cast) reduced the lesion-induced loss of TH-IR cells in SNc such that it was not statistically significant. * indicate significant differences between lesioned and non-lesioned sides as assessed by analyses for simple main effects.


SNc
Lesioned Side
Non-Lesioned Side
TH-IR cells per mm2
400
300
200
100
0
*
*
No-Cast
Ipsi-Cast
Contra-Cast
No-Cast
Cast
Lesioned Groups
Sham Groups


VTA
TH-IR cells per mm2
400
300
200
100
0
No-Cast
Ipsi-Cast
Contra-Cast
No-Cast
Cast
Lesioned Groups
Sham Groups

lesioned and non-lesioned sides in the No-Cast and Contra-Cast groups ($p < .05$, Bonferroni corrected), the effect of lesion was not significant in the Ipsi-Cast group.

In sham-lesioned groups, the ANOVAs revealed no significant effects in any of the regions.

Experiment 6: Effects of forced limb-use on behavioral asymmetry and FGF-2- and TH-IR 28 days after 6-OHDA lesion (21 days after cast removal)

Limb-use asymmetry test

Figure 15 shows that forced use of the impaired limb for 7 days after lesioning prevented the preferential use of the "good forelimb" for vertical exploration for up to 28 days after lesion (21 days after cast removal). The mixed factor ANOVA on post-surgery data revealed a significant *Group* effect ($F(4, 30) = 5.98$, $p < .01$). Comparisons of the means revealed that the Ipsi-Cast group did not differ from either of the sham-lesioned groups, but Ipsi-Cast and sham group means differed from those of the No-Cast and Contra-Cast 6-OHDA groups. The ANOVA revealed no significant effect of *Time* or *Time* by *Group* interaction. There were no differences between groups in limb use before the surgery ($F(4, 30) = 1.70$, $p > .05$).

FGF-2 in SNc, VTA, SNr:

It can be seen in Figure 16 that in the groups with unilateral 6-OHDA lesions there was a greater number of FGF-2-IR cells on the side of the lesion in the SNc ($F(1, 18) = 51.27$, $p < .0001$), VTA ($F(1, 18) = 43.21$, $p < .0001$), and in the SNr ($F(1, 18) =$

Figure 15. Asymmetry of limb-use for vertical and lateral weight shifting movements in the cylinder test tested 14, 21, and 28 days after 6-OHDA lesions (Experiment 6). As in Experiment 1, lesioned rats were either left uncasted (No-Cast) or were forced to use their impaired (Ipsi-Cast) or non-impaired limb (Contra-Cast) for 7 days after lesioning. Sham-lesioned groups were either left uncasted (No-Cast) or were forced to use the limb ipsilateral (Ipsi-Cast) to the sham-operated hemisphere. Limb-use asymmetry was prevented by forced use of the impaired forelimb after lesioning. The Ipsi-Cast group did not differ from either of the sham-lesioned groups , but all differed significantly (*) from the No-Cast and Contra-Cast groups.

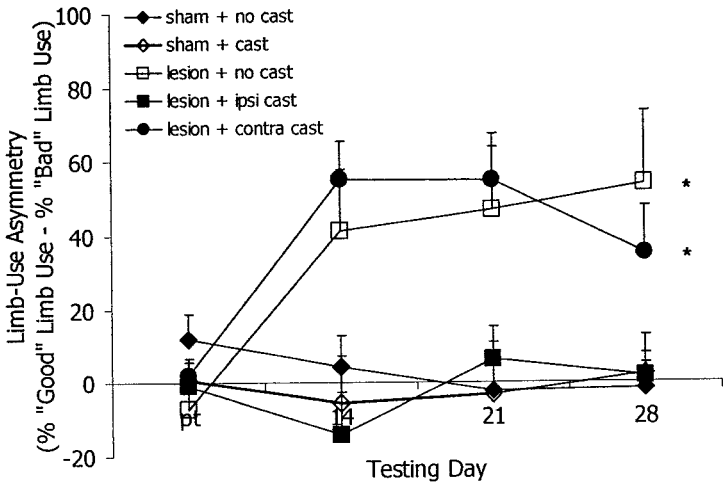
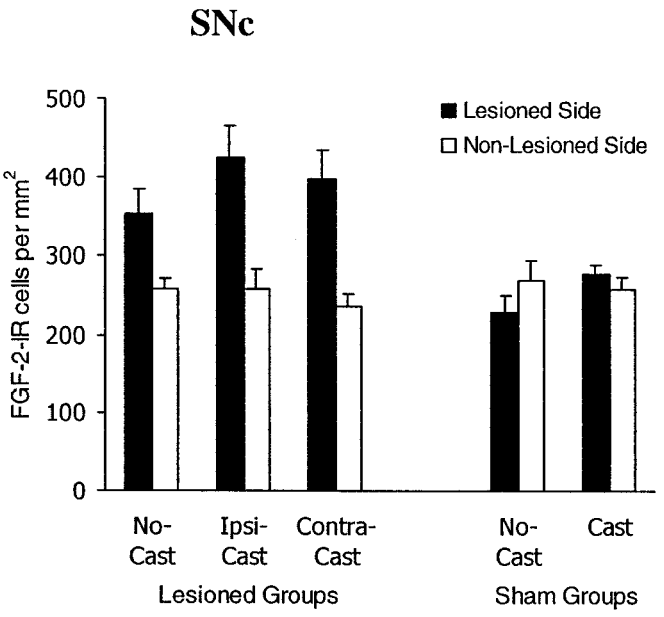
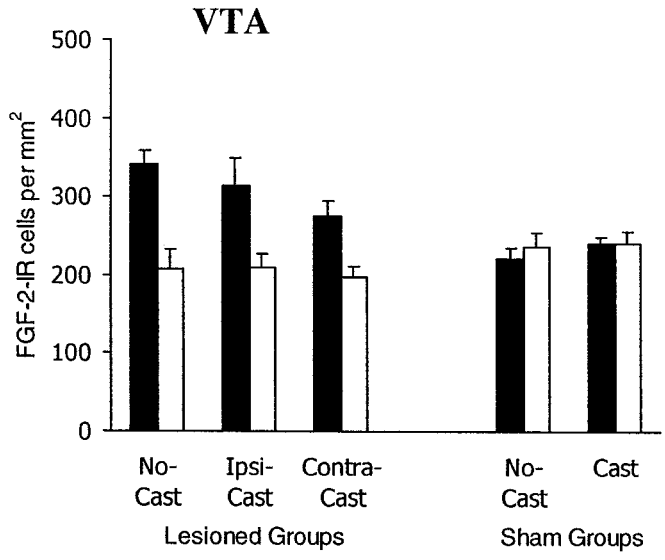

sham + no cast
sham + cast
lesion + no cast
lesion + ipsi cast
lesion + contra cast
Limb-Use Asymmetry
(% "Good" Limb Use - % "Bad" Limb Use)
100
80
60
40
20
0
-20
pt
14
21
28
Testing Day
*
*

Figure 16. FGF-2-IR in SNc (top panel) and VTA (bottom panel) 28 days after 6-OHDA lesion or sham-lesion surgery for groups shown in Figure 15. 6-OHDA lesions increased the numbers of astrocytes expressing FGF-2 in the lesioned SNc and VTA. No residual effects of casting alone were seen at 28 days. Forced limb-use had no additional effect on the lesion-induced increase in FGF-2 expressing cells.


SNc
Lesioned Side
Non-Lesioned Side
FGF-2-IR cells per mm2
500
400
300
200
100
0
No-Cast
Ipsi-Cast
Contra-Cast
No-Cast
Cast
Lesioned Groups
Sham Groups


VTA
FGF-2-IR cells per mm2
500
400
300
200
100
0
No-Cast
Ipsi-Cast
Contra-Cast
No-Cast
Cast
Lesioned Groups
Sham Groups

23.67, $p < .05$). There was no effect of *Cast* or *Side* by *Cast* interaction in any of the regions. Thus, lesions increased the numbers of FGF-2-expressing cells and forced use had no additional effect on this lesion-induced effect.

In sham-lesioned groups, the ANOVAs revealed no significant effects in any of the regions.

FGF-2 in nucleus accumbens (shell and core) and dorsomedial striatum:

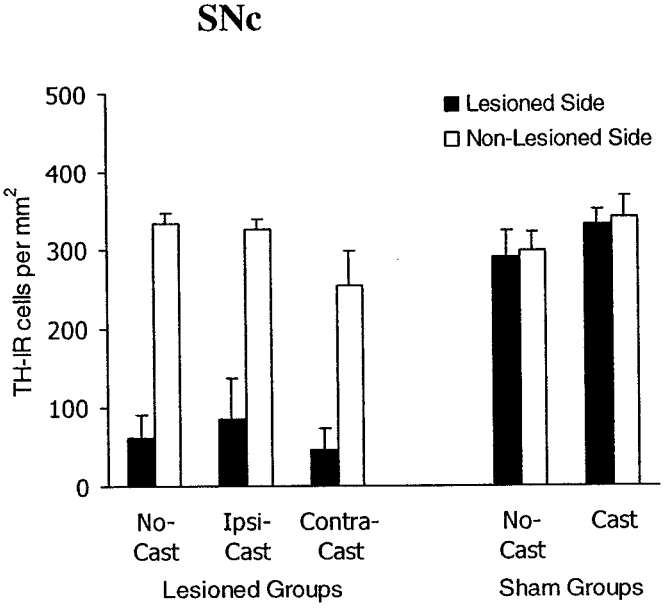
There was a greater number of FGF-2-IR cells on the *Side* of the lesion in the groups with unilateral 6-OHDA lesions in all terminal regions examined (NA shell, $F(1, 18) = 33.34$, $p < .0001$; NA core, $F(1, 18) = 8.82$, $p < .05$; dorsomedial striatum, $F(1, 18) = 24.15$, $p < .0001$). In the sham-lesioned groups the ANOVAs revealed no significant effects in any of the regions.

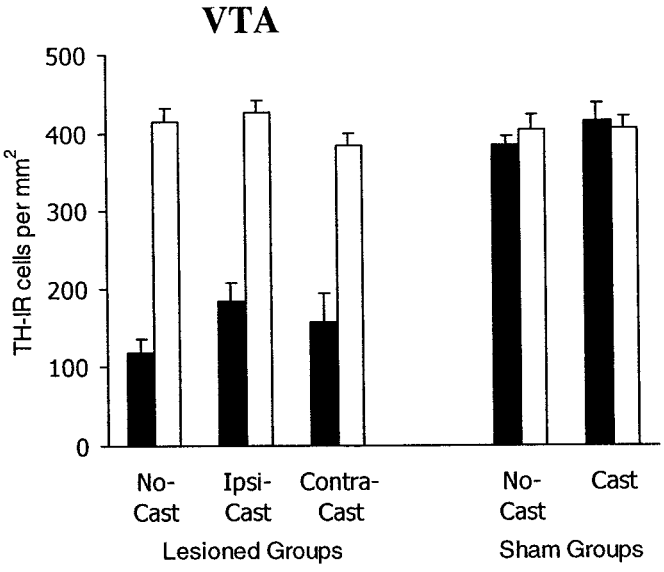
TH in SNc and VTA:

It can be seen in Figure 17 that in the lesioned groups there was a significant reduction in the number of TH-IR cells on the lesioned side in both SNc ($F(1, 18) = 63.77$, $p < .0001$) and VTA ($F(1, 18) = 160.37$, $p < .0001$). As was the case for FGF-2, the ANOVA revealed no effect of *Cast* or *Cast* by *Side* of lesion interaction in either region.

In sham-lesioned groups the ANOVA revealed no significant effects in either region.

Figure 17. TH-IR in SNc (top panel) and VTA (bottom panel) 28 days after 6-OHDA lesion or sham-lesion surgery for groups shown in Figure 15. 6-OHDA lesions significantly reduced the numbers of TH-IR cells in both the SNc and VTA.


SNc
Lesioned Side
Non-Lesioned Side
TH-IR cells per mm2
500
400
300
200
100
0
No-Cast
Ipsi-Cast
Contra-Cast
No-Cast
Cast
Lesioned Groups
Sham Groups

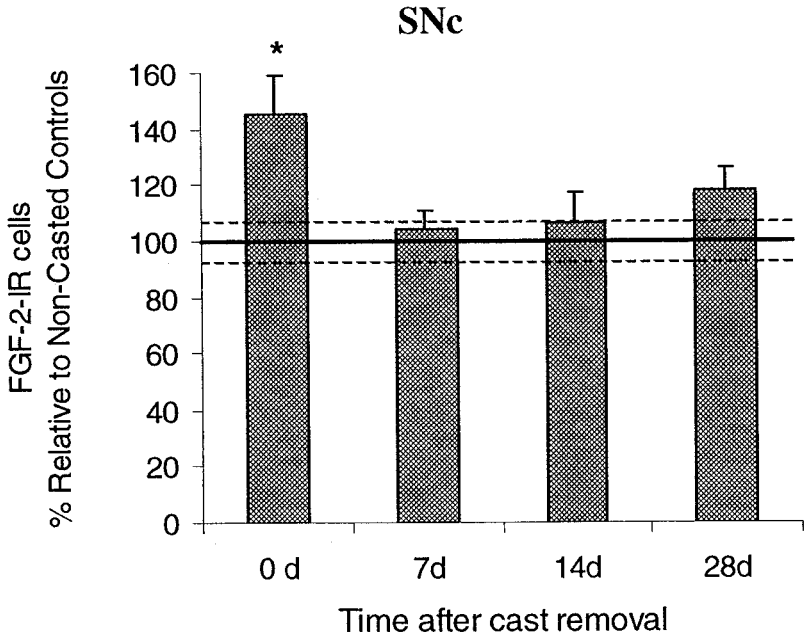

VTA
TH-IR cells per mm2
500
400
300
200
100
0
No-Cast
Ipsi-Cast
Contra-Cast
No-Cast
Cast
Lesioned Groups
Sham Groups

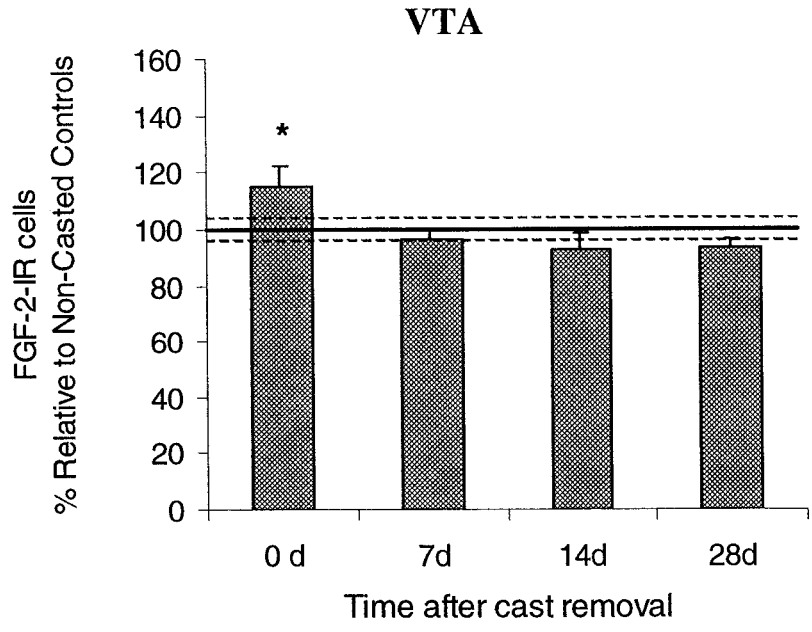
Experiment 7: Time course of FGF-2 expression after forced limb-use in intact rats

The time-course experiment was conducted in two replications each containing animals from all groups. There were, however, noticeable differences in stain quality in the two separate immunocytochemistry runs for FGF-2-IR that were apparent even in the non-casted controls. In this latter group the mean number of FGF-2-IR cells counted in SNc, VTA, and SNR was 1.5-2 times higher in the second run than during the first. For this reason, the analysis of the data from each run was carried out on transformed scores. The raw numbers of FGF-2-IR cells in each region of each animal were expressed as the percent increase or decrease relative to appropriate means of the non-casted controls from the same run. These transformed values from each run were then analyzed together in a single mixed factor ANOVA. As shown in Figure 18, an increase in FGF-2-IR of about 45 % in the SNc and 15 % in the VTA over that seen in non-casted controls was observed immediately after cast removal, but not at any other time point. The analysis revealed a significant effect of *Time* in both the SNc ($F(3, 28) = 3.50$, $p < .05$) and VTA ($F(3, 28) = 4.12$, $p < .05$) reflecting the fact that the number of FGF-2-IR cells immediately after cast removal differed significantly from the other time points. No significant effect of *Side* or *Time* by *Side* interactions were found in either region.

No significant effects of forced use were found in the SNr, nucleus accumbens (shell and core) and dorsomedial striatum at any time point after cast removal.

Figure 18. Mean (± SEM) percent increase in the number of FGF-2-IR cells relative to the non-casted controls in the SNc (top panel) and VTA (bottom panel) in groups of intact rats casted for seven days and killed either immediately or 7, 14, and 28 days after cast removal (Experiment 7). One hundred percent (100%) represents the mean (solid line) and SEM (dashed lines) counts from non-casted controls. * indicates that the group killed immediately after cast removal was significantly different from all other groups.


SNc
*
160
140
120
100
80
60
40
20
0
FGF-2-IR cells
% Relative to Non-Casted Controls
0 d
7d
14d
28d
Time after cast removal


VTA
*
160
140
120
100
80
60
40
20
0
FGF-2-IR cells
% Relative to Non-Casted Controls
0 d
7d
14d
28d
Time after cast removal

Discussion

In rats with partial unilateral 6-OHDA lesions of the nigrostriatal pathway, forced use of the impaired forelimb for the first seven days after lesioning has been shown to result in sparing of limb use (Tillerson et al., 2001). The fact that such behavioral sparing was accompanied by reduced tissue loss of striatal DA and TH, as well as VMAT and DAT, suggests that forced use induces factors that can protect against DA neuron degeneration and/or enhance sprouting of DA terminals (Tillerson et al., 2001; Tillerson et al., 2002). The primary purpose of the present experiments was to examine the impact of forced use of the impaired forelimb on expression of the neurotrophic and neuroprotective factor FGF-2 in rats with similar lesions. In agreement with previous results, we found that forced use of the impaired forelimb for seven days after unilateral 6-OHDA infusion prevented asymmetry of forelimb use for vertical and lateral weight shifting movements. This effect was evident as early as 9 days after lesioning (or 2 days after cast removal) in Experiment 5 and was maintained for 28 days after lesioning (or 21 days after cast removal) in Experiment 6. In contrast, forced non-use of the impaired forelimb for seven days after lesioning led to long-lasting asymmetry of limb use similar to that observed in rats that did not receive forced limb-use intervention after lesioning (see also Tillerson et al., 2002).

As expected based on our previous findings (Moroz, Rajabi, et al., 2002), 6-OHDA lesions led to increases in the number of FGF-2-IR astrocytes in the SNc and VTA on the lesioned side. These increases were evident 10 days after the lesion and were greatest 28 days after the lesion. Neither forced use nor forced non-use of the impaired forelimb after lesioning modified this lesion-induced increase in FGF-2-IR

cells, suggesting that the therapeutic effects of forced use in this context cannot be explained simply by the levels of FGF-2 expression. Interestingly, however, in rats without 6-OHDA lesions, forced use of one limb resulted in an increase in FGF-2-IR cells in SNc and VTA in both hemispheres measured 10 days after sham-lesion surgery or two days after cast removal. Examination of this effect in rats that did not undergo surgery, but that were given casts forcing them to use one limb for seven days (Experiment 7), revealed an increase in FGF-2-IR cells in SNc and VTA in both hemispheres. This effect was transient and was seen in rats killed immediately after cast removal but not at later time points.

Consistent with our earlier findings (Moroz, Rajabi, et al., 2002), 6-OHDA lesions of the MFB led to time dependent decreases in the number of TH-IR cells in the SNc and VTA on the side of the lesion. Ten days after lesioning, the number of TH-IR cells was significantly reduced to about 50 % in rats without casts. In rats forced to use their impaired forelimb after lesioning, however, TH-IR was reduced, but not significantly, suggesting at least a transient partial neuroprotective effect of forced limb-use on the degenerating DA neurons. This finding of a modestly attenuated loss of TH-IR cells in SNc 10 days, but not 28 days after the lesion in rats forced to use their impaired limb, suggests that forced limb-use delays the gradual loss of TH-IR cells. This delayed loss of TH-IR cells, may provide a window for the operation of factors that lead to compensatory changes in the remaining DA cells and that are responsible, at least in part, for the sparing of behavior. It has been found, for example, that there is substantial sparing of striatal DA and TH, as well as VMAT and DAT, in rats forced to use their impaired forelimb after 6-OHDA lesions (Tillerson et al., 2001; Tillerson et al., 2002).

Although these original findings were obtained in rats killed 45-80 days after the lesions, it has been found recently that such sparing of striatal DA and TH can be seen as early at 12 days after the lesion (Tillerson, Caudle, Reveron, & Miller, 2003). These findings from the terminal regions of the DA neurons suggest that in spite of the continuing loss of TH-IR cells seen following MFB 6-OHDA lesions, compensatory sprouting must be occurring within the remaining terminals and may be promoted by the induction of neurotrophic factors such as FGF-2 (Chadi et al., 1994; Moroz, Rajabi, et al., 2002), GDNF (Cohen, Tillerson, Smith, Schallert, & Zigmond, 2003) and BDNF (Aliaga, Carcamo, Abarca, Tapia-Arancibia, & Bustos, 2000; Bustos et al., 2002).

The finding that forced use of one forelimb, in itself, induced increased expression of FGF-2-IR in the SNc and VTA in both hemispheres is interesting in view of the previous finding that a period of seven days of forced use of one forelimb given before 6-OHDA lesions led to sparing of limb use and greatly attenuated loss of striatal DA and its metabolites (Cohen et al., 2003). Furthermore, it was found that forced use of one forelimb, in itself, increased glial derived neurotrophic factor (GDNF) in the striatum contralateral to the overused forelimb. These findings suggest that experience-dependent induction of neurotrophic factors even before brain injury has the potential to protect against subsequent insults. This idea is supported by the finding that an injection of adenoviral vector encoding human GDNF, into regions of the SNc seven days before 6-OHDA lesions leads to behavioral sparing and reduced loss of DA neurons (Choi-Lundberg et al., 1997; Choi-Lundberg et al., 1998). Thus, treatments and therapies known to have the capacity to induce neurotrophic factors in the brain may have both prophylactic and therapeutic effects in neurodegenerative diseases. Forced use of one

limb may initiate the induction of neurotrophic factors, in part, via its effects on neurotransmitter release or reuptake. There is evidence that physical exercise such as running or walking stimulates DA synthesis (Hattori, Naoi, & Nishino, 1994), release (Meeusen et al., 1997; Ouchi et al., 2002), and metabolism in the terminal regions of DA neurons in rats (Hattori et al., 1994; Sabol, Richards, & Freed, 1990). Moreover, gait training reduces dopamine reuptake in humans (Ouchi et al., 2001), an effect that conceivably could render DA neurons resistant to functional or physical degeneration caused by neurotoxins that enter through the dopamine transporter. Interestingly, the indirect dopaminergic agonist, amphetamine, which enhances locomotor activity, has been shown to induce large increases in FGF-2-IR in cell body and terminal regions of the midbrain DA neurons (Flores et al., 1998; Flores & Stewart, 2000b) and more recently, treatment with the DA receptor agonists, apomorphine and quinpirole, has been reported to increase the expression of FGF-2 in the terminal regions of DA neurons (Roceri et al., 2001). Thus, activation of the DA system through means of exercise or forced limb-use could be functionally neuroprotective and might be capable of reducing cell vulnerability due to its effects on endogenous production of neurotrophic factors.

The finding that in lesioned rats, forced use of one forelimb did not increase the expression of FGF-2 above that seen after lesions alone, is consistent with previous work showing that FGF-2-IR induced by denervation of the transcallosal afferents to the motor cortex was not further increased by forced use of the impaired limb (Bury, Eichorn, Kotzer, & Jones, 2000). One possible explanation for the lack of additive effects on FGF-2 expression induced by forced limb-use and lesions is that there may be some maximal level of FGF-2 expression associated with the lesion- and experience-induced

plasticity. In other words, having responded to 6-OHDA-induced degeneration of nigrostriatal neurons, FGF-2 expressing astrocytes may simply not be able to further respond to forced use.

In conclusion, we examined the effects of forced limb-use after partial unilateral lesions of the MFB on behavioral asymmetry, and FGF-2- and TH-IR 10 and 28 days after lesioning. Forced use of the impaired forelimb for seven days after 6-OHDA insult led to complete behavioral sparing of limb use for vertical and lateral weight shifting movements in the cylinder test. Both 6-OHDA lesions and forced limb-use, in itself, led to increases in the numbers of astrocytes expressing FGF-2 in SNc and VTA, but the effects of each manipulation on FGF-2-IR were not additive suggesting that the effects of forced limb-use cannot be accounted for simply by the levels of FGF-2 expression. It was also found that forced use of the impaired forelimb led to reduced loss of TH-IR cells in SNc 10 days, but not 28 days after the lesion, suggesting a delay but not a prevention of lesion-induced loss of TH-IR cells.

Acknowledgements

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CHAPTER V

BEHAVIORAL SPARING AFTER 6-HYDROXYDOPAMINE LESIONS OF THE NIGROSTRIATAL PATHWAY IN RATS EXPOSED TO A PRE-LESION SENSITIZING REGIMEN OF AMPHETAMINE OR TO POST-LESION EXERCISE TREATMENT

Isabella Anna Moroz[1*], Susana Peciña[2*], Timothy Schallert[3,4], and Jane Stewart[1]

1. Center for Studies in Behavioral Neurobiology, Concordia Univ., Montreal, PQ, Canada;
2. Department of Psychology, Univ. Michigan, Ann Arbor, MI, USA
3. Dept of Psychology and Institute for Neuroscience, Univ. Texas, Austin, TX, USA
4. Department of Neurosurgery, Univ. Michigan, Ann Arbor, MI, USA

- **These authors contributed equally to these independent experiments.**

Submitted to the *Journal of Neuroscience, Brief Communications*

Abstract

Repeated administration of amphetamine leads to enduring augmentation of its behavioral activating effects, its effect on dopamine (DA) release in striatal regions, and to long-lasting morphological changes in DA target neurons. It also increases astrocytic expression of basic fibroblast growth factor (FGF-2) immunoreactive cells near midbrain dopamine (DA) neurons, and, the number of FGF-2-immunoreactive astrocytes in these regions correlated with the magnitude of sensitization to amphetamine. Here we show that exposure to a two-week escalating-dose regimen of amphetamine, known to increase FGF-2 expression in the cell body and terminal regions of DA neurons, prevents behavioral asymmetries of forelimb use and turning behavior after subsequent 6-OHDA lesions of the nigrostriatal pathway. The prophylactic effect of this pre-lesion amphetamine treatment was as effective as post-lesion exercise. Exposure to three injections of amphetamine before 6-OHDA lesions did not lead to behavioral sparing. There were no significant differences between treatment groups in postmortem tissue levels of DA and its metabolites. There were however, significant negative correlations between DA levels in striatal tissue and the magnitude of asymmetries. These data suggest that the increases in amphetamine-induced endogenous neurotrophic factors may play a role in the behavioral sparing observed after subsequent 6-OHDA lesions.

Introduction

Exposure to psychostimulant drugs leads to changes in brain and behavior that outlast their acute neuropharmacological effects. Repeated administration of amphetamine results in an enduring enhancement of its behavioral activating effects that is associated with enhanced DA overflow in striatal regions in response to acute drug challenges (Kalivas & Stewart, 1991; Robinson & Becker, 1986). The behavioral and neurochemical effects of amphetamine develop gradually and have been observed for months after termination of drug treatment (Castner & Goldman-Rakic, 1999; Paulson, Camp, & Robinson, 1991). This gradual and long-lasting nature of behavioral sensitization has been proposed to occur as a result of structural modifications in neural circuitry and alterations in patterns of synaptic connectivity (Robinson & Kolb, 1997; Robinson & Kolb, 1999), most probably brought about by the actions of neurotrophic factors (Flores & Stewart, 2000a). We hypothesized that repeated administration of amphetamine given before 6-hydoxydopamine (6-OHDA) lesions of the nigrostriatal DA system may mobilize neurotrophic factors and other precursors to structural adaptations that could enhance DA functioning and promote behavioral sparing.

Repeated treatment with amphetamine has been shown to increase dendritic length, density of dendritic spines, and the number of branched spines on the major DA output neurons in the nucleus accumbens (NAcc) and prefrontal cortex (Robinson & Kolb, 1997; Robinson & Kolb, 1999). Repeated administration of amphetamine has also been shown to induce increases in expression of neurotrophic factors, known to affect survival, maintenance and morphological plasticity of adult neurons. As few as three

injections of amphetamine have been found to induce increased expression of the neurotrophic and neuroprotective factor, basic fibroblast growth factor (FGF-2), in astrocytes in the DA cell body regions, ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), evident for up to one month after the last injection of the drug (Flores et al., 1998). Furthermore, a two-week escalating-dose regimen of amphetamine increases FGF-2 in the DA striatal terminal regions, as well (Flores & Stewart, 2000b). More importantly, it was found that the number of FGF-2-immunoreactive astrocytes in the VTA and SNc was strongly and positively correlated with the magnitude of behavioral sensitization, and that infusions of a neutralizing antibody to FGF-2 into the VTA prior to amphetamine administration prevented the development of sensitization (Flores et al., 2000). Repeated administration of amphetamine has also been found to increase the expression of brain-derived neurotrophic growth factor (BDNF) in the basolateral amygdala and its projections targets, including medial NAcc and dorsal medial striatum (Meredith, Callen, & Scheuer, 2002). Thus, neurotrophic factors, such as FGF-2 and BDNF appear to be involved in the long-lasting behavioral and neurochemical changes induced by repeated administration of amphetamine.

In view of findings that increases in neurotrophic factors expression before brain injury have the potential to protect against subsequent insults (Altar, Boylan, Fritsche, Jones, et al., 1994; Choi-Lundberg et al., 1998; Connor et al., 1999; Georgievska et al., 2002; Mandel et al., 1997; Shults et al., 1996; Shults et al., 2000), we hypothesized that exposure to amphetamine before 6-OHDA lesions might serve to attenuate behavioral and neurochemical deficits. We report here two independent experiments, conducted in separate laboratories, using different strains of rats and different placements of 6-OHDA

infusions within the nigrostriatal pathway, both showing that exposure to an escalating-dose regimen of amphetamine prevents subsequent 6-OHDA lesion-induced asymmetries of forelimb use and turning behavior.

Materials and Methods

Experiment 8

Subjects. Adult male Long Evan rats (Charles River, Wilmington, MA; 250-300g), housed on a reversed light-dark cycle with free access to food and water served as subjects.

Amphetamine Regimen. D-amphetamine (Sigma, St. Louis, MO), dissolved in physiological saline, was given in a 2-week escalating-dose regimen (esc/amph; n = 10), as described previously (Flores et al., 2000b). This regimen involved 2 daily injections of amphetamine in the colony room, 7-8 hours apart, five days a week, for 2 weeks. The dose of amphetamine began with 1 mg/kg and escalated to 4 mg/kg for the last 4 days of treatment. Saline-treated control group (sal; n = 12) received 0.9% saline (1 mg/kg). To control for amphetamine-induced increases in motor activity, four rats from the sal group were housed 24h/day in cages with running wheels (34 cm diameter) during the amphetamine administration period (Robinson and Kolb, 1999).

Intrastriatal 6-OHDA Lesions. Unilateral 6-OHDA (6-hydroxydopamine hydrochloride, Sigma) infusions (10µg/4µl in 0.05% ascorbic acid solution; 0.5 µl/min) into the striatum (stereotaxic coordinates with flat skull: 1.7 mm posterior, 2.9 mm lateral to bregma, and 4.0 mm ventral to skull) were performed under equithesin (25 mg/kg

pentobarbital; Sigma, St. Louis, MO) and 150 mg/kg chloral hydrate anesthesia (0.35 ml/100 g., i.p.) followed by atropine sulfate (0.1 mg/kg i.p.; Sigma).

Behavioral testing and limb-use observation. Use of each forelimb for upright support and for landing on when descending from a rearing position was analyzed both pre- and post-operatively in the cylinder test, as previously described (Tillerson et al., 2001). Briefly, occurrences of forelimb use for wall exploration and landing were determined separately and each was expressed in terms of (1) the percent ipsilateral limb use [(ipsi/ipsi+contra+both) x 100] and (2) the percent contralateral limb use [(contra/ipsi+contra+both) x 100]. The percent contralateral limb use was then subtracted from the percent ipsilateral limb use for both the wall behavior and for landing. These two scores (wall and landing) were averaged to obtain a single overall limb use asymmetry score.

Designs and Procedures. Two weeks after termination of the two-week escalating-dose regimen of amphetamine, rats received unilateral intra-striatal 6-OHDA infusions. Asymmetry of forelimb use was tested in the cylinder test 1, 3, 7, and 14 days after lesioning.

Statistical Analyses. Data were analyzed by repeated measures ANOVA with *Drug* (sal, esc/amph) as the between factor and *Day* (1, 3, 7, 14) as the repeated factor.

Experiment 9

Subjects. Adult male Wistar rats (Charles River, QC; 325-350g), housed on a reversed light-dark cycle with free access to food and water served as subjects.

Amphetamine Regimens. D-amphetamine (SmithKline Beecham Pharma, Oakville, ON), dissolved in physiological saline, was given either in a two-week escalating-dose regimen (esc/amph; n = 5) or once a day, every second day, for a total of 3 injections (3inj/amph; n = 5). Saline-treated animals for each amphetamine regimen (sal, n = 4) received 0.9% saline (1.0 mg/kg).

Intra-MFB 6-OHDA Lesions. Unilateral 6-OHDA (6-hydroxydopamine hydrochloride, Sigma, Oakville, ON) infusions (4 µl of 8 µg 6-OHDA/4 µl of 0.9 % saline containing 0.05 % ascorbic acid solution; rate: 0.4 µl/min) into the medial forebrain bundle, MFB, (stereotaxic coordinates with flat skull: 2.9 mm posterior, 1.7 mm lateral to bregma, and 7.6 mm ventral to dura) were performed under sodium pentobarbital anesthesia (30 mg/kg i.p.) supplemented with Isoflurane (Biomeda MTC, Cambridge, ON). Atropine sulfate (0.5 mg/ml, 0.2 ml/rat, s.c.) was given to reduce bronchial secretions. Desmethylimipramine (15 mg/kg, i.p.; RBI Biochemicals, Oakville, ON), a norepinephrine reuptake inhibitor, was given 30 min before infusion of 6-OHDA to protect the noradrenergic cells from 6-OHDA.

Behavioral testing and limb-use observation. Use of each forelimb for upright support and for landing on when descending from a rearing position was analyzed both pre- and post-operatively in the cylinder test, as described in Experiment 8. Rats were also tested for spontaneous ipsiversive turning (turning toward the side of the lesion) in a novel environment, as previously described (Moroz, Rajabi, et al., 2002). Briefly, the number of compact (within the diameter of approximately 20 cm) 360 degree turns and 180 degree half-turns ipsilateral and contralateral to the side of the lesion were recorded and

summed across 5 min testing sessions. The number of ipsilateral turns was presented as a percent of the total number of turns displayed by an animal (ipsi/[ipsi + contra] x 100%).

Postmortem Tissue Analysis. Rats were killed by decapitation and their brains were rapidly removed, placed in isopentane, cooled on dry ice, and frozen at –80 degrees C. The brains were then sliced on a cryostat into 200 μm sections. Punches were taken from the dorsal striatum (1, 2 mm) of the lesioned and non-lesioned hemisphere, from two sections about 1.2 and 1.5 mm posterior to bregma, and from the SNc (2, 1 mm) of the lesioned and non-lesioned hemisphere, from two sections about 5.2 and 5.5 posterior to bregma. The neurochemical assessment of DA, DOPAC, and HVA was then performed as described previously (Moroz, Rajabi, et al., 2002).

Design and Procedures. Seven days after the last amphetamine injection of each regimen, rats received unilateral 6-OHDA infusions into the MFB. An additional group of rats given comparable saline injections before the 6-OHDA lesions received post-lesion exercise treatment (exercise, n = 8), for the purposes of comparing efficacy of amphetamine pretreatment to the well-established beneficial effects of post-injury exercise intervention (Tillerson et al., 2001; Tillerson et al., 2002; Tillerson et al., 2003). Post-lesion exercise consisted of walking or running while inside commercially available transparent, ventilated, shatter-resistant plastic balls (30 cm diameter) that permitted a 360 degree range of motion (Jumbo Kritter Krawler, Lee's Aquarium and Pet Products, San Marcos, CA). Rats that did not voluntarily run inside the balls were forced to do so by the experimenter, who manually moved the ball in random directions. The exercise treatment begun 4-5 hours after lesioning, with a 15 min session, and continued for the next 7 days with 30 min/day sessions and then for additional 7 days with 15 min/day

sessions. Starting 7 days after lesioning, rats were tested once a week for forelimb use in the cylinder test and for turning behavior in the novel environment. Twenty-eight days after lesioning, rats were killed and their brains were taken for DA, DOPAC, and HVA tissue assays.

Statistical Analyses. Data were analyzed by one-way ANOVAs as required; post hoc comparisons were made using the Fisher's PLSC test ($p < .05$).

Results

Exposure to the two-week escalating-dose regimen of amphetamine before intrastriatal (Experiment 8) or intra-MFB infusions of 6-OHDA (Experiment 9) prevented the preferential use of the ipsilateral forelimb for vertical exploration (Fig. 19 and 20 A) and ipsilateral turning (Fig. 20 B) at all time points tested after lesioning. Remarkably, the esc/amph groups from both Experiments 8 and 9 displayed no detectable asymmetry of limb use when compared to the sal group at any time point (Fig. 19 and 20 A). In addition, in Experiment 9, the esc/amph group, but not the 3inj/amph group, showed no asymmetry turning, nor did the esc/amph group differ from the post-lesion exercise group (Fig. 20). In Experiment 8, rats housed in cages with free access to running wheels before lesioning showed high levels of motor activity, running an average of 9.4 km/24 h, but their post-lesion asymmetry of forelimb use was not different from that of the sal group. There was a positive and significant correlation between asymmetry of forelimb use and asymmetry of turning (day 7 and 14 tests: $r = .66$, $p < .05$; day 21 and 28 tests: $r = .75$, $p < .0001$).

Figure 19. Experiment 8: Asymmetry of limb-use (mean $\pm$ SEM) in the cylinder test assessed 1, 3, 7 and 14 days after intrastriatal 6-OHDA lesions was prevented by the esc/amph treatment ($F_{(1, 109)} = 13.51$, $p < .01$). The saline treated rats housed in cages with free access to running wheels did not differ from the saline treated rats that did not run ($F_{(1, 59)} = .17$, ns), thus these rats were combined to form the sal group.

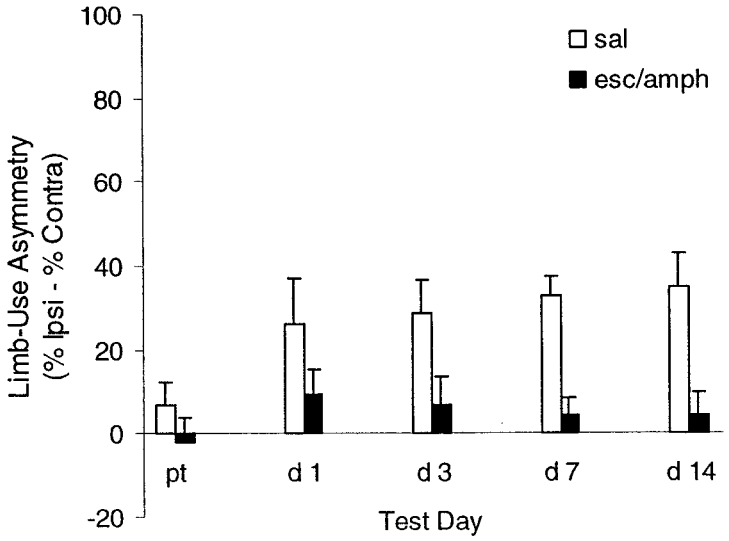
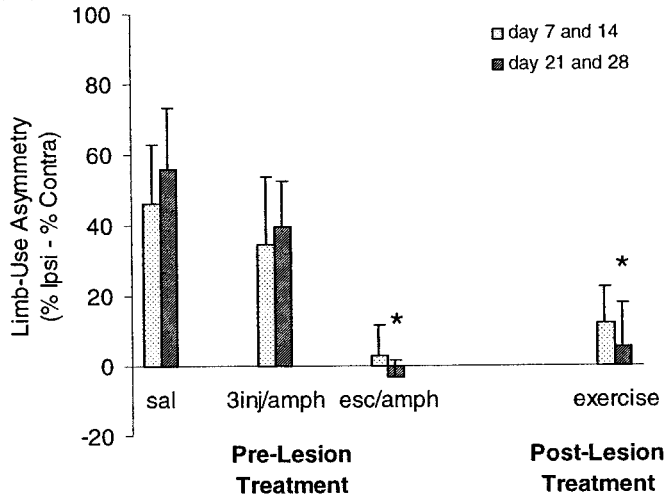

100
80
60
40
20
0
-20
Limb-Use Asymmetry
(% Ipsi - % Contra)
sal
esc/amph
pt
d 1
d 3
d 7
d 14
Test Day

Figure 20. Experiment 9: Mean ($\pm$ SEM) asymmetry of (A) limb-use and (B) spontaneous turning, tested 7 and 14, and 21 and 28 days after 6-OHDA lesions of the MFB. Behavioral observations for days 7 and 14 were combined to produce single scores (one for limb-use and one for turning). The observations for days 21 and 28 were treated similarly. ANOVAs on the data from tests at 21 and 28 days after lesions revealed significant *Group* effects (limb-use asymmetry test, $F_{(3, 18)} = 4.19$, $p < .05$; turning, $F_{(3, 18)} = 2.77$, $p = .07$). The esc/amph and the exercise groups displayed no asymmetry of limb-use or turning and did not differ from each other; both differed significantly from the sal group (* , $p < .05$). Note in (B), a dashed line at 50 % represents no asymmetry, or an equal number of ipsilateral and contralateral turns.

A.


100
80
60
40
20
0
-20
Limb-Use Asymmetry
(% Ipsi - % Contra)
day 7 and 14
day 21 and 28
*
*
sal
3inj/amph
esc/amph
exercise
Pre-Lesion
Treatment
Post-Lesion
Treatment

B.

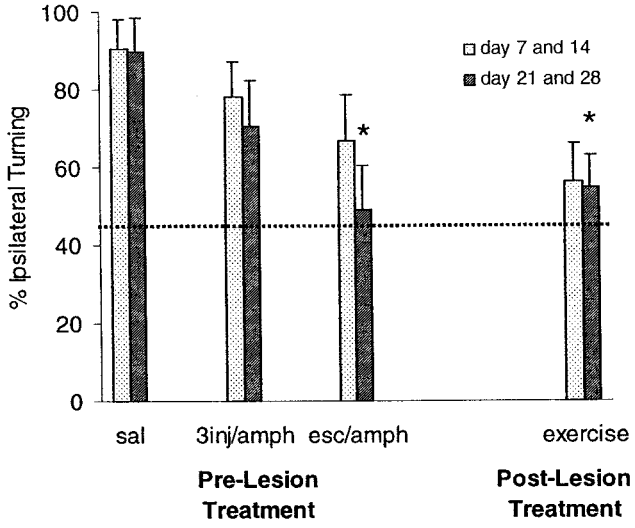
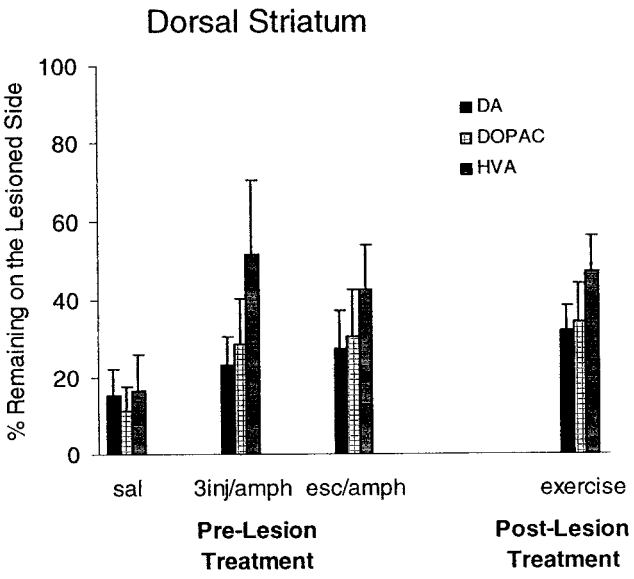

100
80
60
40
20
0
% Ipsilateral Turning
day 7 and 14
day 21 and 28
*
*
sal
3inj/amph
esc/amph
exercise
Pre-Lesion
Treatment
Post-Lesion
Treatment

Figure 21. Experiment 9: Mean ($\pm$ SEM) percent of DA, DOPAC, and HVA remaining in the ipsilateral dorsal striatum (A) and SN (B), 28 days after 6-OHDA lesion of the MFB, calculated by dividing the content in the lesioned hemisphere by the content in the non-lesioned hemisphere x 100.

A.


Dorsal Striatum
% Remaining on the Lesioned Side
100
80
60
40
20
0
DA
DOPAC
HVA
sal
3inj/amph
esc/amph
exercise
Pre-Lesion Treatment
Post-Lesion Treatment

B.

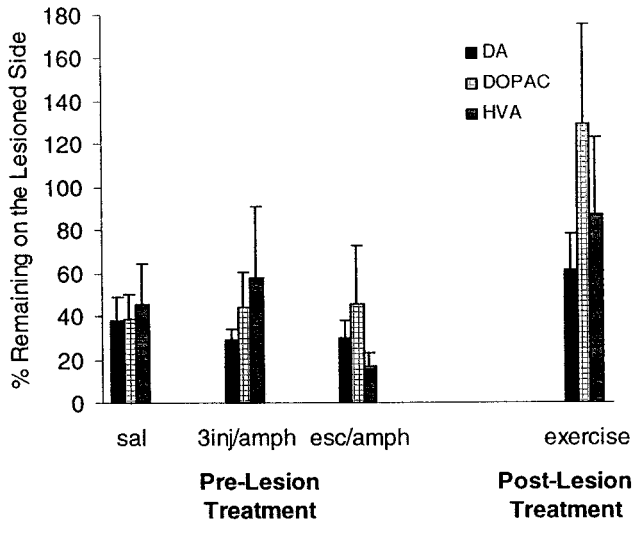

Substantia Nigra
% Remaining on the Lesioned Side
180
160
140
120
100
80
60
40
20
0
DA
DOPAC
HVA
sal
3inj/amph
esc/amph
exercise
Pre-Lesion Treatment
Post-Lesion Treatment

Table 1. Experiment 9: Correlations between the percent DA remaining in the striatum and SN of the lesioned hemisphere and behavioral asymmetries on tests of limb use and turning conducted 7 and 14 days, and 21 and 28 days after lesioning. * $p < .05$.

Test Day	Day 7 and 14		Day 21 and 28	
Type of Test	Limb Use	Turning	Limb Use	Turning
Striatal DA Content	r = .52 p = .01*	r = .37 p = .09	r = .69 p = .0003*	r = .51 p = .01*
SN DA Content	r = .17 p = .46	r = .06 p = .80	r = .48 p = .02*	r = .35 p = .11

Figure 21 shows the tissue levels of DA, DOPAC, and HVA in the dorsal striatum (Fig. 21 A) and SN (Fig. 21 B) observed 28 days after lesioning in the four groups of Experiment 9. Although there were no statistically significant differences between the groups on any of the measures, it can be seen that in comparison to the sal group, striatal DA levels remaining on the side of the lesion were highest in the esc/amph group and the group that received post-lesion exercise, whereas SN DA levels were higher only in the group that received post-lesion exercise. To explore further the relation between tissue levels of DA and behavioral asymmetries, a series of correlations were run using data from all four groups. In general there were strong negative correlations between the percent DA remaining in the striatum ipsilateral to the lesion and behavioral asymmetries of forelimb use and turning. The relation between SNc DA content and behavior tended to be significant only for turning (see Table 1).

Discussion

The results of the present experiments, conducted independently in separate laboratories, using different strains of rats and different placements of 6-OHDA infusions within regions of the nigrostriatal pathway, show that exposure to the two-week escalating-dose regimen of amphetamine before lesioning prevents asymmetries of forelimb use and turning behavior. Rats housed in cages with free access to running wheels for two weeks did not show behavioral sparing, suggesting that the effect of the exposure to escalating-dose amphetamine was not a consequence of increased locomotor activity (Experiment 8). Exposure to three injections of amphetamine before lesioning did not lead to behavioral sparing (Experiment 9). The prophylactic protective effect of the escalating-dose regimen was comparable to the effect of post-lesion exercise as seen here in

Experiment 9 and as reported previously (Tillerson et al., 2001; Tillerson et al., 2002; Tillerson et al., 2003). Note, however, that the effects seen here were obtained with a novel and less intense exercise program than the ones used previously.

The idea that manipulations before brain injury can be protective is not unprecedented. Numerous studies have shown that exogenous delivery of FGF-2, BDNF, or GDNF before 6-OHDA lesions, prevents the development of lesion-induced behavioral deficits and protects against loss of DA neurons and striatal DA innervation (Altar et al., 1994; Choi-Lundberg et al., 1997; Choi-Lundberg et al., 1998; Connor et al., 1999; Georgievska et al., 2002; Kearns & Gash, 1995; Mandel et al., 1997; Shults, Kimber, & Altar, 1995; Shults et al., 1996; Shults et al., 2000). Recently, it was found that forced use of one forelimb for seven days before 6-OHDA lesions led to sparing of limb use and attenuation of striatal DA loss (Cohen et al., 2003). Interestingly, forced use of one forelimb, in itself, increases FGF-2-IR in the SNc and VTA in both hemispheres (Moroz, Cohen, et al., 2002) and GDNF in the striatum contralateral to the overused forelimb (Cohen et al., 2003). In the present experiment, exposure to the escalating-dose regimen of amphetamine known to increase the expression of FGF-2 in both the DA cell bodies and terminals (Flores & Stewart, 2000b) also led to behavioral sparing after 6-OHDA lesions. The fact that exposure to three injections of amphetamine, found previously to increase FGF-2 expression only in the cell body regions of DA neurons, did not lead to behavioral sparing suggests that increased expression of FGF-2, and possibly other neurotrophic factors, in the terminal regions of DA neurons is critical. Support for this idea comes from studies showing that preservation of normal motor functions in the 6-OHDA lesion model requires

intrastriatal, but not nigral, administration of GDNF (Connor et al., 1999; Connor, 2001; Kirik, Rosenblad, & Bjorklund, 2000; Shults et al., 1996; Sullivan, Opacka-Juffry, & Blunt, 1998). We emphasize, however, that the direct involvement of neurotrophic factors in the beneficial effects of pre-lesion exposure to the escalating-dose regimen of amphetamine or pre-lesion forced use of one limb remains to be determined.

Although, in the present study, the percent tissue levels of DA remaining in the striatum ipsilateral to the lesion in the escalating-dose group was twice as high as in the saline group, this trend was not statistically significant. Thus, the behavioral sparing cannot be attributed simply to higher levels of DA remaining in the striatal tissue on the side of the lesion. This finding is in agreement with earlier microdialysis studies showing that the gradual process of behavioral recovery from partial 6-OHDA lesions was accompanied by normalization of extracellular DA levels, but not tissue DA levels, in the striatum (Robinson & Whishaw, 1988; Robinson et al., 1994). Extracellular DA levels in the striatum were reduced 4 days after the lesion when ipsilateral turning predominated, but were normalized 3-4 weeks later, when turning was no longer asymmetric. This enduring enhancement of function within the remaining DA neurons is reminiscent of the enhanced DA response seen in sensitization to amphetamine. Long after repeated administration of amphetamine, rats show increases in extracellular DA in striatal regions in response to subsequent challenges, such as exposure to drugs or stressors, in the absence of changes in tissue levels of DA (Robinson & Becker, 1986). Although it remains to be tested, rats exposed to the escalating-dose regimen of amphetamine before receiving 6-OHDA lesions may have higher extracellular DA levels in the striatum in spite of the severe depletions in the tissue DA levels.

Another explanation for the behavioral sparing induced by previous exposure to the escalating-dose regimen of amphetamine is its effect on dendritic morphology of post-synaptic neurons (Robinson & Kolb, 1997; Robinson & Kolb, 1999). Such morphological changes could represent a reorganization of synaptic inputs onto these neurons. Consequently, the prophylactic effects of exposure to the escalating-dose regimen of amphetamine before 6-OHDA lesions of the nigrostriatal DA neurons found in the present experiments, could be mediated by the amphetamine-induced increases in neurotrophic factors (Flores & Stewart, 2000b) and the enhanced synaptic transmission between the surviving DA input neurons and their striatal and cortical targets.

Interestingly, post-injury administration of amphetamine has been shown to enhance functional recovery in patients suffering from stroke and in animals following cortical lesions or ischemia (Feeney, 1997; Gladstone & Black, 2000; Goldstein, 2000). Multiple mechanisms have been proposed to underlie this therapeutic effect of amphetamine, including enhancement of noradrenergic transmission (Feeney, 1997; Feeney & Westerberg, 1990; Gladstone & Black, 2000) and neuritogenesis and synaptogenesis (Stroemer, Kent, & Hulsebosch, 1998). Furthermore, behavioral recovery observed in ischemic animals treated with amphetamine correlates with increased expression of markers of neuronal remodeling, such as growth associated protein-43 (GAP-43) and synaptophysin (Stroemer, Kent, & Hulsebosch, 1995). As argued earlier, modifications of neuronal architecture and synaptic connectivity are likely to be mediated via actions of neurotrophic factors. In fact, there is evidence that exogenous administration of FGF-2 after the onset of ischemia enhances behavioral recovery without reducing the infarct volume (Kawamata, Alexis, Dietrich, & Finklestein, 1996).

Instead, FGF-2 administration is associated with a selective increase in GAP-43 expression (Kawamata et al., 1997).

In summary, we report that exposure to a two-week escalating-dose regimen of amphetamine before unilateral 6-OHDA lesions prevents asymmetries of forelimb use and turning behavior in the absence of significant protection of striatal tissue DA levels. Although the mechanisms mediating this effect remain to be elucidated, the ability of a sensitizing regimen of amphetamine to render the DA neurons more responsive to subsequent challenges may underlie the observed behavioral sparing. Furthermore, the endogenous increases in the expression of neurotrophic growth factors previously observed after exposure to the two-week escalating-dose regimen of amphetamine may play a key role in the enhanced responsiveness of the DA system to a subsequent 6-OHDA insult and the resulting behavioral sparing.

GENERAL DISCUSSION

The general idea guiding the experiments presented in this thesis is that the enduring enhancements in functioning of the midbrain DA system seen in behavioral sensitization to psychostimulant drugs and in the recovery from partial lesions of nigrostriatal DA neurons are mediated by similar mechanisms. A focus on FGF-2 expression after partial 6-OHDA lesions and the role of this neurotrophic factor in behavioral recovery following this type of lesion was adopted because of previous studies on behavioral sensitization. In these studies, increases in the endogenous expression of astrocytic FGF-2 in the DA cell body regions were found after repeated exposure to amphetamine (Flores et al., 1998), and, behavioral sensitization was prevented when intra-VTA infusions of a neutralizing antibody to FGF-2 preceded amphetamine injections (Flores et al., 2000). It has been suggested that these amphetamine-induced increases in FGF-2 expression mediate the profound changes in the morphology of the DA striatal and cortical output neurons observed after repeated administration of psychostimulant drugs (Robinson & Kolb, 1997; Robinson & Kolb, 1999). Thus, it was expected that partial 6-OHDA lesions would induce FGF-2 expression which would, it turn, have neuroprotective effects on the midbrain DA neurons.

As expected, 6-OHDA lesions led to time-dependent increases in astrocytic FGF-2 expression in the DA cell body regions similar to those seen after amphetamine treatment. In spite of these increases in FGF-2, however, there was a gradual loss of TH-IR cells in SNc and VTA and no behavioral recovery. Furthermore, behavioral asymmetry after lesions correlated with both the percent loss of TH-IR cells and the

percent increase in FGF-2-IR cells in the DA cell body regions. In other words, the greater the behavioral asymmetry, the greater the TH-IR cell loss, and the greater the number of FGF-2-IR astrocytes. Since the 6-OHDA lesions in the present investigation were made in the MFB, the resulting extensive damage may have prevented behavioral recovery in spite of the increases in FGF-2 expression. Consequently, it was not possible to conclude that the previously reported enhancements in functioning of the midbrain DA system after partial 6-OHDA lesions were due to increases in endogenous FGF-2. Nonetheless, the fact that FGF-2 expression is increased in the vicinity of DA neurons after both repeated administration of amphetamine and after 6-OHDA lesions supports the idea that FGF-2 expression is functionally involved in drug-induced and injury-induced plasticity of the midbrain DA neurons.

Following the initial study, experiments were done to explore the effect of variables known to affect behavioral sensitization to amphetamine on the FGF-2 response to 6-OHDA lesions. Next, the effects of manipulations known to facilitate recovery from 6-OHDA lesions on the expression of FGF-2 were studied. It was reasoned that, if indeed, the mechanisms by which sensitization- and lesion-induced enhancements in functioning of the midbrain DA system are mediated via enhanced expression of endogenous FGF-2, then manipulations that affect behavioral sensitization to amphetamine would affect FGF-2 expression after 6-OHDA lesions. Consequently, one would expect that manipulations that affect behavioral recovery would also affect FGF-2 expression after lesions. In the final experiment, the effects of amphetamine treatments known to increase endogenous levels of FGF-2 were examined on recovery from

subsequent 6-OHDA lesions. The principal findings of these experiments are discussed below.

Effects of Gonadal Hormones and Perinatal Anoxia on Behavioral and Cellular Outcomes of 6-OHDA Lesions of the MFB

Neonatal exposure to testosterone, circulating levels of estradiol, and perinatal anoxia, all affect the behavioral and neurochemical responsiveness to psychostimulant drugs, hence the effects of each of these manipulations on 6-OHDA lesion-induced loss of TH-IR expressing cells and increases in FGF-2 expressing astrocytes were examined. It was found that rats exposed to testosterone at birth exhibited somewhat greater loss of TH-IR cells after 6-OHDA lesions and greater astrocytic FGF-2 response. As in the first study, it was found that the greater the behavioral asymmetry, the greater the TH-IR cell loss, and the greater the number of FGF-2-IR astrocytes. These findings suggest that neonatal exposure to testosterone may increase the vulnerability to the effects of 6-OHDA lesions and that the higher levels of FGF-2 expression may be a consequence of the increased toxicity.

Interestingly, in animals not exposed to testosterone at birth but subsequently treated with estradiol benzoate in adulthood, the 6-OHDA lesion-induced loss of TH-IR cells was significantly reduced and the increases in FGF-2 expression were modest. Thus, gonadal hormone manipulations known to increase the responsiveness of rats to psychostimulant drugs (lack of testosterone neonatally and circulating estradiol in adulthood: Camp & Robinson, 1988; Forgie & Stewart, 1993; Forgie & Stewart, 1994)

reduced the effect of lesions on both the loss of TH-IR cells and the increase in FGF-2-IR astrocytes, reflecting perhaps a decreased vulnerability to the effects of lesions.

The effects of neonatal exposure to testosterone on the vulnerability to the effects of 6-OHDA lesions observed in the present experiments and on the responsiveness to psychostimulant drugs reported in previous research may result from the ability of gonadal hormones to affect the development of the midbrain DA system. Dopaminergic activity develops earlier in females than in males and manipulating neonatal exposure to testosterone by either gonadectomy of males or neonatal testosterone administration to females shifts the pattern of development to that of the other sex (Stewart, Kuhnemann, & Rajabi, 1991; Stewart & Rajabi, 1994). As already mentioned, DA containing neurons synapse with soma, and dendritic shafts and spines on the medium spiny neurons in the striatum and on pyramidal cells in the DA cortical projections areas (Berger, et al., 1991; Freund, et al., 1984; Goldman-Rakic, et al., 1989; Smith & Bolam, 1990). It is reasonable to expect, therefore, that neonatal gonadal hormone manipulations may alter the time-course of development of DA innervation and the final interconnectivity and synaptic transmission between the DA input and output neurons. That, in turn could affect synaptic transmission between the DA neurons and their targets and contribute to the vulnerability of the midbrain DA neurons to the effects of 6-OHDA lesions or to the responsiveness to psychostimulant drugs.

Although the effects of neonatal hormonal manipulations on morphological changes in the DA output neurons in the adult animal have not been studied, there is evidence that neonatal gonadectomy in males decreases the horizontal span of intrahemipsheric circuits of the primary motor cortex in the rat (Venkatesan & Kritzer,

1999). Interestingly this effect is observed in the motor cortex, which receives dense DA inputs, but not in the visual cortex, which is only sparsely innervated by DA afferents. Furthermore, neonatal gonadectomy of males profoundly diminishes TH innervation of the cingulate cortex, and the primary somatosensory and motor cortices (Kritzer, 1998). Thus, neonatal hormonal manipulations exert significant effects on dopaminergic innervation of the cortex and on cortical interconnectivity. These effects are believed to be mediated via genomic mechanisms of hormone action during the neonatal period, when there is a transient peak in cortical intracellular estrogen receptors (Shughrue, Stumpf, MacLusky, Zielinski, & Hochberg, 1990), androgen receptors (Lieberburg, MacLusky, & McEwen, 1980), and aromatase levels (MacLusky, Walters, Clark, & Toran-Allerand, 1994). Furthermore, subsets of DA neurons in the SN and VTA, which provide the major source of DA input to the highly hormone-sensitive cortical regions, contain intracellular estrogen and androgen receptors (Kritzer, 1997; Shrughrue, Lane, & Merchenthaler, 1997).

It is interesting to speculate about how the alterations in DA innervation of the cortex and cortical interconnectivity after neonatal hormonal manipulations might contribute to the effects of neonatal exposure to testosterone on responsiveness to psychostimulant drugs or to the effects of 6-OHDA lesions of the nigrostriatal neurons. The dendritic arbor of pyramidal neurons in various regions of the prefrontal cortex of adult rats has been previously shown to vary as a function of neonatal exposure to testosterone (Kolb & Stewart, 1991; Stewart & Kolb, 1994). In general, the cortical pyramidal neurons in animals neonatally exposed to testosterone have more dendritic branches, higher overall dendritic length, and higher density of dendritic spines.

Furthermore, the morphology of cortical pyramidal cells has also been shown to be influenced by other factors, such as circulating levels of estrogen, injury (cortical lesions), age, and environment (Kolb et al., 1998; Schallert et al., 2000). Interestingly, however, neonatal exposure to testosterone seems to suppress the morphological changes of these neurons later in life in response to the other factors, as if the earlier growth and development limit their structural plasticity.

In view of findings demonstrating enhancements in dendritic morphology of post-synaptic DA neurons after repeated exposure to psychostimulant drugs, it is reasonable to hypothesize that this plastic response would be compromised in animals neonatally exposed to testosterone, accounting for the reduced sensitivity of these animals to psychostimulant drugs. On the other hand, the previously observed decreases in horizontal span of intrahemispheric circuits (Venkatesan & Kritzer, 1999) and TH-innervation in certain cortical areas (Kritzer, 1998) in males gonadectomized at birth could serve to allow for the enhancement in dendritic morphology and synaptic connectivity in response to administration of psychostimulant drugs. This enhancement could contribute to the increased responsiveness to psychostimulant drugs observed in animals not exposed to testosterone neonatally. Similarly, in the case of 6-OHDA lesions, neonatal testosterone may limit the ability of the surviving DA neurons and their postsynaptic target neurons to change their morphology following lesions in order to compensate for the damage. This, in turn, may reduce synaptic transmission between the surviving DA input neurons and their striatal and cortical targets, contributing to greater degeneration and loss of DA neurons seen in groups exposed to testosterone neonatally.

In this latter case, the higher astrocytic FGF-2 response to lesions in the DA cell body regions may simply reflect greater damage.

In contrast to the effects of gonadal hormone manipulations, perinatal anoxia, a manipulation reported to result in enhanced responsiveness of the midbrain DA system to stressors and stimulant drugs later in life, appeared to result in more pronounced TH-IR cell loss and behavioral impairment than those seen in animals born vaginally or by a C-section. This greater cell loss was not accompanied by increases in astrocytic FGF-2 expression in the anoxic group that were enhanced relative to those observed in the other groups. Interestingly, it has been shown previously that rats exposed to anoxia at birth have lower basal numbers of FGF-2 expressing astrocytes in the vicinity of DA neurons at 2 weeks and at 3 ½ months after birth (Flores et al., 2002). Further, in mice, lower levels of FGF-2 have been associated with greater vulnerability to neuronal cell death following nerve crush injury (Kuzis et al., 1999). Thus, it is possible that perinatal anoxia may render the midbrain DA neurons more vulnerable to insults later in life.

Importantly, certain responses observed to be enhanced in adult rats exposed to anoxia at birth, such as the NAcc DA response after tail-pinch stress, do not typically sensitize, or become augmented with repeated exposure (Brake et al., 1997a), suggesting pathological, rather than sensitized responding in rats born with anoxia. The effects of perinatal anoxia on DA agonist-elicited behaviors (Brake, Boksa, et al., 1997; El-Khodor & Boksa, 1998; El-Khodor & Boksa, 2000), TH-IR and TH mRNA expression (Andersson et al., 1995; Bjelke et al., 1991; Gross et al., 2000), and DA receptor expression and binding (Chen et al., 1997) resemble the effects of intrastriatal 6-OHDA lesions performed on the day of birth or postnatal day 1 (Frohna et al., 1993; Frohna et

al., 1997; Neal-Beliveau & Joyce, 1997; Neal-Beliveau & Joyce, 1992; 1993; Thomas et al., 1998). Thus, it is possible that the behavioral, neurochemical, and cellular effects seen in the adult animal after perinatal anoxia arise from altered development of the DA system. As such, the mechanisms underlying the enhancement in functioning of the DA system seen in adult rats exposed to anoxia at birth may be different from those underlying behavioral sensitization to the effects of psychostimulant drugs. They may reflect pathological development leading to the increased vulnerability of the midbrain DA system to neurotoxic insults later in life.

Effects of Forced-Limb Use on Behavioral and Cellular outcomes of 6-OHDA Lesions

In the second part of the thesis, the effects of forced use of the impaired limb on lesion-induced increases in FGF-2 were examined. Forced limb-use is a behavioral manipulation shown previously to stimulate behavioral and neurochemical sparing after extensive 6-OHDA lesions of the MFB (Tillerson et al., 2001; Tillerson et al., 2002). In view of findings showing that motor activity can upregulate expression of FGF-2 and BDNF (Gomez-Pinilla et al., 1997; Gomez-Pinilla & Kesslak, 1998; Neeper et al., 1996), it was predicted that the beneficial effects of forced use in the 6-OHDA lesioned rat, may have been due, in part, to the ability of this manipulation to enhance the FGF-2 expression after injury. It was found that although forced use of the impaired limb after lesioning led to behavioral sparing, the increases in the number of astrocytes expressing FGF-2 were not greater than those induced by the lesion alone. Although by 28 days after the lesions all rats suffered extensive loss of TH-IR cells, at 10 days after the lesion there was some evidence of sparing (or delay of loss) of TH-IR cells in those forced to use the impaired limb. Thus, while the behavioral sparing seen after forced use cannot be

accounted for by significant sparing of TH-IR cells, it may be that the delayed loss allows for compensatory sprouting of DA terminals. As previously shown, the behavioral sparing observed in these rats is accompanied by reduced tissue loss of striatal DA and TH, as well as VMAT and DAT (Tillerson et al., 2001; Tillerson et al., 2002), suggesting that forced use may either preserve the existing striatal DA innervation or enhance sprouting of DA terminals. Several studies demonstrated that preservation of normal motor functions in the 6-OHDA lesioned rat requires preservation of striatal DA innervation, with or without preservation of nigral DA cell bodies (Connor et al., 1999; Connor, 2001; Kirik et al., 2000; Shults et al., 1996; Sullivan et al., 1998). Thus, although this possibility remains to be tested, the beneficial effects of forced limb-use in the 6-OHDA lesioned rat may be due to its ability to preserve functional connectivity between the remaining DA neurons and their postsynaptic targets.

The idea that structural changes in the brain after injury depend on activity is not new and has been extensively studied in models of cortical lesions. Unilateral damage to the forelimb representation area of the sensorimotor cortex leads to preferential use of the forelimb ipsilateral to the damage (Jones & Schallert, 1992). Importantly, such use is associated with increases in dendritic growth and surface area of dendritic processes as well as in marked synaptogenesis, including multiple synaptic boutons and perforated synapses, in layer V pyramidal neurons of the intact homotopic cortex (Jones & Schallert, 1994; Jones, Kleim, & Greenough, 1996; Jones, 1999). These structural changes are not only use-dependent but also injury-dependent. In particular, restricting the use of the nonimpaired forelimb interferes with these structural changes, whereas forced use of one forelimb in sham operated animals has no effect (Jones & Schallert, 1994). Furthermore,

forced use of one limb has also been found to induce astrocyte proliferation and FGF-2 expression in layer V astrocytes in the corresponding sensorimotor cortex (Bury et al., 2000), suggesting that FGF-2 may mediate the use-dependent changes in neuronal morphology. Similarly to the present findings, however, forced use did not enhance the endogenous FGF-2 increases observed after mild denervation of afferents to the motor cortex. In summary, in the 6-OHDA lesioned rat, the ability of forced limb-use to increase astrocytic FGF-2 expression, could serve to stimulate structural changes and synapse formation in striatal and cortical projection areas of DA neurons. These changes could, in turn, lead to enhanced synaptic transmission between the remaining DA neurons and their postsynaptic targets.

Effects of a Pre-Lesion Treatment with a Sensitizing Regimen of Amphetamine on Behavioral and Neurochemical Outcome after Subsequent 6-OHDA Lesions of the MFB

In the final section of the thesis, the effects of pre-lesion treatment with two different sensitizing regimens of amphetamine on behavioral and neurochemical outcome after subsequent 6-OHDA lesions were examined. These manipulations were carried out in an attempt to increase endogenous expression of FGF-2 (and possibly other neurotrophic factors) before injury. Pre-lesion exposure to an escalating-dose regimen of amphetamine, previously shown to increase FGF-2 expression in the DA cell body and terminal regions, led to behavioral sparing after subsequent 6-OHDA lesions. On the other hand, pre-lesion exposure to three injections of amphetamine, previously shown to increase FGF-2 expression only in the DA cell body regions, did not lead to behavioral sparing. The analysis of striatal tissue levels of DA revealed significant depletions in all lesioned groups, suggesting that behavioral sparing after pre-lesion treatment with the

escalating-dose regimen of amphetamine cannot be attributed simply to higher levels of DA remaining in the striatal tissue on the side of the lesion.

The principal finding of this experiment, although correlational in nature, is that having increased levels of endogenous FGF-2, and possibly other neurotrophic factors, in the vicinity of DA neurons at the time of injury is very important, if not critical, for behavioral recovery. As discussed earlier, numerous studies revealed that delivery of exogenous neurotrophic factors, such as FGF-2, BDNF, or GDNF before 6-OHDA lesions can prevent the development of lesion-induced behavioral deficits and can provide significant protection against loss of DA neurons and/or striatal DA innervation (Altar et al., 1994; Choi-Lundberg et al., 1997; Choi-Lundberg et al., 1998; Connor et al., 1999; Georgievska et al., 2002; Kearns and Gash, 1995; Mandel et al., 1997; Shults et al., 1995; Shults et al., 1996; Shults et al., 2000). Furthermore, preservation of normal motor function in the 6-OHDA lesioned rat requires preservation of striatal DA innervation, which, at least in the case of GDNF, depends on the intrastriatal delivery of this neurotrophic factor (Connor et al., 1999; Connor, 2001; Kirik et al., 2000; Shults et al., 1996; Sullivan et al., 1998).

In the case of GDNF, the importance of intrastriatal delivery for behavioral sparing may be explained by the well-established target-derived action of this neurotrophic factor. Several target regions of the midbrain DA system express GDNF receptors, and GDNF has been shown to be transported from the DA target field in the striatum to the dopaminergic cell bodies in the SNc, in a receptor-mediated fashion (Tomac et al., 1995; Lapchak et al., 1996). On the other hand, the expression patterns of FGF-2 and its receptor, both of which reside on DA cell bodies themselves as well as on

the astroglia within the DA cell body and terminal regions, suggest multiple mechanisms of action. There is evidence for retrograde transport of FGF-2 from the striatum to SN (Ferguson & Johnson, 1991), as well as for anterograde transport of FGF-2 from SN to striatum (McGeer, Singh, & McGeer, 1992). The anterograde transport has been also proposed to play a role in the autocrine effects of FGF-2 on DA terminals and in the paracrine effects on local neurons and glia cells in the striatum. The fact that pre-lesion exposure to three injections of amphetamine, found previously to increase FGF-2 expression only in the cell body regions of DA neurons, did not lead to behavioral sparing suggests that increased expression of FGF-2 in the cell body regions is not sufficient to lead to behavioral sparing. Rather, the behavioral sparing observed after pre-lesion treatment with the escalating-dose regimen of amphetamine may be due to the ability of this regimen to increase the number of astrocytes expressing FGF-2 in both DA cell body and terminal regions.

Behavioral sparing after pre-lesion escalating-dose regimen of amphetamine did not preserve striatal tissue DA levels. It is proposed, therefore, that the beneficial effects of amphetamine may be mediated by structural changes in neuronal morphology previously observed after similar amphetamine treatment (Robinson & Kolb 1997; Robinson & Kolb, 1999). Support for this idea comes from studies on post-injury treatment with amphetamine following cortical lesions or ischemia. In particular, behavioral recovery in amphetamine-treated rats correlates with increased expression of markers of neuronal remodeling, such as GAP-43 and synaptophysin (Stroemer et al., 1995). Furthermore, the beneficial effects of amphetamine in these animals have been shown to depend on deficit-specific motor training (Feeney, Gonzalez, & Law, 1982;

Schmanke, Avery, & Barth, 1996; Schmanke & Barth, 1997), which in the case of forced limb-use has also been shown to enhance dendritic growth and synaptogenesis (Jones & Schallert, 1994; Jones et al., 1996; Jones 1999). Thus, the endogenous increases in the expression of neurotrophic growth factors previously observed after exposure to the two-week escalating-dose regimen of amphetamine may lead to structural changes that allow for enhanced synaptic transmission between the surviving DA input neurons and their striatal and cortical targets. This enhanced synaptic transmission might serve to preserve the integrity of circuits involved in control of motor behavior and account for the observed behavioral sparing after 6-OHDA lesions, despite the extensive losses of striatal tissue DA levels.

Contradictions and Paradoxes

There are several apparently contradictory and paradoxical aspects to the findings obtained in the present thesis that need to be considered before one can come to any conclusions about the role of the induction of endogenous FGF-2 recovery of function after 6-OHDA lesions.

First, it was found that in spite of large increases in endogenous FGF-2 expression after lesions, there was progressive and extensive loss of TH-IR cells and no behavioral recovery. While it was expected that cell damage would induce FGF-2 expression, it was also expected that the greater expression would prove neuroprotective. In contrast, it was found that the greater the lesion-induced TH-IR cell loss and behavioral deficits, the higher the FGF-2 expression on the side of the lesion. This result was somewhat surprising in view of the finding that exogenous delivery of FGF-2 led to preservation of

TH-IR cell bodies in the SNc and TH-IR innervation in the striatum, and attenuation of drug-induced turning asymmetries after 6-OHDA-induced degeneration of the nigrostriatal DA system (Shults et al., 2000). Thus, increases in the expression of endogenous FGF-2 after 6-OHDA lesions appear to reflect a magnitude-dependent response to degeneration of DA neurons, possibly in an attempt at neuroprotection/or restoration. It would be useful, therefore, to study the endogenous expression of FGF-2 in response to smaller 6-OHDA lesions, and in particular, whether in these situations it would prevent the lesion-induced loss of TH-IR cell bodies and lead to behavioral recovery.

Another paradoxical finding was that although forced limb-use, in itself, increased FGF-2 expression in non-lesioned rats, when combined with lesions, the forced limb-use did not further increase FGF-2 expression in the DA cell body regions. Thus, it was not possible to attribute the effect of forced use of the impaired limb to additional increases in FGF-2 expression. One likely explanation for this paradox is that the increases in endogenous expression of FGF-2 are effective in promoting recovery only when combined with behavioral demand, such as forced use, which can serve to actively engage the restorative potential of FGF-2. This may be particularly true when large lesions are made, such as those used in the present studies, which lead to severe sensorimotor neglect of the contralateral side. Furthermore, it may be that forced use engages additional neurotrophic mechanisms or factors to promote recovery under these conditions.

Another apparently paradoxical finding it that although behavior was spared after forced-use of the impaired limb, TH-IR cell bodies in the SNc and VTA were not spared.

This again requires us to think about the alternative mechanisms to account for the effects on behavior. Similarly, in the final experiment where it was found that pre-lesion treatment with amphetamine led to behavioral sparing, striatal tissue levels of DA were not different from those seen in saline pretreated rats. It was suggested earlier in this thesis that events occurring in the terminal regions of the DA might account for these effects, but that the mechanisms underlying these events might be different in the two cases. However, it is possible that because both forced limb-use and amphetamine treatment enhance neuronal growth and cell morphology, their beneficial effects may be mediated by structural changes and enhanced synaptic connectivity between the surviving DA input neurons and their striatal and cortical targets.

Finally, although the present investigation focused strictly on examination of FGF-2 expression after 6-OHDA lesions, the role of other neurotrophic factors in the effects obtained here cannot be discounted. As described in the general introduction, GDNF and BDNF have also been shown to be potent neurotrophic and neuroprotective factors of the midbrain DA neurons in vitro and in vivo. Their exogenous administration protects against the behavioral and neurodegenerative effects of 6-OHDA lesions. Furthermore, increases in expression of endogenous BDNF and in BDNF mRNA have been reported after intrastriatal 6-OHDA lesions and were proposed to reflect compensatory plastic processes within the degenerating DA neurons (Aliaga et al., 2000; Bustos et al., 2002; Venero, Beck, & Hefti, 1994). Increases in endogenous BDNF have also been observed after sensitizing regimen of amphetamine (Meredith et al., 2002) and in GDNF after forced limb-use (Cohen et al., 2003). Thus, BDNF, GDNF, and possibly

other neurotrophic factors in addition to FGF-2, are likely to play a role in processes occurring after 6-OHDA lesions of the nigrostriatal neurons.

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