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Role of Basic Fibroblast Growth Factor in the Development of Sensitization to the Effects of Amphetamine

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A Thesis

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ABSTRACT

Role of Basic Fibroblast Growth Factor in the Development of Sensitization to the Effects Amphetamine

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Concordia University, 2000

Repeated exposure to the stimulant drug, amphetamine, results in long-lasting increases in its effects on locomotor activity, reward and dopaminergic function. The precise mechanisms responsible for this persistent drug-induced sensitization are unknown, but evidence suggests that neuroadaptations initiated in the cell body region of midbrain dopaminergic neurons and dependent on glutamatergic transmission underlie its development. In this thesis, the hypothesis that basic fibroblast growth factor (bFGF), a neurotrophic factor produced by astrocytes, plays a crucial role in the development of sensitization was tested. A series of experiments was conducted to assess whether bFGF expression in cell body regions of dopaminergic neurons is altered by repeated administration of amphetamine, whether this effect is dependent on glutamatergic activation, and whether blockade of bFGF actions in midbrain dopaminergic cell body regions prevents the development of sensitization. It was found that repeated administration of amphetamine induces prolonged increases in the expression of astrocytic bFGF in midbrain dopaminergic cell body regions and that this effect depends on glutamatergic activation. Furthermore, it was

shown that, in animals that exhibit behavioral sensitization, bFGF expression in midbrain dopaminergic cell body regions is strongly and positively correlated with the magnitude of sensitization that develops. In a final experiment it was found that when amphetamine injections are preceded by infusions of a neutralizing antibody to bFGF into the cell body region of dopaminergic cells, the development of behavioral sensitization is prevented indicating that bFGF plays a critical role in the development of sensitization to amphetamine.

It is argued that repeated exposure to stimulant drugs increases the demands on dopaminergic cell functioning and, thus, stimulates the recruitment of neurotrophic and neuroprotective substances, such as bFGF. The actions of bFGF, in turn, give rise either directly or indirectly to enduring neuronal changes that underlie sensitized responding to further drug exposure. Although, the mechanisms whereby bFGF exerts its effects to induce the kinds of long-lasting neuroadaptations underlying sensitization to the effects of amphetamine remain to be unraveled, the findings from this thesis provide the first evidence that the neurotrophic factor bFGF is critically involved.

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DEDICATION

I dedicate this thesis to my beloved mother Bebé, to my sister Carolina, and to my brothers Guillermo and Federico. I thank them for supporting me in innumerable ways throughout these years and for their love and encouragement.

I also dedicate this work to the memory of Guillermo Flores-Parkman and Tomás Noriega.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

OVERVIEW

A fundamental property of the mammalian brain is its continuous capacity to change. Experience with internal and external events influence the function of the developing and adult brain by altering its neuronal connections or by creating new ones. The idea that experience can induce reorganizational changes in neuronal connections, already asserted by Ramón y Cajal in 1928, was explicitly argued for by Hebb who showed that environmental factors have a significant influence on behavior: Rats that were reared in complex environments were better at learning tasks than laboratory-raised rats (Hebb, 1947). To explain such findings, Hebb proposed that in order for experience to induce lasting changes in brain functioning and behavior, structural alterations resulting in increased contact (i.e. neuronal growth) between synapses, and thus, in synaptic strengthening, must occur (Hebb, 1949).

It was not until later that the effect of experience on behavior was related to changes in brain weight, cortical thickness, dendritic structure, and synaptic number in adult rats (for reviews see Rosenzweig, 1996; Rosenzweig & Bennett, 1996). From this point onward, not only a variety of internal and external stimuli have been found to shape the adult brain, but also a great deal has been learned about the neurochemical and molecular mechanisms that underlie plastic events. Of importance is the relatively new finding that neurotrophic factors, a particular type of proteins that control neuronal

differentiation, growth, survival, and synaptic formation during brain development, are key mediators of plastic changes in adult neurons.

A compelling example of experience-dependent plasticity in the adult is the phenomenon of behavioral sensitization. When animals, including humans, are repeatedly exposed to stimulant drugs such as amphetamine and cocaine, or to the opioid drug morphine, an increased sensitivity in the locomotor activating effects of these drugs is produced. Behavioral sensitization appears to be mediated, in part, by drug-induced enhancement in the functioning of midbrain dopaminergic neurons. Importantly, behavioral and neurochemical alterations induced by drug exposure develop gradually and can last for years, suggesting reorganization of neuronal structure. The implications of drug-induced plasticity may be profound, particularly in light of evidence showing that a single exposure to amphetamine is sufficient to induce long-lasting behavioral sensitization in rats (Vanderschuren et al., 1999).

Considerable effort has been expended to unravel the processes that give rise to the development of behavioral sensitization to stimulant drugs. The neuroadaptations underlying the increased sensitivity to the effects of these drugs may well be implicated in the development of drug-induced psychopathologies and drug addiction. However, the precise cellular and molecular events mediating sensitized responding to stimulants remain unknown. The experiments undertaken in this thesis were aimed at exploring the idea that neurotrophic factors are released in response to

repeated drug exposure and are responsible for the development of enduring sensitization. Specifically, I tested the hypothesis that repeated exposure to stimulant drugs induces the expression of the neurotrophic and neuroprotective substance basic fibroblast growth factor which, in turn, produces long-lasting changes in neuronal functioning.

In the first chapter of the thesis, I describe basic concepts of behavioral sensitization and provide the rationale behind the idea that basic fibroblast growth factor could be involved in this process. In Chapter 2, 3 and 4, I present experimental evidence showing that basic fibroblast growth factor is necessary for the development of sensitization to the effects of amphetamine. Finally, in Chapter 5, I discuss the idea that basic fibroblast growth factor mediates amphetamine-induced neuronal plasticity by promoting neuronal growth, and thus, by altering neuronal circuits.

CHAPTER I

INTRODUCTION

1.1. SENSITIZATION TO THE EFFECTS OF STIMULANT DRUGS

1.1.1. Behavioral Effects

Stimulant drugs, such as amphetamine and cocaine, produce a broad spectrum of effects including behavioral activation. When administered repeatedly and intermittently, the behavioral activating effect of these drugs become sensitized (see Kalivas & Stewart, 1991; Robinson & Becker, 1986). Behavioral sensitization is usually assessed in laboratory animals employing paradigms that consist of two phases, the induction phase and the test phase. During the induction phase, adult rats are treated with intermittent injections of amphetamine or saline and the behavioral activating effects elicited by each injection are measured. Following a determined amount of time (see below) after the last injection, a test for sensitization is conducted during which *all* rats are challenged with amphetamine. Although in this test all rats receive exactly the same dose of amphetamine, rats that were given amphetamine during the induction phase show significantly more behavioral activation (behavioral sensitization) than those receiving the drug for the first time. It is important to keep in mind that at low doses amphetamine increases locomotor activity, including horizontal and vertical movements, but at higher doses produces stereotypy. To characterize behavioral changes induced by repeated amphetamine treatment accurately, paradigms are chosen according to the dose used.

In similar experimental settings, prior exposure to amphetamine has been shown to result in sensitized behavioral activation in humans. For instance, in a double-blind placebo-controlled study, human subjects that did not have prior history of stimulant drug use were exposed to two systemic injections of amphetamine or saline, separated by 48 h. Each amphetamine injection was associated with elevated activity, mood, rates of speech, and increased rate of eye blinks. In comparison to the first amphetamine challenge and to saline injections, the second amphetamine injection produced greater increases in all behavioral measures (Strakowski, Sax, Setters, & Keck, 1996).

In addition to the sensitization of behavioral activating effects, repeated exposure to stimulant drugs has been associated with enduring increases in their rewarding properties, suggesting a possible relation between the two effects (see Wise & Bozarth, 1987). Previous exposure to cocaine, amphetamine, or to the opioid drug morphine have been shown to potentiate acquisition and expression of place preferences (Lett, 1989; Shippenberg & Bals-Kubik, 1995) and the motivation to self-administer these drugs, even after long withdrawal periods (Horger, Giles, & Schenk, 1992; Horger, Shelton, & Schenk, 1990; Lorrain, Arnold, & Vezina, 2000; Mendrek, Blaha, & Phillips, 1998; Piazza, Deminiere, Le Moal, & Simon, 1989; Valadez & Schenk, 1994; Vezina, Pierre, & Lorrain, 1999b; Woolverton, Cervo, & Johanson, 1984).

Behavioral sensitization has two interesting features: It develops gradually and is persistent. When rats are exposed to relatively short amphetamine or cocaine regimens, small sensitization effects are evident 24-48 h after cessation of treatment, but their magnitude increases significantly over time (Heidbreder, Thompson, & Shippenberg, 1996; Kolta, Shreve, De Souza, & Uretsky, 1985). After longer and more severe regimens, behavioral sensitization is not evident until 2 to 3 weeks after the last day of the induction phase (Paulson, Camp, & Robinson, 1991; Paulson & Robinson, 1995; Segal & Kuczenski, 1992a; Segal & Kuczenski, 1992b). These results, together with evidence indicating that injections given widely spaced in time produce more robust sensitization (Post, 1980), suggest that the neuronal changes underlying sensitization continue to develop for some time after discontinuation of drug treatment. The expression of sensitization at early withdrawal times may rely on transient changes in receptor function (see Paulson & Robinson, 1995) whereas long-term sensitization may be maintained by enduring structural modifications.

In rats and monkeys, sensitized behavioral responses to amphetamine have been reported 1 and 3 years after treatment termination, respectively (Castner & Goldman-Rakic, 1999; Paulson et al., 1991). Considering the life span of these animals, this evidence suggests that exposure to amphetamine may cause permanent changes in brain function. It is likely, therefore, that the development of sensitization requires the operation of factors normally involved in neural plasticity and that neurotrophic factors may be involved.

1.1.2. Effects on the Functioning of the Midbrain Dopaminergic System

Stimulant drugs have acute and long-lasting effects on the functioning of the midbrain dopaminergic system. The organization of this system is complex and a detailed neuroanatomical description is beyond the scope of this thesis (for comprehensive reviews see Fallon & Loughlin, 1987; Fallon & Moore, 1978). Essentially, the midbrain dopaminergic system has three major components: mesolimbic, mesocortical and nigrostriatal systems. The mesolimbic system arises from groups of neurons located in the ventral tegmental area (VTA, A10 group) and projects to nucleus accumbens (NAcc), and to other limbic regions including amygdala and septum. The mesocortical system arises from VTA cells and projects to cortical regions in limbic cortex including medial prefrontal cortex (PFC). Finally, the nigrostriatal system originates from cells in the substantia nigra (A9 group) and projects mainly to dorsal striatum. All three components are targets of stimulant drug actions.

Systemic injections of amphetamine, cocaine, or morphine, increase extracellular dopamine in both cell body and terminal regions (Kalivas & Stewart, 1991). Cocaine raises the extracellular concentration of dopamine in an impulse-dependent manner. When dopamine neurons fire and dopamine is released into the synaptic cleft, cocaine competes with dopamine for uptake sites on the plasma membrane dopamine transporter (DAT), decreasing the amount of dopamine that is taken-up form the synaptic cleft

back into the neuron. Morphine enhances concentrations of extracellular dopamine indirectly by increasing dopaminergic cell firing (Johnson & North, 1992).

Amphetamine, like cocaine, competes with dopamine for uptake sites on DAT, but, in addition, it stimulates dopamine release directly without requiring cell firing. Two mechanisms have been proposed to account for this effect and both of them appear to be critical (Jones, Gainetdinov, Wightman, & Caron, 1998). According to the reverse transport model, the DAT is like a carrier with a binding site that allows dopamine transport from one side of the plasma membrane to another. By acting as a substrate for DAT, amphetamine increases the number of times the transporter binding site faces the cytoplasm, stimulating dopamine reverse transport (Fischer & Cho, 1979). The weak base or vesicle depletion model suggests that cytoplasmic amphetamine, internalized via lipophilic diffusion (see Jones et al., 1998) and/or through DAT, enters dopaminergic vesicles and causes displacement of dopamine from vesicles into the cytoplasm by disrupting the pH gradient. Elevated concentrations of cytoplasmic dopamine result in an altered concentration gradient across the plasma membrane and cause dopamine reverse transport (Sulzer et al., 1995).

A large body of evidence has shown that behavioral sensitization is accompanied by increased responsiveness of the mesolimbic and nigrostriatal dopaminergic systems (see Kalivas & Stewart, 1991; Robinson & Becker, 1986). Results from brain slice or from *in vivo* microdialysis studies have revealed

that after repeated intermittent exposure to amphetamine, cocaine, or morphine, the increased dopamine release in the NAcc and dorsal striatum induced by subsequent drug challenge is sensitized (Heidbreder et al., 1996; Kolta et al., 1985; Paulson & Robinson, 1991; Spanagel, Almeida, & Shippenberg, 1993). Enhanced dopaminergic function develops gradually, is long-lasting, and is often associated with behavioral sensitization (Heidbreder et al., 1996; Kalivas & Duffy, 1993; Kolta et al., 1985; Paulson et al., 1991; Paulson & Robinson, 1995; Vezina, 1993; Wolf, White, Nassar, Brooderson, & Khansa, 1993), but see (Kuczenski, Segal, & Todd, 1997).

Dopamine release in the NAcc is known to mediate, at least in part, the behavioral activating effects of these drugs. Direct acute injections of amphetamine into the NAcc result in increased locomotor activity (Kalivas & Stewart, 1991). Hence, neuroadaptations in mesolimbic dopaminergic neurons (i.e. cells projecting from the VTA to NAcc) are likely to underlie behavioral sensitization.

1.2. THE DEVELOPMENT OF SENSITIZATION

There are two sets of findings concerning the initiation of sensitization that are critical for an understanding of the nature of the neuroadaptations that may underlie its development. First, sensitization of both the behavioral and neurochemical effects of stimulant drugs is initiated by drug actions in cell body regions of dopaminergic cells. Second, glutamate transmission

within these regions, during the induction phase, is necessary for the development of behavioral and neurochemical sensitization.

1.2.1. Role of Dopamine in the VTA

Repeated administration of amphetamine into the VTA, but not into the NAcc, is sufficient to induce sensitized behavioral responses and enhanced dopaminergic release in the NAcc in response to subsequent systemic injections of amphetamine, cocaine, or morphine (Bjijou, Stinus, Le Moal, & Cador, 1996; Cador, Bjijou, Cailhol, & Stinus, 1999; Cador, Bjijou, & Stinus, 1995; Hooks, Jones, Hemby, & Justice, 1993; Kalivas & Weber, 1988; Vezina, 1993; Vezina, 1996; Vezina & Stewart, 1990). Repeated intra-VTA amphetamine injections also result in sensitized locomotor responding to an intra-NAcc amphetamine challenge (Perugini & Vezina, 1994). Discrepant results regarding the effects of repeated intra-SNc injections of amphetamine on the development of sensitization have been reported (e.g. Kalivas & Weber, 1988; Perugini & Vezina, 1994).

In vivo microdialysis studies have shown that amphetamine directly administered into the VTA increases cell body dopamine release (Kalivas, Bourdelais, Abhold, & Abbott, 1989). Thus, an immediate effect of amphetamine in this region is activation of dopamine receptors. Dopamine receptors can be classified into two main families, D_1 and D_2. In the VTA, D_2 receptors are associated with dopaminergic cells per se and play an autoreceptor role because their activation inhibits the activity of these

neurons (see White, 1996). In contrast, D_1 receptors are not synthesized by dopaminergic neurons (Mansour et al., 1992) and, in the VTA, are localized on terminals of glutamatergic and GABAergic neurons (Cameron & Williams, 1993; Dewar, Rompré, Stewart, & Warren, 1997; Lu, Churchill, & Kalivas, 1997).

D_1 receptor activation in the VTA is critical in the development of sensitization to amphetamine. When, during the induction phase, rats are exposed to systemic injections of amphetamine preceded by systemic injections of a D_1 receptor antagonist, neither sensitized behavior nor sensitized dopaminergic function (increased dopaminergic release in NAcc) is observed upon subsequent amphetamine challenge. Sensitization is also blocked when, during the induction phase, amphetamine is injected directly into the VTA and D_1 antagonists are given systemically, or when both drugs are administered into the VTA (Bjijou et al., 1996; Stewart & Vezina, 1989; Vezina, 1996; Vezina & Stewart, 1989). D_2 receptor activation, on the other hand, does not play a role in development of sensitization (Bjijou et al., 1996; Vezina, 1996).

1.2.2. Role of Glutamatergic Transmission in the VTA

1.2.2.1. Glutamate Receptors and Glutamate Release in the VTA

Apart from amphetamine-induced D_1 receptor activation in the VTA, glutamatergic transmission in this region is necessary for the development of sensitization. Glutamatergic transmission is mediated by ionotropic

receptors, which have their agonist binding sites and associated ion channels incorporated into the same molecular complex, and by metabotropic receptors which are linked to second messenger pathways (for review see Michaelis, 1998). The development of behavioral sensitization to amphetamine, morphine, or cocaine is prevented when, during the induction phase, injections with these drugs are preceded by systemic administration of antagonists of the ionotropic glutamate receptor *N*-methyl-D-aspartate (NMDA) (Druhan & Wilent, 1999; Jeziorski, White, & Wolf, 1994; Kalivas & Alesdatter, 1993; Karler, Calder, Chaudhry, & Turkanis, 1989; Karler, Chaudhry, Calder, & Turkanis, 1990; Stewart & Druhan, 1993; Wolf & Jeziorski, 1993; Wolf & Khansa, 1991). This effect appears to be produced at the level of the VTA because direct injections of NMDA antagonists into the VTA block the development of sensitization to amphetamine and cocaine (Cador et al., 1999; Kalivas & Alesdatter, 1993).

Blockade of behavioral sensitization has also been observed when injections of antagonists of the other ionotropic glutamate receptor, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), are given concurrently with injections of amphetamine, cocaine or morphine (Carlezon, Rasmussen, & Nestler, 1999; Karler, Calder, & Turkanis, 1991a; Li, Vartanian, White, Xue, & Wolf, 1997). In addition, stimulation of metabotropic glutamate receptors is required for sensitization to develop. Rats pre-exposed to repeated intra-VTA injections of amphetamine and metabotropic glutamate receptor antagonists do not develop locomotor

sensitization (Kim & Vezina, 1998). Glutamate neurotransmission in the VTA has also been shown to be required for the development of sensitization of dopamine release in NAcc following repeated amphetamine exposure (Jake-Matthews, Jolly, Queen, Brose, & Vezina, 1997).

Pertinent to these findings is evidence showing that systemic or intra-VTA injections of amphetamine, cocaine and D_1 receptor agonists increase extracellular glutamate in this region. In experiments using *in vivo* microdialysis in awake rats, Kalivas and Duffy reported that a systemic injection of cocaine produced an immediate elevation in extracellular glutamate in the VTA that lasted approximately 20 min. This effect was abolished by blocking D_1 receptors directly in the VTA. Perfusion of D_1 agonists into the VTA also produced increases in extracellular glutamate in this region (Kalivas & Duffy, 1995a; Kalivas & Duffy, 1998).

Using a similar paradigm, Wolf and colleagues assessed the effects of systemic amphetamine on glutamatergic efflux in the VTA. They found that amphetamine produced a delayed increase in extracellular glutamate in this region that peaked 3 h after drug injection and was still raised at 4 h (Xue, Ng, Li, & Wolf, 1996). Elevated glutamate efflux in the VTA induced by systemic injections of amphetamine was abolished when these injections were preceded by a systemic injection of a D_1 receptor antagonist at a dose known to block the development of behavioral sensitization (Wolf & Xue, 1999). A delayed increase in glutamate efflux in the VTA was observed when amphetamine or D_1 agonists were directly perfused into this region. These

effects were prevented by co-perfusion of D_1 antagonists (Wolf & Xue, 1998; Wolf & Xue, 1999).

Whereas Kalivas's group observed immediate increases in glutamatergic efflux in the VTA following injections of cocaine or D_1 receptor agonists, the increases in glutamate release in the VTA observed by Wolf and colleagues were not evident before at least 1 h after the injection with amphetamine or the D_1 receptor agonist. Despite the discrepancies in the time course observed by the two research groups, which are most probably due to methodological and drug differences (see discussion in Kalivas & Duffy, 1998; Wolf & Xue, 1998), results from both studies show that acute injections of amphetamine and cocaine result in relatively prolonged increases in glutamatergic efflux in the VTA that are mediated by activation of D_1 receptors.

What is the source of glutamate in the VTA? VTA glutamatergic innervation arises from a number of nuclei including prefrontal cortex, pedunculopontine region, and subthalamic nucleus (Christie, Bridge, James, & Beart , 1985; Phillipson, 1979; Scarnati, Proia, Campana, & Pacitti, 1986; Sesack & Pickel, 1992). Special attention has been paid to the dense glutamatergic innervation that arises from the medial prefrontal cortex (PFC) with terminals ending on both dopaminergic and non dopaminergic cells (Sesack & Pickel, 1992). Results from several studies suggest that glutamatergic transmission in this pathway is crucial in the development of sensitization. Excitotoxic lesions of the PFC have been found to prevent the

development of sensitization to systemic and to intra-VTA amphetamine (Cador et al., 1999; Wolf, Dahlin, Hu, Xue, & White, 1995). Moreover, excitotoxic lesions of the PFC have been shown to prevent amphetamine-induced increases in glutamate efflux in the VTA (Wolf & Xue, 1999).

Glutamatergic terminals that originate in the PFC and end in the VTA are known to express D_1 receptors (Dewar et al., 1997; Lu et al., 1997). As mentioned before, D_1 receptor activation in the VTA increases glutamatergic release in this region. Thus, these findings suggest that stimulant drugs increase glutamatergic efflux in the VTA by activating D_1 receptors on PFC glutamatergic terminals. To date, however, direct evidence of D_1 receptor-induced glutamate release from glutamate-containing terminals in the VTA is not available and this issue remains unresolved (Wolf & Xue, 1999). Additional or alternative mechanisms that might explain the D_1-induced increases in VTA glutamate efflux are 1) activation of D_1 receptors located on GABAergic terminals in the VTA; and 2) activation of D_1 receptors located on VTA astrocytes (Bal et al., 1994; Hosli & Hosli, 1993; Luo, Kokkonen, Wang, Neve, & Roth, 1998).

1.2.2.2. Glutamate-Dependent Cellular Adaptations in the VTA

Repeated administration of stimulant drugs has been associated with biochemical and molecular alterations in the VTA that are dependent on glutamatergic transmission in this region. Repeated amphetamine or cocaine administration results in D_2 autoreceptor subsensitivity in the VTA and this

effect is prevented by NMDA antagonists (Wolf, White, & Hu, 1994). In addition, repeated treatment with stimulant drugs has been shown to alter protein expression in VTA dopaminergic and astroglial cells. Repeated cocaine injections or chronically implanted morphine pellets increase tyrosine hydroxylase (TH; the rate-limiting enzyme of dopaminergic synthesis) expression in VTA 24 h after termination of treatment (Beitner-Johnson & Nestler, 1991). TH activity in this region has also been shown to be elevated up to 12 weeks after termination of repeated cocaine treatment (Masserano, Baker, Natsukari, & Wyatt, 1996).

The activity of the signaling transduction protein, extracellular signal regulated kinase (ERK), also known as mitogen-associated protein (MAP) kinase, is up-regulated in the VTA for a few hours after repeated, but not after acute cocaine or morphine injections (Berhow, Hiroi, & Nestler, 1996b). This effect appears to mediate the drug-induced increases in TH immunoreactivity because ERK regulates TH expression (Haycock, Ahn, Cobb, & Krebs, 1992). Concomitant intra-VTA infusions of NMDA antagonists prevent increases in ERK and TH activity and immunoreactivity in this region induced by systemic treatment with amphetamine or cocaine (Berhow et al., 1996b; Masserano et al., 1996).

Morphine and cocaine treatments also result in increased glial fibrillary acidic protein (GFAP) immunoreactivity and activity within the VTA and decrease neurofilament proteins immunoreactivity (Beitner-Johnson,

Guitart, & Nestler, 1992; Beitner-Johnson, Guitart, & Nestler, 1993). Whether these modifications are long-lasting and dependent on glutamate remains to be determined.

1.2.2.3. AMPA Receptors on Cell Body Regions of Dopaminergic Cells

Glutamatergic NMDA, AMPA and metabotropic receptors are expressed in both VTA and SNc and have been found to colocalize with dopaminergic cells (Carlezon et al., 1997; Christoffersen & Meltzer, 1995; Fitzgerald, Ortiz, Hamedani, & Nestler, 1996; Kosinski, Standaert, Testa, Penney, & Young, 1998; Paquet, Tremblay, Soghomonian, & Smith, 1997; Petralia, Yokotani, & Wenthold, 1994). In vivo electrophysiological recordings have shown that iontophoretic administration of glutamate, NMDA, AMPA, or metabotropic receptor agonists into the VTA increases the firing rate of midbrain dopaminergic neurons (White, Hu, Zhang, & Wolf, 1995; Zhang, Hu, White, & Wolf, 1997c) and similar effects are seen with electrical stimulation of the PFC (Tong, Overton, & Clark, 1996a; Tong, Overton, & Clark, 1995).

White et al. (1995) performed recordings from VTA dopaminergic neurons in anesthetized rats 3 days after repeated exposure to systemic amphetamine or cocaine. Both amphetamine and cocaine treatments led to a significant increase in the responsiveness of dopamine neurons to iontophoretically infused glutamate without altering basal levels of activity. Potentiation of the excitatory effects of PFC electrical stimulation on

dopaminergic neurons was also observed after repeated amphetamine treatment (Tong et al., 1995). Increased sensitivity of VTA dopamine cells to glutamatergic activation was found to be mediated by AMPA receptors (Zhang et al., 1997c).

There are four AMPA receptor subunits (GluR1-GluR4) that associate in various combinations to form functional receptor channels with large differences in their Ca^{++} permeability. The presence of GluR2 subunit results in channels relatively impermeable to Ca^{++} whereas GluR1 homomeric receptors are considerably Ca^{++} permeable (Hollman, Hartley, & Heinemann, 1991; Seeburg, 1993). Thus, large increases in levels of GluR1 (without changes in GluR2) would be expected to increase the proportion of Ca^{++} permeable AMPA receptors.

Interestingly, repeated, but not acute, exposure to cocaine has been shown to elevate the expression of the GluR1 subunit of the AMPA receptor in the VTA. Similar results are seen after exposure to repeated morphine injections (Fitzgerald et al., 1996). The changes in GluR1 expression have been seen at least 16 h after the last drug injection and appear to be selective to the VTA because no changes have been observed in other brain regions including NAcc, STR, cortical regions and, surprisingly, SN (Fitzgerald et al., 1996). Preliminary results have shown that the same amphetamine treatment regimen that produces electrophysiological supersensitivity of dopamine cells to AMPA activation is associated with a trend toward GluR1 increase in VTA (Wolf, 1998).

A causal relationship between increased GluR1 in the VTA and sensitization to stimulant drugs has been shown by Carlezon and colleagues. Groups of rats were given VTA microinjections of a defective herpes simplex virus vector encoding the GluR1, GluR2, or a reporter protein. Up-regulation of GluR1 produced a sensitized-like response to the locomotor-activating effects of a subcutaneous injection of morphine given 2 or 4 days after transfection. Double-labeling immunohistochemistry revealed that the large majority of GluR1-positive cells were dopaminergic. Selective increases in GluR1 expression in the VTA were accompanied by increases in Ca^{++} entry, assessed in tissue slices (Carlezon et al., 1997).

1.2.2.4. Glutamatergic-Dopaminergic Interaction in the VTA

What I have attempted to do so far, is to provide a picture of the brain actions of stimulant drugs, and especially amphetamine, that take place during the induction phase of sensitization and that are known to initiate enduring changes in neuronal function. Stimulant drugs act within the VTA region to enhance extracellular levels of both dopamine and glutamate. There is evidence that drug-induced increase in glutamatergic efflux is mediated by activation of D_1 receptors. These receptors are located on glutamatergic and GABAergic terminals and, perhaps, on astroglial cells. Although, most evidence suggest that glutamate release results from direct activation of D_1 receptors on glutamatergic terminals, this issue requires further investigation. What is well established is that glutamate, in turn, acts on NMDA, AMPA and metabotropic receptors in this region, and that

activation of these receptors is required for the development of long-lasting neuroadaptations.

Several cellular and molecular alterations on VTA dopaminergic neurons such as changes in AMPA receptor responsiveness and increased expression of specific proteins (section 1.2.2.2) have been observed after repeated stimulant exposure and have been shown to be dependent on glutamate transmission. At least one of these alterations, increased GluR1 expression, appears to play a role in enduring enhancement of dopaminergic functioning. A glutamatergic-dopaminergic interaction in the VTA, therefore, initiates persistent changes in brain function and behavior. What has yet to be understood is the nature of this interaction and the precise mechanisms through which it brings about persistent neuroadaptations. In this thesis I will focus on the hypothesis that neurotrophic factors and, in particular, basic fibroblast growth factor (bFGF), may mediate this interaction.

1.3. NEUROTROPHIC FACTORS

1.3.1. General Aspects of Neurotrophic Factors

Neurotrophic factors can be defined as proteins that stimulate neuronal growth and differentiation in the developing brain and that are now known to play a critical role in the survival, maintenance, and morphological plasticity of adult neurons (Hefti, Denton, Knusel, & Lapchak, 1993). Neurotrophic factors are synthesized by both neurons and glia. Typically, they are secreted into the extracellular milieu where they diffuse to

act on other cells in a paracrine fashion, or on the same cell that secretes them, in an autocrine way. However, they can also be released when cell death occurs or when the integrity of the plasma membrane is compromised (see MacNiell & Gower, 1982). The actions of neurotrophic factors are mediated by transmembrane receptors which, for the majority of growth factors, are tyrosine kinases. Activation of these receptor tyrosine kinases by specific ligands turns on their enzymatic activity and triggers intracellular signaling transduction pathways that may culminate in gene transcription. The Ras-MAP kinase pathway, known to relay extracellular signals to the nucleus, has been found to be activated by several neurotrophic factors. Indeed, expression of both immediate early genes and delayed response genes have been shown to be enhanced by binding of neurotrophic factor to tyrosine kinase receptors (for reviews see Segal and Greenberger, 1996; Landreth, 1999). It is important to mention that different neurotrophic factors show specificity for different neuronal populations and this seems to be largely determined by developmental stage and/or by cell phenotype.

The function of neurotrophic factors has been studied primarily during neuronal development. Increasing amount of evidence indicates that different stages of development are determined to a great extent by the effects that these factors exert on neurons. At very early stages of brain development, neurotrophic factors seem to enhance differentiation and proliferation of neuronal precursors. Subsequent survival and maturation of differentiated neurons and contact of specific targets by their developing

axons seems to be controlled by neurotrophic agents secreted by *en passant* and final target cells. The massive death of excess neurons that occurs soon after axons reach their targets appears to be dependent on competition for neurotrophic supply provided in limited amounts by target cells. Once synapses are formed, actions of neurotrophic factors seem to control the elimination of weak connections (pruning) and the sprouting of stable ones. Finally, at late stages of development and throughout adulthood (see below), neurotrophic factors enhance functional maturation and promote synaptic efficacy (see Henderson, 1996; Oppenheim, 1996; Pettman & Henderson, 1998; Wang & Tessier-Lavigne, 1999).

Initially, the presence of neurotrophic factors in the adult brain was thought to be limited to brain injury or damage where they are known to promote survival and sprouting of spared neurons (for reviews see Nieto-Sampedro & Cotman, 1985). Within the last decade, however, the known range of effects of neurotrophic factors on injured and intact neurons has been dramatically increased. For instance, neuronal morphology, axonal sprouting, receptor subunit conformation, neurotransmitter release, free radicals concentration, and membrane depolarization have been shown to be modulated by neurotrophic factors (Cheng et al., 1995; Fagan et al., 1997; Kafitz, Rose, Thoenen, & Konnerth, 1999; Kawamata et al., 1997; Li, Zhang, Lester, Schuman, & Davidson, 1998; Pechan, Chowdhury, & Seifert, 1992). Not surprisingly, neurotrophic factors have been found to be not only involved, but also required in various neuronal processes. Of particular

relevance to the topic of this thesis is the evidence showing that neurotrophic factors are required for various types of experience-dependent plasticity such as learning (Kesslak, So, Choi, Cotman, & Gomez-Pinilla, 1998; Mu, Li, Yao, & Zhou, 1999; Zhang, Endo, Cleary, Eskin, & Byrne, 1997a), recovery of behavioral function (Chadi et al., 1993; Rowntree & Kolb, 1997) and the long-lasting increase in synaptic strength that can be induced by repetitive activation of excitatory synapses, known as long-term potentiation (Chen, Kolbeck, Barde, Bonhoeffer, & Kossel, 1999; Ishiyama, Saito, & Abe, 1991; Korte, Kang, Bonhoeffer, & Schuman, 1998; Terlau & Seifert, 1990).

1.3.2. Possible Involvement of Neurotrophic Factors in the Development of Sensitization

The long-lasting, and perhaps permanent, nature of the changes induced by repeated administration of stimulants suggests that behavioral sensitization may involve structural modifications in neuronal circuitry. As mentioned before, repeated injections of morphine or cocaine have been shown to induce changes in the expression of neurofilament proteins and GFAP in the VTA as assessed by immunoblotting procedures (Beitner-Johnson et al., 1992; Beitner-Johnson et al., 1993). Alterations in these proteins that are components of the cytoskeleton may well indicate modifications in neuronal and astrocytic morphology. Changes in the structure of VTA dopaminergic neurons, in fact, have been observed 24 h after daily subcutaneous implantation of morphine pellets for 5 days.

Specifically, a reduction in cell body size and in the length of cell processes of individual dopaminergic neurons isolated from brains of drug-treated rats have been reported (Sklair-Tavron et al., 1996). The changes in neuronal structure and protein expression shown in these studies, however, have been determined immediately after the termination of the drug treatment and interpretations about their relevance to enduring functional changes cannot be made.

To date, the only experiments that have assessed long-term modifications in neuronal structure as a consequence of repeated exposure to stimulant drugs have been those conducted by Robinson and Kolb (1997; 1999). In a first study, Robinson and Kolb examined neuronal morphology in specific brain regions using Golgi-staining, one month after termination of a five-week treatment with escalating doses of amphetamine. Increases in the length of dendrites, in the density of dendritic spines, and in the number of branched spines were observed in NAcc medium spiny neurons (major neuronal output of mesolimbic dopaminergic cells) and in PFC pyramidal neurons (major neuronal output of mesocortical dopaminergic cells). These changes appeared to be specific to regions receiving dopaminergic innervation because they were not observed in two cortical 'control' regions (Robinson & Kolb, 1997). Similar persistent structural modifications were subsequently observed following a milder amphetamine treatment and following repeated cocaine injections (Robinson & Kolb, 1999). Whether

alterations of this nature occur in the cell body region of midbrain dopaminergic cells remains to be determined.

Considering the role that neurotrophic factors play in neuronal plasticity, it is logical to suppose that structural modifications induced by repeated exposure to stimulant drugs may be brought about by the action of neurotrophic factors. Repeated stimulation of dopaminergic neurons and the consequent increases in extracellular glutamate resemble conditions similar to those seen during injury or metabolic stress and may lead to the secretion of neurotrophic factors. These agents, in turn, may induce long-term changes in neuronal function, including morphological alterations, that may manifest as enhanced neurochemical and behavioral responding to subsequent drug challenge. Consistent with this idea is the fact that some of the changes observed following repeated exposure to stimulant drugs have been observed after toxic insults to dopaminergic neurons (see for example Stromberg et al., 1986).

1.3.3. Neurotrophic Factors and Stimulant Drugs

Several investigators have examined whether neurotrophic factors can alter the effects of stimulant drugs by microinfusing neurotrophic factors in the brain and assessing changes in responses to amphetamine, cocaine, or morphine injections. Altar and colleagues used osmotic minipumps to examine the effects of continuous infusion (14 days) of brain derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3) into the SNc on

dopaminergic function. They found that increased locomotor activity elicited by an acute injection of amphetamine given on day 15, was enhanced by treatment with both neurotrophic factors (Altar et al., 1992; Martin-Iverson, Todd, & Altar, 1994). In another study, Altar and colleagues tested whether BDNF infused for 14 days in the SNc would alter the activity of dopaminergic cells in this region. Electrophysiological recordings conducted on day 15 revealed an increased number of spontaneously active dopaminergic neurons, increased average firing rate, and increased number of action potentials contained within bursts in the SNc (Shen, Altar, & Chiodo, 1994).

In a series of experiments, Nestler and colleagues found that chronic infusions of either BDNF, NT-3 or, NT-4 into the VTA prevented and reversed the effects of chronic exposure to morphine and cocaine on TH and GFAP expression and on dopaminergic neuronal size (Berhow et al., 1995; Sklair-Tavron et al., 1996). In another experiment, chronic intra-VTA administration of ciliary neurotrophic factor (CNTF) was found to increase TH expression, but did not alter the effects induced by morphine or cocaine (Berhow et al., 1995). Because of these findings, Nestler and colleagues suggested that neurotrophic factors and stimulant drugs regulate the mesolimbic dopaminergic system through common cellular signal transduction pathways. Indeed, ERK (also known as MAP kinase) activity which, as mentioned earlier, is an important part of the signal transduction cascade activated by many neurotrophic factors, is up-regulated in the VTA after chronic, but not acute, cocaine or morphine injections (Berhow et al.,

1996b). Furthermore, Janus kinase immunoreactivity, a known CNTF-regulated protein, is increased in VTA dopaminergic, non-dopaminergic, and astroglial cells after chronic cocaine treatment (Berhow, Hiroi, Kobierski, Hyman, & Nestler, 1996a).

Two recent studies have investigated long-lasting effects of minipump infusions of exogenous BDNF and NT-3 on sensitivity to cocaine. Horger et. al. (1999) found that a 14-day treatment with continuous infusion of BDNF into the VTA or NAcc (from where it is retrogradely transported to dopamine cell bodies (Mufson et al., 1994)) via osmotic minipumps, significantly potentiated the locomotor effects of an acute systemic injection of cocaine given 7 days after the beginning of the BDNF treatment. In addition, intra-NAcc infusions of BDNF were found to enhance the effect of an acute injection of cocaine on responding for a conditioned stimulus previously paired with food reward. Importantly, this effect was evident during infusions of BDNF as well as more than a month after termination of BDNF infusions (Horger et al., 1999). These results suggest that BDNF induces acute and long-lasting changes in dopaminergic function potentiating the effects of acute and repeated cocaine injections.

In the second study, Pierce, Pierce-Bancroft, and Prasad (1999) found that intra-VTA microinjections of NT-3, but not BDNF, given once a day for 3 days, produced sensitized locomotor responding to an injection of cocaine given two weeks later, but not one day later. In a subsequent experiment, they showed that inhibition of the Ras-MAP kinase signal transduction

pathway prevented the development of behavioral sensitization to cocaine, raising the possibility that the effects of NT-3 may be mediated via this pathway. The discrepancy between the effects of BDNF infusions in this and the study conducted by Horger and colleagues needs further investigation.

Taken together, the findings outlined in this section indicate that infusions of exogenous BDNF, NT-3 and CNTF can alter midbrain dopaminergic cell function, either directly or indirectly and influence the behavioral effects of stimulant drugs. Moreover, the activation of pathways known to be regulated by neurotrophic factors are required for the development of behavioral sensitization to cocaine. Whether endogenous neurotrophic factors play a role in the development of sensitization to the effects of stimulant drugs remains to be elucidated. At present there is little direct evidence concerning this issue. Pierce et al. (1999) assessed the effect of cocaine on the expression of NT-3 or BDNF mRNA in the VTA and found that an acute injection of cocaine induced a short-lived increase in NT-3 mRNA levels in the VTA 4 h, but not 2, 8 or 24 h, later. No increase was detected when the cocaine injection was preceded by repeated cocaine treatment. No changes in BDNF mRNA levels in the VTA were observed after either acute or chronic cocaine treatment. In another study, rats were implanted with morphine pellets (75 mg/day for 5 days) and mRNAs for BDNF, NT-3 and their receptors, *Trk*B and *Trk*C, were assessed 24 later. Under these conditions, no changes in gene expression were seen in the VTA (Numan, Lane-Ladd, Zhang, Lundgren, & Russell, 1998).

1.3.4. A Rationale for Proposing a Role for bFGF in the Development of Sensitization

In our search for neurotrophic factors that might be involved in the processes underlying the development of sensitization, Jane Stewart and I found the neurotrophic factor, basic fibroblast growth factor (bFGF, also known as FGF-2), to be especially interesting. bFGF belongs to the fibroblast growth factor family and can be found in four conformations (18, 22, 23, and 24-kD) expressed from a single mRNA transcript containing multiple translational initiation sites. bFGF, originally isolated from bovine brain and pituitary and found to stimulate fibroblast growth (Hoffman, 1940; Trowell, Chir, & Willmer, 1939) promotes survival, differentiation and growth of many types of mammalian cells, including neurons and glia (for reviews see Thomas, 1993; Unsicker, Grothe, Ludecke, Dorte, & Westermann, 1993). Although, the bFGF protein lacks a secretory signal sequence (short amino acid sequence that determines that the eventual location of a protein is on a secretory vesicle), it has been shown to be released into the extracellular milieu *in vitro*, albeit by an as yet uncharacterized mechanism (Araujo & Cotman, 1992b; Florkiewicz, Majack, Buechler, & Florkiewicz, 1995). Released bFGF binds to specific high and low affinity receptors.

As is the case for many neurotrophic factors, the high affinity bFGF receptor, FGFR1, is a receptor tyrosine kinase that is dimerized and activated upon ligand recognition. In vitro studies have shown that activation of

FGFR1 results in activation of signaling proteins such as protein kinase C-γ and phospholipase C, and of the Ras-MAP pathway (Chew et al., 1997; Hill & Treisman, 1995; Thomas, 1993). The low affinity receptor, heparan sulfate-proteoglycan (HSPG), is a large hydrophilic glycoprotein that is anchored to a core protein of the extracellular matrix. HSPG has been shown to protect bFGF from proteolytic inactivation and its presence appears to be critical for bFGF-FGFR1 binding. (Lam, Rao, & Qasba, 1998; Thomas, 1993).

There are several reasons to think that bFGF may participate in the development of long-lasting changes induced by stimulant drugs. First, bFGF is a major neuroprotective and neurotrophic factor of midbrain dopaminergic cells. In culture, it promotes their growth and survival (Bouvier & Mytilineou, 1995; Ferrari, Toffano, & Skaper, 1991; Hou, Cohen, & Mytilineou, 1997; Knusel, Michel, Schwaber, & Hefti, 1990; Krieglstein, Reuss, Maysinger, & Unsicker, 1998; Park & Mytilineou, 1992). In adult animals, bFGF increases dopaminergic cell survival following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) lesions, attenuates subsequent locomotor behavioral impairment and enhances growth and survival of implanted dopaminergic neurons (Chadi et al., 1993; Date et al., 1993; Otto, Frotscher, & Unsicker, 1990; Takayama et al., 1995). Exceptionally high levels of mRNA and protein for the high affinity bFGF receptor, bFGFR1, are found in midbrain dopaminergic regions (Gonzalez, Berry, Maher, Logan, & Baird, 1995). Interestingly, bFGF and FGFR1 mRNAs are induced by glutamate *in vitro* (Pechan, Chowdhury, Gerdes, & Seifert, 1993) and, importantly, bFGF

up-regulates the GluR1 subunit in cultured hippocampal neurons and increases the Ca^{++} permeability of AMPA receptors (Cheng et al., 1995). Finally, sustained and strong increases in bFGF immunoreactivity in the VTA and SNc have been observed after 6-hydroxydopamine (6-OHDA) lesions in the adult rat (Chadi, Cao, Pettersson, & Fuxe, 1994) suggesting that neurotoxic events or metabolic stressors stimulate bFGF expression.

In this thesis it is proposed that the large increases in extracellular dopamine and glutamate in the VTA induced by repeated exposure to stimulant drugs places excessive demands on the functioning of dopaminergic neurons and leads to the recruitment of the neurotrophic, neuroprotective substance bFGF. The actions of bFGF in this region in turn initiate long-lasting changes in dopaminergic function that lead to enduring changes in dopaminergic functioning and connectivity.

CHAPTER II

EFFECTS OF REPEATED EXPOSURE TO AMPHETAMINE ON BASIC FIBROBLAST GROWTH FACTOR EXPRESSION: ROLE OF GLUTAMATE

2.1. Experiment 1: Effects of Amphetamine on bFGF Expression in Dopaminergic Cell body Regions.

2.1.1. Introduction

The first step toward exploring the idea that bFGF may participate in the development of sensitization is to investigate whether repeated exposure to stimulant drugs alters the expression of this trophic factor. A considerable amount of work in the area of sensitization to stimulants has been focused on the effects of amphetamine and, as a result, there is substantial information regarding the events involved in the development of enduring changes induced by its repeated administration. It was decided, therefore, to investigate whether repeated exposure to amphetamine would result in changes in bFGF immunoreactivity.

Sensitization to the effects of amphetamine takes time to develop and the underlying neuronal adaptations appear to continue to develop for some time after discontinuation of drug treatment (Heidbreder et al., 1996; Kolta et al., 1985; Paulson et al., 1991; Paulson & Robinson, 1995; Segal & Kuczenski, 1992a; Segal & Kuczenski, 1992b). Therefore, alterations in bFGF expression were assessed at different time intervals after the last amphetamine injection:

24h, 72h, one week, and one month. Because sensitization is initiated by amphetamine actions in dopaminergic cell body regions (Bjijou et al., 1996; Cador et al., 1995; Hooks et al., 1993; Kalivas & Weber, 1988; Vezina, 1993; Vezina, 1996; Vezina & Stewart, 1990) bFGF expression was first examined within the VTA and SNc.

The results of this work have been published (Flores, Rodaros, & Stewart, 1998).

2.1.2. Materials and Methods

2.1.2.1. Subjects

Male Wistar rats weighing 300-350 g at the beginning of the experiment served as subjects. Rats were housed individually in standard stainless steel hanging boxes with free access to tap water and rat chow, and were maintained on a 12 h light/dark cycle.

2.1.2.2. Drugs and Antibodies

D-amphetamine sulfate was obtained from SmithKline Beecham Pharma (Oakville, Ontario) and was dissolved in physiological saline. Basic fibroblast growth factor (bFGF) immunoreactivity was detected using a mouse, monoclonal antibody purchased from Upstate Technology (Lake Placid, NY). This antibody recognizes the biologically active conformation of bFGF (Matsuzaki, Yoshitake, Matuo, Sasaki, & Nishikawa, 1989).

2.1.2.3. Immunohistochemistry

Animals received an overdose of sodium pentobarbital (120 mg/kg) and were perfused transcardially with 200 ml of ice-cold phosphate buffered saline followed by 100 ml of a solution of 4% paraformaldehyde (w/v) and 14% picric acid (v/v) in 0.1 M phosphate buffer (PB, pH 6.9). When the perfusion was completed, the brains were removed and placed overnight in the fixative solution at 4 ^{0}C. Coronal sections, 50 µm thick, were cut on a vibratome and stored overnight in PB at 4 ^{0}C. bFGF immunoreactivity was then detected according to the ABC method (Hsu, Raine, & Fanger, 1981). Free-floating tissue sections were incubated for 24 h at 4 ^{0}C with the anti-bFGF antibody diluted to 1:500 with 0.3% Triton X-100 (Sigma) in PB and 1% normal horse serum (Vector, Burlingame, CA). Following incubation in the primary antibody, sections were rinsed 3 times in cold PB and incubated for 1 h at room temperature (RT) in a solution of rat adsorbed biotinylated anti-mouse antibody (Vector) diluted 1:200 with PB and 1 % normal horse serum. After 3 5-min washings in cold PB, sections were then incubated in an avidin-horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector) for 30 min at RT, and rinsed again 3 times (5 min each) in cold PB. Sections were then incubated for 10 min, at RT and under constant agitation, in a solution of 0.05% 3,3'-diaminobenzidine (DAB, Sigma) in PB. Without washing, the sections were then transferred to a DAB/PB solution (pH 7.8) containing 0.01% H_2O_2 which catalyzed the reaction, and 8% $NiCl_2$, which darkened the reaction product. Sections were incubated in this solution at RT and under

constant agitation for 8 min. Special care was taken to maintain this time rigorously constant for all sections processed within one single experiment and throughout the entire study. Three 10-min washes with cold PB terminated this final incubation.

Processed sections were wet-mounted onto gelatin-coated slides and were allowed to dry for at least one day before being hydrated in distilled water and gradually dehydrated through a series of graded alcohol solutions. Sections were counterstained with 0.1% cresyl violet to demonstrate anatomical landmarks. Slides were cleared in xylene and coverslipped with Permount.

2.1.2.4. Image Analysis

Immunostained sections were examined under a Leica microscope (Leitz DMRB). For quantitative analysis of bFGF immunoreactivity, images of sampling areas of the VTA, substantia nigra compacta (SNc) were digitized using a computerized image-analysis system (NIH Image 1.6). Structure boundaries were defined according to the Paxinos and Watson stereotaxic atlas (Paxinos & Watson, 1997). Sampling areas of VTA and SNc were taken from sections corresponding to plates 38 and 39.

For each brain, three images from each structure, taken from three different sections, were digitized and were assigned code names. The number of bFGF positive cells in each image was then counted by two individuals who were blind to the code assignment. The mean cell counts of the three

sections for each structure in each animal were calculated by each observer. Correlation coefficients were calculated between the mean cell counts obtained by each observer. When these correlation coefficients were equal or greater than 90%, the cell counts obtained by the two observers were averaged; the resulting values were used to calculate the group means ± SEM per area. Importantly, brains of subjects from each of the experimental conditions were always processed in parallel from perfusion to image analysis.

2.1.4. Statistical Analysis

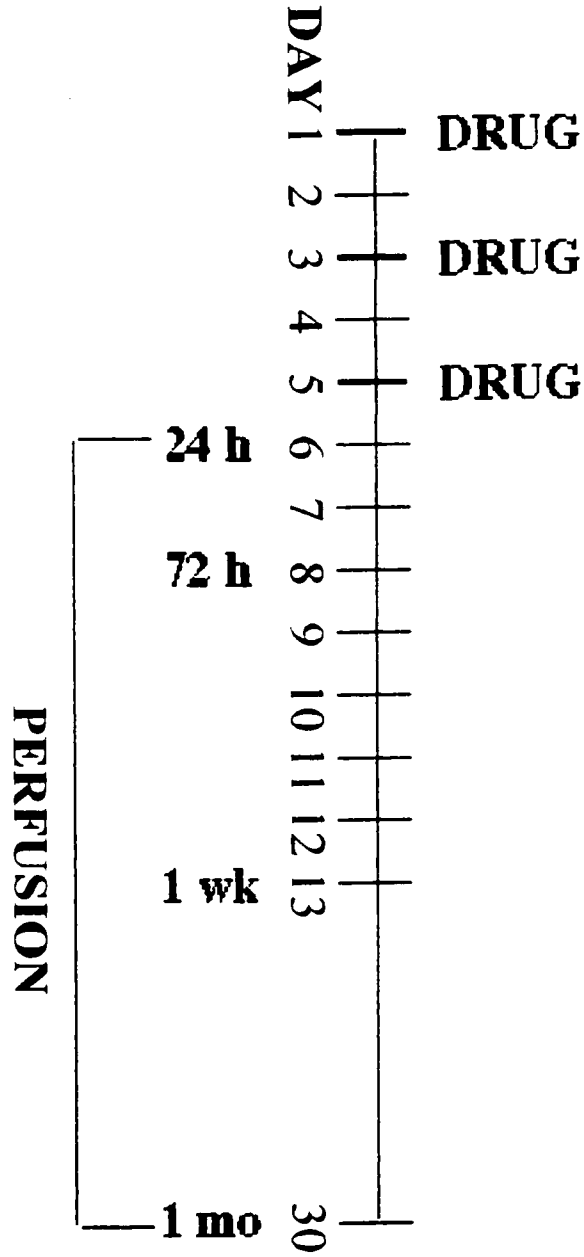
The data were analyzed with 1-way analyses of variance (ANOVA). Post-hoc analyses of significant effects were made using Fisher's Protected LSD tests ($p \leq .05$). The analyses were done using the number of bFGF immunoreactivity cells per square mm. The data in the figures are presented as percent of controls. Because no differences in bFGF expression were found between the saline-treated groups at the different time periods in either the VTA or SNc, the data from all saline-treated groups were combined and compared to the amphetamine-treated groups at each time interval.

2.1.5. Procedures

Fig. 2.1 outlines the timing of the treatment and experimental manipulations in this experiment.

The goal of this experiment was to determine levels of bFGF immunoreactivity in midbrain dopaminergic regions (VTA and SNc) at

Fig. 2.1. Diagram outlining the timing of the treatment and experimental manipulations in Experiment 1.

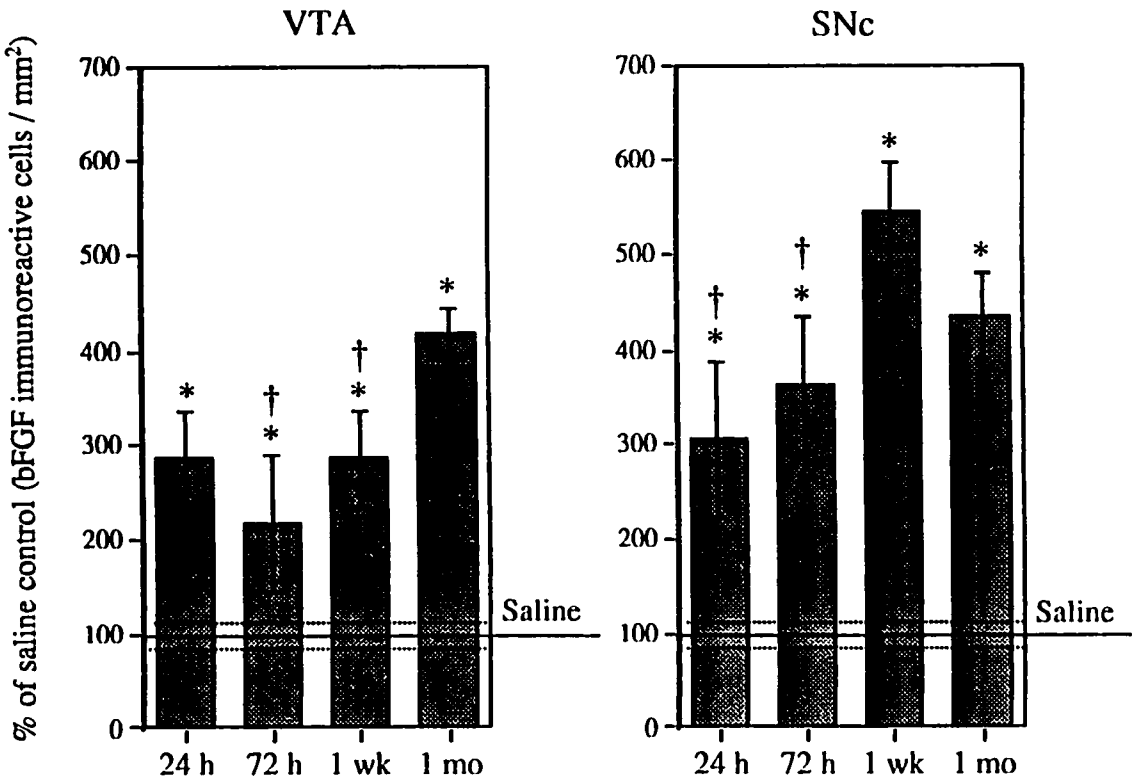

DAY
1
2
3
4
5
6
7
8
9
10
11
12
13
30
DRUG
DRUG
DRUG
24 h
72 h
1 wk
1 mo
PERFUSION

several time points after repeated administration of amphetamine. In the preexposure phase, different groups of rats were given injections of either amphetamine (3 mg/kg intraperitoneally, i.p.) or saline in the morning (10:00 AM) on days 1, 3, and 5, in the colony room. This or similar treatment regimens are known to produce behavioral sensitization and this was confirmed under the conditions used in the present experiments. Groups of amphetamine-treated (n=5) and saline-treated (n=4) rats were killed and perfused at each time period, 24 h, 72 h, one week, and one month, after the last injection and their brains were processed for bFGF immunohistochemistry.

2.1.6. Results

Repeated intermittent amphetamine administration (3.0 mg/kg i.p., three injections, once every other day) resulted in an increase in bFGF immunoreactivity in both VTA and SNc over that seen in saline-treated animals. As shown in Fig. 2.2, a significant increase in bFGF immunoreactivity in the VTA was observed 24 h (274 ±46%) after the last amphetamine injection. This increase was sustained 72 h (206 ± 68%) and one week (272 ± 49%) after the last drug injection and was further increased one month (400 ± 24%) after treatment termination. Within the SNc, a significant increase in bFGF immunoreactivity was observed 24 h (324 ± 89%) and 72 h (426 ± 77%) following the last drug injection (Fig. 2.2). Levels of bFGF immunoreactivity within this region appeared to peak at one week (578

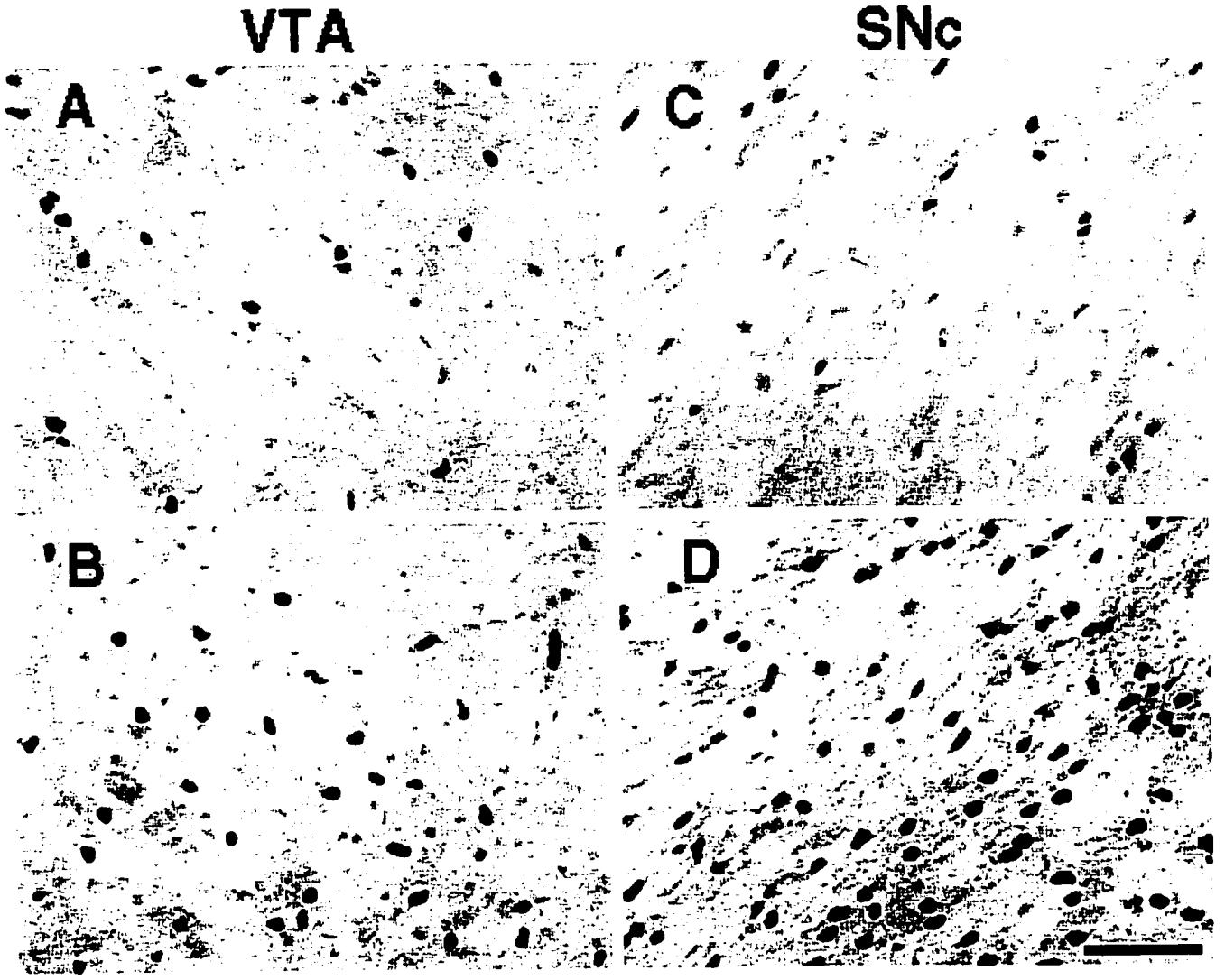
Fig. 2.2. Time course: Mean (± SEM) percent of saline control (bFGF immunoreactive cells per square millimeter) in the VTA and SNc in groups (n=5/group) of amphetamine-treated animals (3.0 mg/kg, i.p., on three occasions, once every other day) killed 24 h, 72 h, one week and one month after the last injection. 100% represents the mean (solid line) and SEM (dashed lines) counts from saline-treated animals that were killed at each of the time interval after the last injection (n=4/time interval). ANOVAs performed on the actual counts showed a significant effect of drug treatment (VTA: $F(4,20) = 6.39$; $p = 0.001$; SNc: $F(4,20) = 8.2$; $p < 0.001$). Asterisks indicate significant differences from the saline group $p < 0.01$; daggers indicate significant differences from the peak, $p < 0.05$.


VTA
SNc
% of saline control (bFGF immunoreactive cells / mm²)
700
600
500
400
300
200
100
0
Saline
24 h
72 h
1 wk
1 mo
*
†

± 57%) and remained elevated (463 ± 48%) one month after treatment termination. The maximum number of bFGF immunoreactive cells observed within the VTA of amphetamine-treated animals was three times higher than that of saline-treated animals and was observed one month after the last drug injection. In the case of the SNc, maximum levels of bFGF immunoreactivity, observed one week after the last amphetamine injection, were almost five times higher than those observed in saline-treated rats.

Fig. 2.3 shows examples of bFGF-IR in the VTA and SNc of animals that were treated with either saline or AMPH and that were killed one week after the last injection. It can be seen that there was a greater number of bFGF positive cells in AMPH-treated animals in these areas. Previous studies have shown that the antibody used in this study detects bFGF-IR in astrocytic nuclei (Flores, Salmaso, Cain, Rodaros, & Stewart, 1999; Szele, Alexander, & Chesselet, 1995). In agreement with those observations, strong immunostaining for bFGF in both the VTA and SNc was observed in nuclei of small cells (Fig. 2.3; see also Fig. 2.7).

Fig. 2.3. Digitized images showing darkly labeled bFGF-immunoreactive cells in tissue lightly counterstained with cresyl violet (revealing both glia and larger neurons). Images were taken from representative animals that were injected, intraperitoneally, with either saline or amphetamine (3.0 mg/kg), on three occasions, once every other day, and killed one week later. Images **A** (saline) and **B** (amphetamine) were taken from the VTA, and images **C** (saline) and **D** (amphetamine) were taken form the SNc. Scale bar = 50 μm. Cell counts included only darkly labeled profiles that were in the same plane of focus.


VTA
SNc
A
C
B
D

2.2. Experiment 2. Role of Glutamate

2.2.1. Introduction

The development of sensitization to amphetamine depends on glutamatergic transmission within cell body regions of dopaminergic cells. Previous studies have shown that amphetamine injections increase glutamate release in the VTA (Kalivas & Duffy, 1995a; Kalivas & Duffy, 1998; Wolf & Xue, 1998; Wolf & Xue, 1999; Xue et al., 1996) and that both NMDA and AMPA receptor antagonists abolish the development of behavioral sensitization as well as some of its cellular correlates (see section 1.2.2). Interestingly, it has been shown that the mRNA of bFGF and of its receptor, FGFR1, are induced by glutamate in cultured astrocytes (Pechan et al., 1993). It was tested, therefore, whether blockade of ionotropic glutamate receptors, AMPA and NMDA, would prevent the effects of amphetamine on bFGF expression observed in the VTA and SNc. This was examined by treating animals with the nonselective ionotropic glutamate receptor antagonist kynurenic acid (Stone, 1993; Taber & Fibiger, 1995) In addition, bFGF expression was examined in dopaminergic terminal regions in NAcc and STR.

There is evidence showing that even a single injection of amphetamine can produce an increase in glutamatergic efflux in the VTA (Xue et al., 1996). Therefore, if glutamate is responsible for the effects of amphetamine on bFGF expression, then the processes involved in bFGF

expression might be initiated after each amphetamine injection. To ensure that glutamate receptors would be blocked not only while amphetamine is acting, but also in the period between injections, animals were injected with kynurenic acid twice a day. One injection was given in the morning, 1 h before each amphetamine injection, and the second one at night. While amphetamine was given three times, once every other day, kynurenic acid injections were given every day. In addition, kynurenic treatment was continued for a few days after amphetamine treatment termination because in Experiment 1 the expression of bFGF increased over time after the last injection of amphetamine.

Although, the primary purpose was to obtain information about the effects of blockade of ionotropic receptors in the VTA on bFGF expression, kynurenic acid was administered systemically rather than directly into the VTA. Up-regulation in bFGF expression occurs after injury (Fagan et al., 1997; Nieto-Sampedro & Cotman, 1985; Rowntree & Kolb, 1997) and penetration of the brain would be likely to induce bFGF expression itself.

The results of this work have been published (Flores et al., 1998).

2.2.2. Materials and Methods

2.2.2.1. Subjects

Male Wistar rats weighing 300-350 g at the beginning of the experiment served as subjects. Rats were housed individually in standard stainless steel

hanging boxes with free access to tap water and rat chow, and were maintained on a 12 h light/dark cycle.

Drugs and antibodies, and procedures for immunohistochemistry, image and statistical analysis were similar to those described in Experiment 1. Only those aspects that differ are described below.

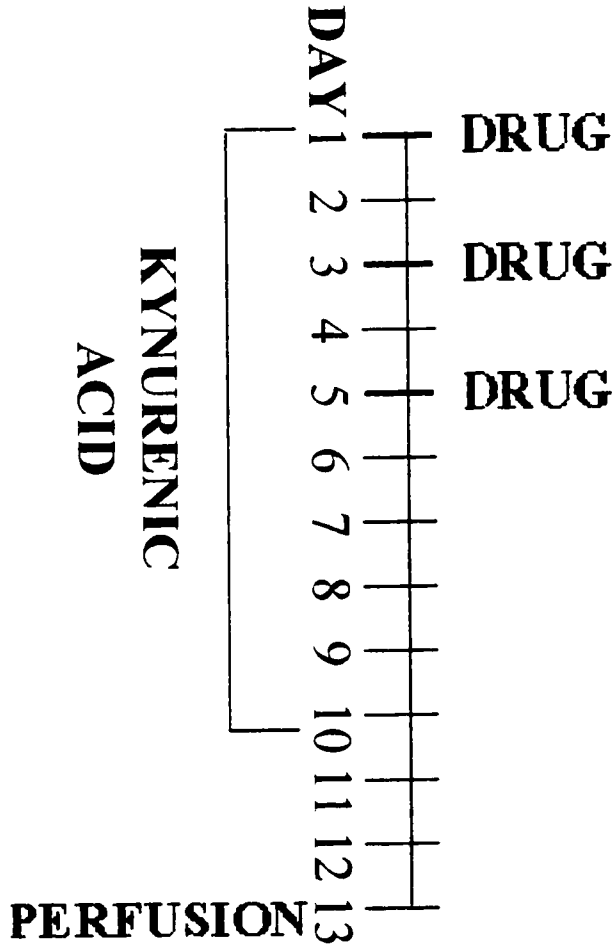
1) Kynurenic acid (4-hydroxyquinoline-2-carboxylic acid) was obtained from Sigma (St. Louis, MO) and was suspended in distilled water containing 2% Tween-80 (vehicle solution). 2) Only midbrain sections processed for bFGF immunoreactivity were counterstained with 0.1% cresyl Violet. 3) For quantitative analysis of bFGF immunoreactivity, images of sampling areas of the VTA, SNc, NAcc shell, NAcc core, and dorsal region of the striatum (STR) were digitized using a computerized image-analysis system. 4) Sampling areas of NAcc shell, NAcc core, and STR were taken from sections corresponding to plates 11, 12, and 13 (Paxinos & Watson, 1997).

2.2.3. Procedures

Fig. 2.4 outlines the timing of the treatment and experimental manipulations in this experiment.

Different groups of rats undergoing the same amphetamine or saline regimen as in experiment 1 on days 1, 3 and 5, were treated twice daily, on days 1-10, with the non-selective ionotropic glutamate receptor antagonist kynurenic acid (100 mg/kg i.p. at 9:00 AM and 6:00 PM) or with vehicle (either

Fig. 2.4. Diagram outlining the timing of the treatment and experimental manipulations in Experiment 1.


DAY
1
2
3
4
5
6
7
8
9
10
11
12
13
DRUG
DRUG
DRUG
KYNURENIC
ACID
PERFUSION

2% Tween 80 or saline). Animals from each of the four groups: vehicle-amphetamine (VTA, n=8; SNc, n= 7), vehicle-saline (n=8), kynurenic-amphetamine (n=4), and kynurenic-saline (n=4) were killed and perfused one week after the last amphetamine or saline injection (72 h following the last kynurenic or vehicle injection) and their brains were processed for bFGF-immunoreactivity. This time point was chosen on the basis of the substantial increase in bFGF immunoreactivity observed in experiment 1 in both VTA and SNc. In this experiment, brain sections from both dopaminergic cell body regions (VTA and SNc) and dopaminergic terminal regions (NAcc shell and core and STR) were analyzed.

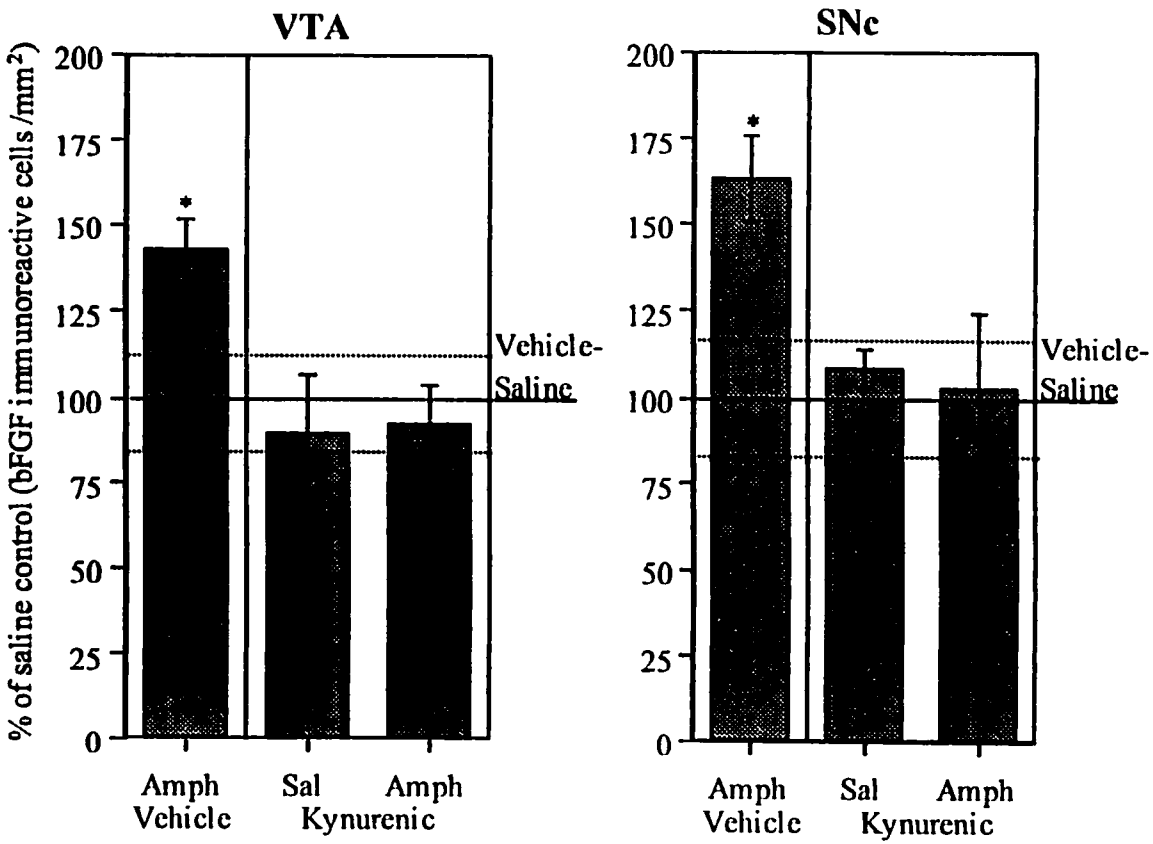
2.2.4. Results

Dopaminergic Cell Body Regions:

As can be seen in Fig. 2.5, once again, animals treated with amphetamine showed a significant increase in bFGF immunoreactivity in both the VTA and SNc compared to saline-treated animals. This difference between amphetamine- and saline-treated animals was prevented by the coadministration of the glutamate receptor antagonist kynurenic acid. In neither area was there any effect on bFGF immunoreactivity of kynurenic acid alone (group kynurenic-saline).

It will be noted that bFGF immunoreactivity levels in the VTA and SNc in the vehicle control group were higher in experiment 2 than they were in the control group in the time course experiment (Experiment 1). Thus, the

Fig. 2.5 Role of glutamate in dopaminergic cell body regions: Mean (± SEM) percent of vehicle-saline control (bFGF-immunoreactive cells per square millimeter) in VTA and SNc in groups of animals treated daily with kynurenic acid (n= 4/group) or vehicle (VTA, n= 8/group; SNc, n=7/group) and given three injections of saline or amphetamine (3.0 mg/kg) and killed one week after the last injection of saline or amphetamine; 100% (dotted line) represents the mean and SEM (dashed lines) counts from vehicle-saline treated animals (n=8). ANOVAs performed on the actual counts showed significant group difference in each area (VTA: $F(3, 20) = 3.77$; $p < 0.05$; SNc: $F(3, 19) = 4.11$; $p < 0.05$). Asterisks indicate that the vehicle-amphetamine group was significantly different from all other groups ($p < 0.05$).


VTA
SNc
% of saline control (bFGF immunoreactive cells /mm²)
200
175
150
125
100
75
50
25
0
*
Vehicle-
Saline
Amph
Vehicle
Sal
Kynurenic
Amph

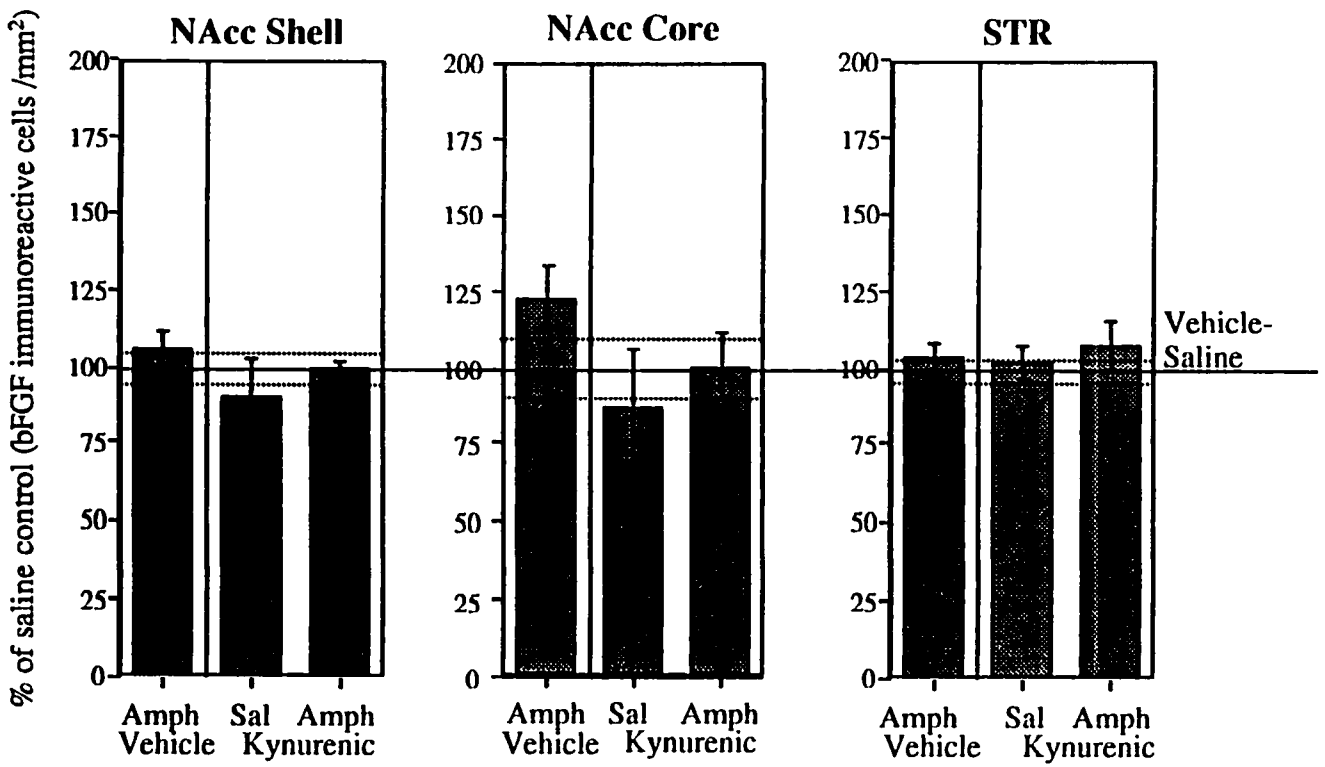
percent increase in bFGF immunoreactivity induced by amphetamine in experiment 2 was smaller than the increase observed in the amphetamine group one week after the last injection in experiment 1 (see Figs. 2.2 and 2.5). It is likely that stress was the source of the difference observed in bFGF immunoreactivity levels between the two control groups. In Experiment 2, kynurenic acid was administered to rats chronically. Whereas animals in the time course experiment were injected only 3 times, animals in Experiment 2 were given two or three injections a day for 10 days. Numerous studies have shown that repeated exposure to stress can lead to changes in the responsiveness of the midbrain dopaminergic system (for example see Kalivas & Duffy, 1995b).

Thus studies of the effects of stress on bFGF immunoreactivity in midbrain dopaminergic regions appear warranted.

Dopaminergic Terminal Regions

As shown in Fig. 2.6, levels of bFGF immunoreactivity in the STR and in the NAcc shell were not affected by amphetamine treatment. No changes in bFGF immunoreactivity within these regions were observed in either vehicle- or kynurenic-treated rats. Within the NAcc core, the amphetamine group exhibited a small but non-significant increase in bFGF immunoreactivity in comparison to the saline group. This elevation was not observed in amphetamine-treated rats that received kynurenic acid administration (Fig. 2.6).

Fig. 2.6. bFGF immunoreactivity in dopaminergic terminal regions: Mean (± SEM) percent of vehicle-saline control (bFGF immunoreactive cells per square millimeter) in NAcc shell, NAcc core and STR in groups (n=4/group) of animals treated daily with kynurenic acid or vehicle (n=8/group) and given three injections of saline or amphetamine (3.0 mg/kg) and killed one week after the last injection of saline or amphetamine; 100% represents the mean (solid line) and ± SEM (dashed lines) counts from vehicle-saline treated animals (NAcc core and STR, n=7; NAcc shell, n=8). See text for details. ANOVAs performed on the actual counts showed no significant group differences in any of the areas (NAcc shell: $F(3, 20) = 0.89$; NAcc core: $F(3, 19) = 1.52$; STR: $F(3, 19) = 0.21$).


NAcc Shell
NAcc Core
STR
% of saline control (bFGF immunoreactive cells /mm²)
200
175
150
125
100
75
50
25
0
Vehicle-
Saline
Amph
Vehicle
Sal
Kynurenic
Amph

2.3. Double-Labeling Experiment

To determine the phenotype of the cells expressing bFGF in the regions analyzed, double-labeling for bFGF/GFAP and for bFGF/TH was conducted on the brains of two rats that received three injections of either amphetamine or saline in the same regimen used in Experiment 1 and that were killed and perfused one week after the last amphetamine or saline injection.

The results of this work have been published (Flores et al., 1998).

2.3.1. Materials and Methods

For glial fibrillary acidic protein (GFAP) and tyrosine hydroxylase (TH) immunoreactivity, a mouse monoclonal antibody obtained from Sigma and a rabbit polyclonal antibody obtained from Eugene Tech (Ramsay, NJ), respectively, were used. Double-labeling for bFGF/GFAP and for bFGF/TH was carried out by processing the sections, first, for bFGF immunohistochemistry (using the same method as the one described in Experiment 1) and then for either GFAP or TH immunohistochemistry. GFAP and TH immunolabeling was performed by using the ABC method. The anti-GFAP antibody was used at a concentration of 1:500 and the anti-TH one at a concentration of 1:2000. For TH immunohistochemistry, sections were preincubated in 0.3% Triton X-100 PB and 1 % normal goat serum for 1 h, at RT. For both GFAP and TH immunohistochemistry no $NiCl_2$ was added to the DAB/PB/ H_2O_2 solution so as to obtain a lighter reaction product.

Histology and qualitative image analysis were conducted as described in Experiment 1, except that brain sections were not counterstained with cresyl violet.

2.3.2. Results

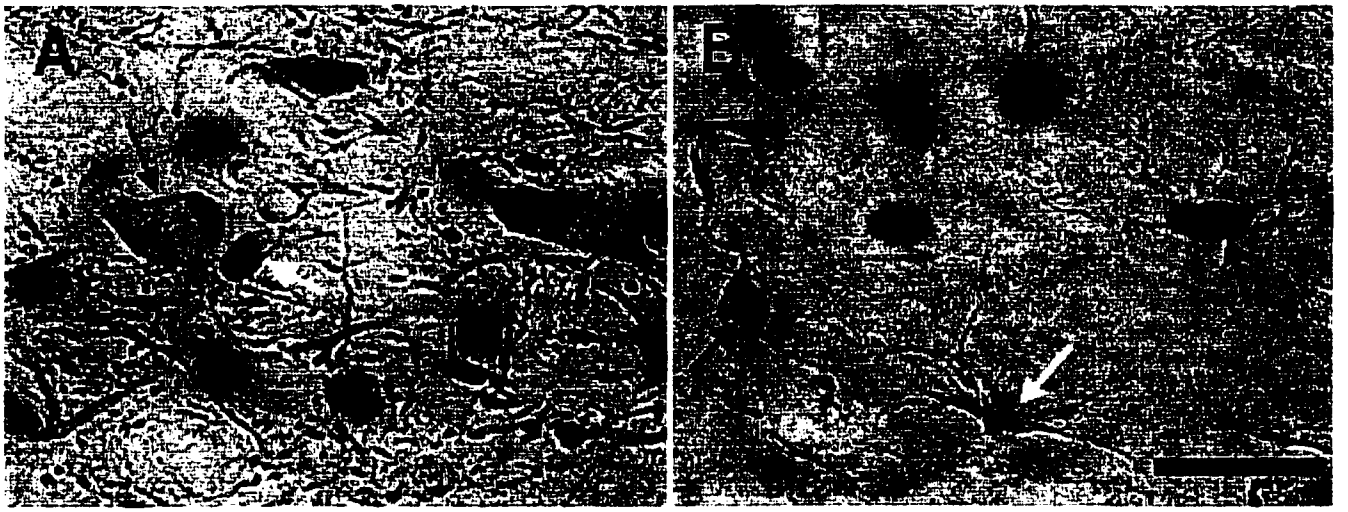
In both, amphetamine and saline-treated rats, double-label experiments indicated that in all areas examined bFGF-positive cells were also GFAP positive Many, but not all, astrocytes expressed bFGF immunoreactivity, and TH-positive cells did not exhibit bFGF immunoreactivity. These observations are illustrated in Fig. 2.7 which shows TH/bFGF (Fig. 2.7.A) and GFAP/bFGF (Fig. 2.7.B) double-labeling in a region of the VTA of one amphetamine-treated animal that was killed one week after the last drug injection. Similar findings were observed in the brain of a saline-treated animal.

2.4. Discussion

The primary purpose of these experiments was to determine whether repeated injections of amphetamine would induce increases in bFGF expression in midbrain dopamine regions following termination of treatment. It was found that after three intermittent injections of 3.0 mg/kg of D-amphetamine sulfate administered i.p., once every other day, there were large increases in bFGF immunoreactivity levels in the cell body region of the midbrain dopamine neurons in the VTA and SNc. These increases occurred as early as 24 h after the last injection, increased over a period of one week,

Fig. 2.7. Digitized images of VTA sections of an animal treated with amphetamine as in experiment 1 and killed one week after the last injection. The large black blobs indicate bFGF immunoreactivity.

A, Section labeled with both TH and bFGF. bFGF immunoreactivity (indicated by the white arrow) was not found within the large TH-positive cells (see black arrow). B, Section labeled with both GFAP and bFGF. Arrows point to three GFAP-immunoreactive astrocytes. The white arrow points to an astrocyte that was not labeled for bFGF. The black arrows point to two astrocytes that were labeled with both bFGF and GFAP. (Oil immersion: Scale bar, 25 μm).


A
B

and were clearly evident at one month. The results from the double-labeling experiments revealed that bFGF immunoreactivity in these regions was associated with astrocytes and not with dopamine neurons themselves.

As discussed in Chapter 1, enhanced dopaminergic function, including increased dopamine overflow in NAcc and dorsal striatum in response to drug challenge, follows repeated administration of stimulant drugs (Kalivas & Stewart, 1991; Robinson & Becker, 1986). These changes take time to develop, appearing 1-3 weeks after the last drug injection (Heidbreder et al., 1996; Kolta et al., 1985; Paulson et al., 1991; Paulson & Robinson, 1995; Segal & Kuczenski, 1992a; Segal & Kuczenski, 1992b). Interestingly, bFGF expression in dopaminergic cell body regions increased during the period that is usually needed for sensitization of the dopamine response to develop. Furthermore, the enhancement in bFGF immunoreactivity was persistent, as are the changes in dopamine function and behavior induced by stimulant drugs. The findings of these experiments raise the possibility, therefore, that increased bFGF expression in the dopaminergic cell body region is one of the events that leads to the development and maintenance of the neural changes underlying sensitization.

The possibility that bFGF expression is related to the development of sensitization to amphetamine is increased by the findings from Experiment 2, showing that the nonselective ionotropic glutamate receptor antagonist, kynurenic acid, blocked the amphetamine-induced increase in bFGF expression in VTA and SNc. These results, suggesting that the enhancement

of bFGF immunoreactivity in dopaminergic cell body regions seen after amphetamine treatment is mediated by glutamate, are supported by data showing that glutamate induces bFGF mRNA expression in astrocytes (Pechan et al., 1993) ,that glutamate overflow in dopamine cell body regions increases during (Kalivas & Duffy, 1995a; Kalivas & Duffy, 1998) or following (Wolf & Xue, 1998; Wolf & Xue, 1999; Xue et al., 1996) amphetamine administration; and that NMDA and AMPA antagonists prevents sensitization to amphetamine (Wolf, 1998) Therefore, in view of the present findings, one may hypothesize that glutamate antagonists block the development of sensitization to amphetamine by preventing increased expression of bFGF.

The results of this study also revealed that the increases in bFGF immunoreactivity induced by amphetamine occurred in the cell body regions of dopaminergic neurons and not in terminal regions in the NAcc core, Nacc shell, or STR. As mentioned earlier, it is well established that the events that lead to sensitization are initiated by the actions of amphetamine in the cell body region of dopaminergic neurons, and not in these terminal regions (Bjijou et al., 1996; Cador et al., 1995; Hooks et al., 1993; Kalivas & Weber, 1988; Vezina, 1993; Vezina, 1996; Vezina & Stewart, 1990). Moreover, injections of glutamate antagonists directly into the VTA have been found to prevent the development of sensitization to amphetamine (Cador et al., 1999; Vezina and Queen, 2000; Kim & Vezina, 1998). Finally, it is known that the development of sensitization to amphetamine can be blocked by systemic

injections of protein synthesis inhibitors (Karler, Finnegan, & Calder, 1993; Robinson, 1991) and that their direct application in the VTA, but not NAcc, can block sensitization to cocaine (Sorg & Ulibarri, 1995). This evidence, together with the finding that bFGF expression was increased in dopamine cell body regions, but not in terminal regions, increases the probability that the enhanced astrocytic bFGF expression seen in the VTA and SNc regions is part of the cascade of intra- and inter-cellular events that leads to long-lasting changes in the control and functioning of dopamine neurons.

It should be pointed out, however, that bFGF immunoreactivity was assessed in striatal terminal regions only at one week after the last amphetamine injection. Thus, increases in bFGF immunoreactivity within these regions either at earlier or later times cannot be ruled out. This issue is important in view of recent studies on persistent changes in postsynaptic neurons in NAcc induced by repeated drug treatment, including modifications in neuronal structure and increased expression of immediate early genes (Kelz et al., 1999; Robinson & Kolb, 1997; Robinson & Kolb, 1999).

An interesting finding of this study is that bFGF expression was observed only in astrocytic cells. The importance attributed to the role of astrocytes in neuronal function has changed considerably in the past years. These cells have been found to do more that just ensheath synapses throughout the brain. They provide most of the glucose to the CNS (Tsacopoulos & Magistretti, 1996), play a major role in the regulation of the extracellular concentration of glutamate (Duan, Anderson, Stein, & Swanson,

1999), release glutamate in a Ca^{++} dependent manner (Bezzi et al., 1998), and appear to modulate synaptic efficacy (Pfrieger & Barres, 1996). Thus, the fact that repeated exposure to amphetamine results in persistent changes in astrocytic function in the VTA may provide a novel mechanism involved in the development of sensitization.

As indicated in the methods section, the mouse monoclonal bFGF antibody that was used in these and following studies recognizes the bFGF protein conformation that is essential for its biological activity (Matsuzaki et al., 1989). We and others have found that this antibody detects bFGF in astrocytes, except for a few restricted brain regions (Flores et al., 1999; Matsuzaki et al., 1989; Szele et al., 1995). There is another bFGF antibody, a rabbit polycolonal, that has been shown to detect bFGF in both neurons and glia (Belluardo, Blum, Mudo, Andbjer, & Fuxe, 1998; Gonzalez et al., 1995). Whether bFGF expression in neurons is altered by amphetamine treatment remains to be tested and cannot be excluded. Most of the bFGF protein and mRNA observed in the VTA and SNc, however, are primarily localized to astrocytes and, in fact, bFGF mRNA is not expressed by SNc neurons (see Gonzalez et al., 1995).

In summary, repeated exposure to amphetamine elicits marked increases in astrocytic bFGF immunoreactivity in dopaminergic cell body regions that appear to be mediated by glutamate. Importantly, these increases are long-lasting, continuing to be evident one month after the last exposure to amphetamine. These findings suggest that increased astrocytic expression

of bFGF in dopaminergic cell body regions is an important mechanism by which stimulant drugs exert their long-term effects on midbrain dopaminergic function.

CHAPTER III

AMPHETAMINE TREATMENT INDUCES BOTH bFGF EXPRESSION AND BEHAVIORAL SENSITIZATION. ROLE OF NMDA RECEPTORS

3.1. Experiment 1: Effects of NMDA Receptor Antagonists on Amphetamine-Induced bFGF Expression

3.1.1. Introduction

The experiments described have shown so far, that three injections of amphetamine given once a day, every other day, induce increased expression of the neurotrophic, neuroprotective factor, bFGF, in astrocytes in the VTA and SNc. Increased bFGF-immunoreactivity in these regions is evident 24 h after the last amphetamine injection and remains elevated for at least one month. The effects of amphetamine on bFGF expression appear to be dependent on glutamatergic activation because coadministration of kynurenic acid, a nonspecific antagonist of ionotropic glutamate receptors, prevents the enhancement in bFGF immunoreactivity induced by amphetamine.

Because kynurenic acid blocks both AMPA and NMDA receptors, it remains to be determined whether activation of both types of receptors is needed for bFGF to be expressed. NMDA and AMPA receptor activation have been shown to be required for the development of sensitization to amphetamine. Furthermore, NMDA receptor activation within the VTA

itself has been shown to be required for the development of sensitization to amphetamine (Cador et al., 1999; Jake-Matthews et al., 1997). In this experiment the effects of the specific NMDA receptor antagonist, 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP) on amphetamine-induced bFGF expression were assessed. CPP is known to prevent the development of sensitization to amphetamine when administered directly into the VTA (Cador et al., 1999).

3.1.2.1. Materials and Methods

3.1.2.1. Subjects

Male Wistar rats (Charles River, Quebec; 325-350g), housed in a colony room on a normal light-dark schedule with free access to food and water, served as subjects.

3.1.2.2. Drugs and Antibodies

D-amphetamine sulfate was obtained from SmithKline Beecham Pharma (Oakville, Ontario), 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP), a competitive NMDA receptor antagonist, was purchased from Tocris Cookson. Both drugs were dissolved in saline and injected i.p. bFGF immunoreactivity was detected using a mouse, monoclonal antibody (Upstate Technology) that recognizes the biologically active conformation of bFGF (Matsuzaki et al., 1989).

Tissue processing and image analysis were carried out in an identical manner to that described in sections 2.1.2.3, 2.1.2.4, 2.1.2.5.

3.1.3. Statistical Analysis

The data were analyzed with 1-way ANOVAs. Post-hoc analyses of significant effects were made using Fisher's Protected LSD tests ($p \leq .05$). The analyses were done using the number of bFGF immunoreactivity cells per square mm. The data in the figures are presented as percent of controls.

3.1.4. Procedures

Fig. 3.1 outlines the timing of the treatment and experimental manipulations in this experiment.

In the induction phase of this study, different groups of rats were given injections of either amphetamine (3 mg/kg intraperitoneally, i.p.) or saline in the morning (10:00 AM) on days 1, 3, and 5, in the colony room. These animals also received on days 1-10, i.p. injections of 0.5 mg/kg or 1.0 mg/kg of the NMDA receptor competitive antagonist, CPP, or saline (9:00 AM and 6:00 PM). Animals from each of the four groups: saline-amphetamine (VTA, n=8; SNc, n= 7), saline-saline (n=8), CPP(0.5)-amphetamine (n=4), CPP(1.0)-amphetamine (n=4), CPP(0.5)-saline (n=4), and CPP(1.0)-saline (n=4) were killed and perfused one week after the last amphetamine or saline injection (72 h following the last CPP or saline injection) and their brains were processed for bFGF-immunoreactivity. This time point was chosen on the

Fig. 3.1. Diagram outlining the timing of the treatment and experimental manipulations in Experiment 1.

DAY 1 2 3 4 5 6 7 8 9 10 11 12 13
PERFUSION
DRUG
DRUG
DRUG
CPP

basis of the substantial increase in bFGF immunoreactivity observed in results from experiments described in Chapter 2. Brain sections from VTA and SNc.

3.1.5. Results

As shown in Fig. 3.2, animals treated with amphetamine (saline-amphetamine) showed a significant increase in bFGF immunoreactivity in both the VTA (78 ± 15%) and SNc (60 ± 13%) compared to saline-treated animals (saline-saline), one week after the last amphetamine injection. The increased in bFGF expression after amphetamine treatment was prevented by the twice daily 1.0 mg/kg CPP injections (CPP(1.0)-amphetamine), but not by 0.5 mg/kg CPP injections (CPP(0.5)-amphetamine). CPP alone did not have any effect on bFGF immunoreactivity in VTA or SNc (groups CPP(0.5)-CPP(1.0)-saline).

Fig. 3.2. CPP effects on bFGF expression. Mean (± SEM) percent of saline-saline control (bFGF-immunoreactivity cells per square millimeter) in the VTA and SNc in groups of animals that received three i.p. injections of 3 mg/kg of D-amphetamine or saline, once every other day, and that were coadministered, twice a day for 10 days, with i.p. injections of 0.5 mg/kg, 1.0 mg/kg of CPP (n=4/group) or saline (VTA: n=8/group; SNc: n=7/group). Animals were killed one week after the last amphetamine injection (72 h after the last CPP injection). 100% represents the mean (solid line) and SEM (dashed lines) counts from saline-saline treated animals. ANOVAs performed on the actual counts revealed significant group differences (VTA, $F(5,26) = 4.58$, $p < .01$; SNc, $F(5,25) = 4.38$, $p < .01$). In both VTA and SNc, bFGF immunoreactivity in the CPP (1.0 mg/kg)-amphetamine group was significantly less than in the saline-amphetamine group ($p < .01$). The CPP (0.5 mg/kg)-amphetamine group did not differ from the saline-amphetamine group.

bFGF positive cells per mm²
(% of saline-saline)
250
200
150
100
50
0
VTA
SNc
sal/sal
CPP (0.5) sal
CPP (1.0) sal
sal amph
CPP (0.5) amph
CPP (1.0) amph
amph amph

3.2. Experiment 2: bFGF Expression in Rats Exhibiting Behavioral Sensitization. Role of Glutamate.

3.2.1. Introduction

The results of the studies that have been presented point to bFGF as a potential factor implicated in the development of sensitization to amphetamine. First, actions of stimulant drugs in the VTA, but not in NAcc, are required for sensitization to develop; increased astrocytic bFGF expression is observed in cell body, but not terminal regions. Second, bFGF expression is maintained for considerable time after the last injection of amphetamine suggesting that bFGF could participate in neural adaptations that need time to develop and that are responsible for the gradual emergence of sensitized responding to amphetamine. Finally, both behavioral sensitization and bFGF induction depend on NMDA receptor activation.

Up to this point, however, no behavioral tests for sensitization have been conducted in any of the experiments described. Thus, it is not known if the enhanced bFGF expression induced by repeated injections of amphetamine is related to the development of sensitization. To assess this relation, it was asked whether bFGF expression would be increased in animals that exhibit behavioral sensitization and whether levels of bFGF expression would be related to the magnitude of sensitization induced. Therefore, a typical behavioral sensitization study was conducted in which the locomotor-activating effects of amphetamine were determined in an induction phase

and a test phase. Immediately after termination of the behavioral sensitization test, animals were perfused and their brains were processed for bFGF immunoreactivity. To assess the role of NMDA receptor activation, animals were treated with CPP during the induction phase of the experiment.

The result from this experiment have been published (Flores, Samaha, & Stewart, 2000).

3.2.2. Material and Methods

3.2.2.1. *Subjects*

Male Wistar rats (Charles River, Quebec; 325-350g), housed in a colony room on a normal light-dark schedule with free access to food and water, served as subjects. Behavioral testing was conducted in the light phase of the cycle.

3.2.2.2. *Apparatus*

Locomotor activity was measured in 12 activity boxes (20 x 41 x 25 cm), each built of 3 pressed-wood walls, one Plexiglas front wall, a wire screen top and a stainless steel rod floor. Four photocells were located around the perimeter of the box. Two were positioned 3.5 cm above the floor along the front and rear walls (separated by 20 cm) and estimated horizontal activity ('horizontal photocells'). The other two photocell lights sets were positioned on the side walls, 20 cm above the floor and measure vertical movements. Separate interruptions of photocell beams were detected and recorded via an

electrical interface by a computer located in an adjacent room. The activity boxes were kept in the dark throughout all activity session and a white noise generator (75 dB) was used to mask extraneous noise.

The drugs and antibody used in this experiment, as well as the immunohistochemistry and image analysis procedures, were as described in Experiment 1.

3.2.3. Statistical Analysis

Data were analyzed by 1-, 2- and 3-way ANOVAs as required (this is indicated in Results). Post hoc comparisons were made using Fisher's protected LSD tests ($p \leq .05$). Linear correlation analyses were performed by calculating *r* coefficients. All analyses were done using the number of bFGF immunoreactivity cells per square mm.

3.2.4. Procedures

Behavioral Sensitization

Before the start of the induction phase of sensitization, a pretest was done to ensure that there were no a priory differences in spontaneous activity levels among treatment groups. All rats were taken to the activity room for the first time and placed in the activity boxes where their activity was monitored for 2 h. Activity counts were obtained by recording the number of interruptions of horizontal photocells beams. Animals were then assigned to

matched groups on the basis of these original scores (four different groups: CPP-amphetamine, CPP-saline, saline-amphetamine, and saline-saline).

Induction Phase:

On days 1, 3, 5, 7, and 9 rats were given i.p. injections of CPP (4.0 mg/kg, i.p.) or saline in the colony room. This CPP dose was chosen on the basis of a pilot study in which treatment with 1 mg/kg of CPP during the induction phase was not found to prevent the development of behavioral sensitization to amphetamine. Thirty min later, animals were taken to the activity room where they received injections of either saline or amphetamine (1.5 mg/kg, i.p.) and were placed in the activity boxes for 2 h.

Test:

To determine whether pretreatment with CPP blocked the development of sensitization to amphetamine, a test for behavioral sensitization was conducted one week after the last day of the induction phase. For this test, all animals, whether exposed previously to amphetamine or saline, were given a single i.p. injection of 0.75 mg/kg amphetamine immediately before being placed in activity boxes for 2 h. For this test, no prior injections of CPP or saline were given. The dose used in this test was half of that I used in the induction phase to prevent the development of stereotypy in sensitized animals (see Chapter 1).

bFGF immunoreactivity:

Immediately after the sensitization test, animals were perfused and brains were processed for bFGF immunoreactivity. Brain sections from both cell body and terminal regions of dopaminergic cells were analyzed.

3.2.5. Results

Test for Sensitization:

Coadministration of CPP during the induction phase blocked the development of sensitization (Fig. 3.3). Animals given CPP injections and either amphetamine or saline during the induction phase did not differ in their response to amphetamine on the test day (Fig. 3.3, right panel). In contrast, those animals that had been exposed only to amphetamine during the induction phase, showed sensitized responding compared to saline control animals when challenged with amphetamine on the test day (Fig. 3.3, left panel).

Induction Phase:

Fig. 3.4 shows the total activity counts recorded in the test chambers during each of 5 days of the induction phase. A clear locomotor-activating effect of each amphetamine injection can be seen in animals that had been given either CPP or saline 30 min before. However, the effects of amphetamine on locomotor activity were significantly decreased by CPP.

Fig. 3.3. Test for sensitization: CPP experiment. All animals received 0.75 mg/kg amphetamine, i.p. before being placed in the activity boxes. Left panel: mean ± SEM activity counts in animals exposed during the induction phase to amphetamine or saline after saline pretreatment, ANOVA ($F(1,10) = 4.9$, $p = .05$). Right panel: ± SEM activity counts in animals exposed during induction to amphetamine or saline after CPP. ANOVA ($F(1,10) = .07$, ns). n= 6/group.

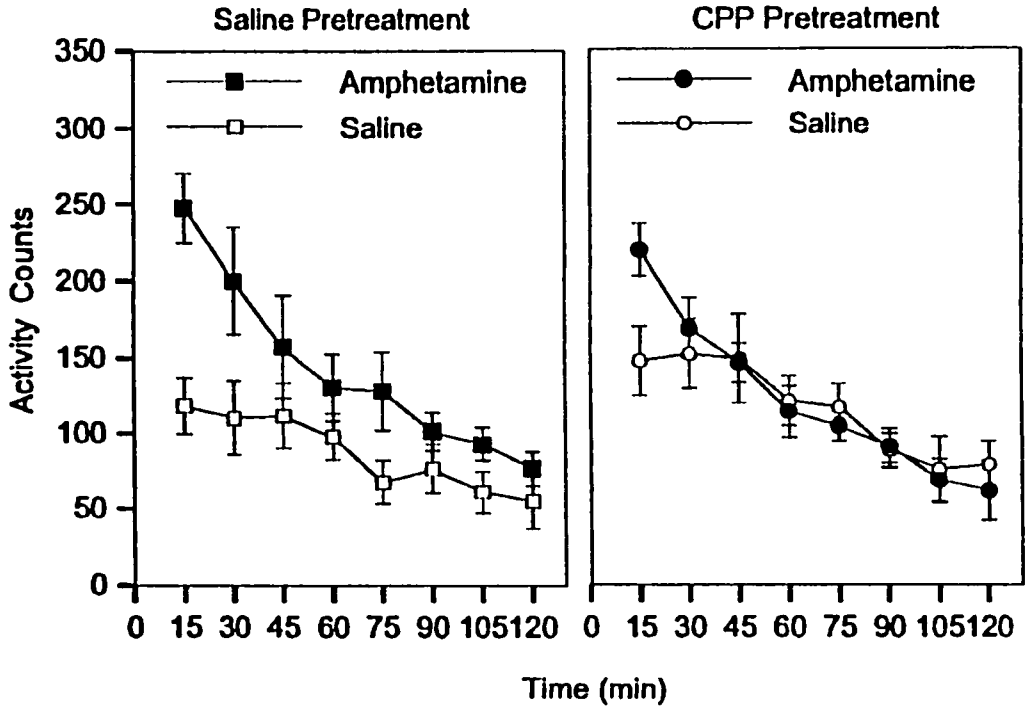
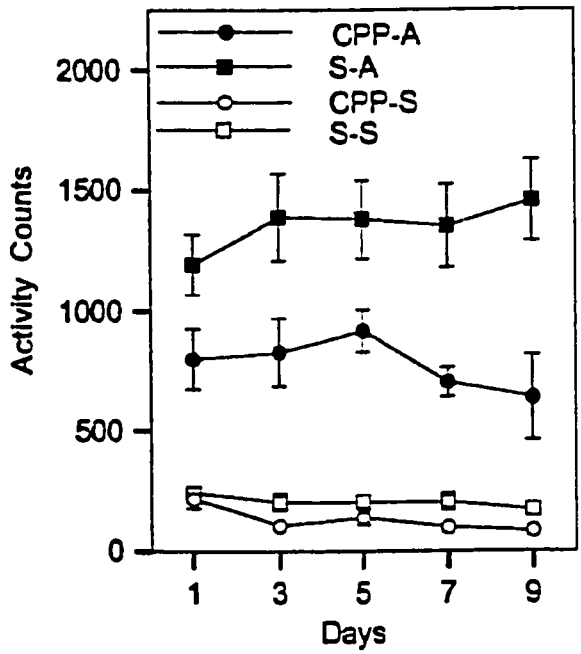

Saline Pretreatment
CPP Pretreatment
Amphetamine
Saline
Amphetamine
Saline
Activity Counts
350
300
250
200
150
100
50
0
0 15 30 45 60 75 90 105120
0 15 30 45 60 75 90 105120
Time (min)

Fig 3.4. Mean ± SEM activity counts during the induction phase. Animals were injected i.p. with CPP or saline before each amphetamine (A) or saline (S) injection (N=6/group). A mixed-design 3-way ANOVA (amphetamine and CPP as between factors; days as repeated measure) revealed: significant amphetamine treatment ($F(1, 80) = 107.9$, $p = .0001$); significant amphetamine by CPP interaction ($F(1, 80) = 8.4$, $p = .008$). A 2-way ANOVA revealed significant difference between CPP-saline and saline-saline groups ($F(1,40) = 8$, $p = .01$).


CPP-A
S-A
CPP-S
S-S
2000
1500
1000
500
0
Activity Counts
1
3
5
7
9
Days

bFGF Expression:

Animals that received amphetamine injections during the induction phase, developed behavioral sensitization (Fig. 3.3 left panel), and, as depicted in Fig. 3.5a, exhibited elevated number of bFGF-immunoreactive astrocytes in the VTA and SNc. However, animals that received both CPP and amphetamine pretreatment in the induction phase, did not develop behavioral sensitization (Fig. 3.3 right panel), and did not exhibit increased bFGF expression. In agreement with the previous results, no differences in bFGF expression were observed in dopaminergic terminal regions within NAcc core, NAcc shell and STR (Fig. 3.6)

Qualitative analysis revealed that, in all areas examined, bFGF immunoreactivity was confined to the nuclei of small cells, presumably glia. These observations are in agreement with the previous findings showing, by means of double-label immunohistochemistry, that the antibody used in this study detects bFGF immunoreactivity in astrocytic nuclei but not in dopaminergic neurons.

Correlation between bFGF expression and behavioral sensitization:

As shown in Figure 3.5b, in the group of animals that received amphetamine alone during the induction phase, highly significant positive correlations ($r = 0.9$) were found between locomotor activity induced by the amphetamine challenge during the sensitization test and the number of bFGF immunoreactive astrocytes in the VTA and SNc. No significant correlations

Fig. 3.5 Effects of CPP on bFGF expression in dopaminergic cell body regions. **a**: mean ± SEM bFGF-labeled cells in each group as a percentage of the saline-saline group. ANOVAs performed on raw scores: VTA, $F(3,19) = 3.9$, $p = .02$; SNc, $F(3, 19) = 2.1$, $p = .13$. In VTA, * significantly different from all other groups; in SNc, † significant difference between CPP-amphetamine and saline-amphetamine (ps <.05). **b, c, d** and **e**: correlations between activity counts during the first 60 min of the sensitization test (see Fig. 3) and number of bFGF-labeled cells in each of the groups. * ps <.05. Abbreviations: S-S, saline-saline; S-A; saline-amphetamine; CPP-A, CPP-amphetamine; CPP-S, CPP-saline.

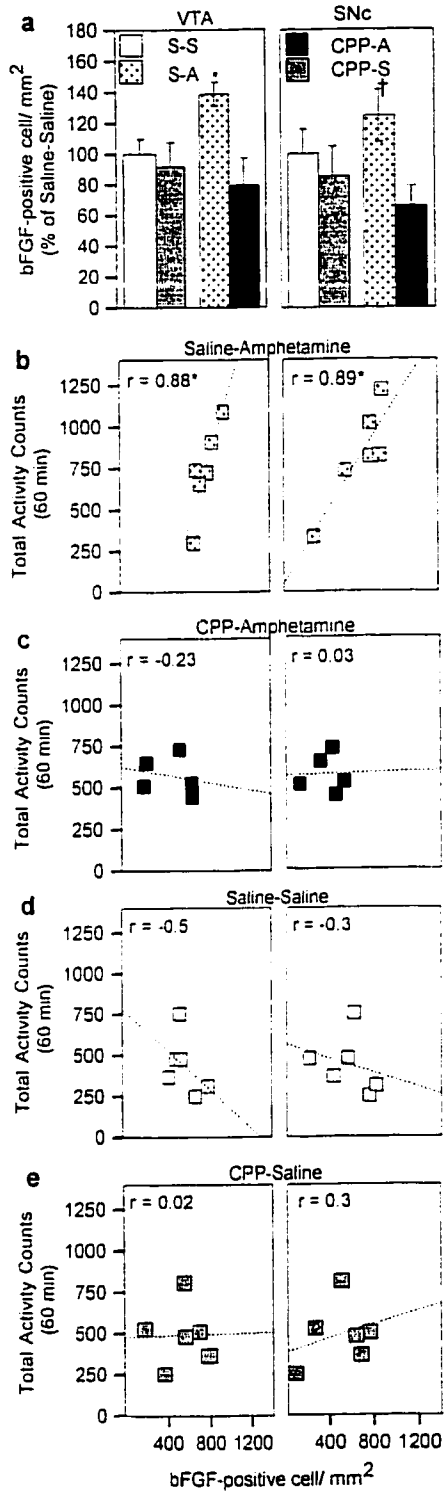

a
VTA
SNc
S-S
S-A
CPP-A
CPP-S
bFGF-positive cell/ mm2
(% of Saline-Saline)
b
Saline-Amphetamine
r = 0.88*
r = 0.89*
Total Activity Counts
(60 min)
c
CPP-Amphetamine
r = -0.23
r = 0.03
d
Saline-Saline
r = -0.5
r = -0.3
e
CPP-Saline
r = 0.02
r = 0.3
400 800 1200
bFGF-positive cell/ mm2

Fig. 3.6. Effects of CPP on bFGF expression in dopaminergic terminal regions. **a**: mean ± SEM bFGF-labeled cells in each group as a percentage of the saline-saline group. Abbreviations: S-S, saline-saline; S-A; saline-amphetamine; CPP-A, CPP-amphetamine; CPP-S, CPP-saline.

bFGF-positive cells per mm2
(% of Saline-Saline)
180
160
140
120
100
80
60
40
20
0
NAcc Shell
NAcc Core
STR
S-S
S-A
CPP-A
CPP -S

were found between locomotor activity and bFGF expression in the other groups (Fig. 3.5c, d & e).

3.3. Discussion

Two main findings emerged from this study. First, bFGF expression in dopaminergic cell body regions, but not in terminals, is increased in animals that exhibit behavioral sensitization. In response to an amphetamine challenge (.75 mg/kg i.p.) given one week after the last day of an induction phase (5 injections of saline or amphetamine, 1.5 mg/kg i.p., once every other day), animals that are treated with amphetamine in the induction phase show increased locomotor activity. Remarkably, the number of bFGF-positive astrocytes in the VTA and SNc of amphetamine-pretreated animals is strongly and positively correlated with the magnitude of locomotor activity elicited by the amphetamine injection during the sensitization test.

Second, both the increased expression of bFGF and the induction of behavioral sensitization depend on activation of NMDA receptors. Animals that are exposed to amphetamine in the presence of CPP in the induction phase, do not exhibit behavioral sensitization in response to amphetamine challenge; nor do they show increased bFGF immunoreactivity in the VTA and SNc. The effects of CPP on both sensitization and bFGF expression are dose dependent.

These results demonstrate that the long-lasting increase in sensitivity to the locomotor effects of amphetamine that follows repeated exposure is

positively related to the number of bFGF-immunoreactive astrocytes in dopaminergic cell body regions. These findings support the idea that bFGF is implicated in drug-induced neuronal plasticity and suggest that glutamate's role in the development of sensitization is mediated by bFGF.

bFGF Expression in Dopaminergic Cell Body Regions as an Index of the Magnitude of Sensitization

Because the effects of a single amphetamine injection on bFGF expression have not been examined, one could speculate that bFGF immunoreactivity in the VTA and SNc measured in Experiment 2 reflects the effectiveness of the amphetamine challenge to produce locomotor activity rather than the magnitude of sensitized responding that developed during, and perhaps after, the induction phase. In the present experiment, animals were perfused after the sensitization test, 2-3 h after receiving an acute injection of amphetamine. Thus, there might have been enough time for bFGF synthesis to be induced. In fact, increased astroglial bFGF immunoreactivity in the VTA and SNc has been reported as early as 2 h after an injection of 6-OHDA (Chadi et al., 1994).

If bFGF expression on the test day were merely an effect of the acute amphetamine injection, then positive correlations between bFGF immunoreactivity and locomotor activity should be observed in all groups (remember that in the sensitization test all animals received amphetamine). However, there were *no* significant correlations between bFGF expression and

locomotor activity during the test day in the group that did not develop behavioral sensitization either because rats were pre-treated with both CPP and amphetamine (CPP-amphetamine group), or because animals were receiving amphetamine for the first time (CPP-saline and saline-saline groups).

The results from this study, therefore, clearly show that the higher levels of bFGF seen in the VTA and SNc in animals that developed behavioral sensitization are not responsible for the high activity scores elicited by the amphetamine challenge on the test day, but reflect underlying cellular processes subserving the development of sensitization.

During the induction phase of this study, i.p. injections of CPP reduced the acute locomotor-activating effects of amphetamine. It is unlikely, however, that this effect was responsible for the lack of sensitization seen on the test day. Considerable evidence shows that increased locomotor activity in response to amphetamine injections during the induction phase is not required for the development of sensitization. Intra-VTA injections of amphetamine do not increase locomotor activity, but are sufficient to induce sensitized behavioral or neurochemical responding to subsequent systemic injections; conversely, amphetamine injections into the NAcc that induce locomotor activity do not lead to the development of sensitization (Cador et al., 1995; Kalivas & Weber, 1988; Vezina, 1993; Vezina, 1996; Vezina & Stewart, 1990). Finally, blockade of the acute effects of amphetamine on locomotor activity is not sufficient to prevent the development of sensitized

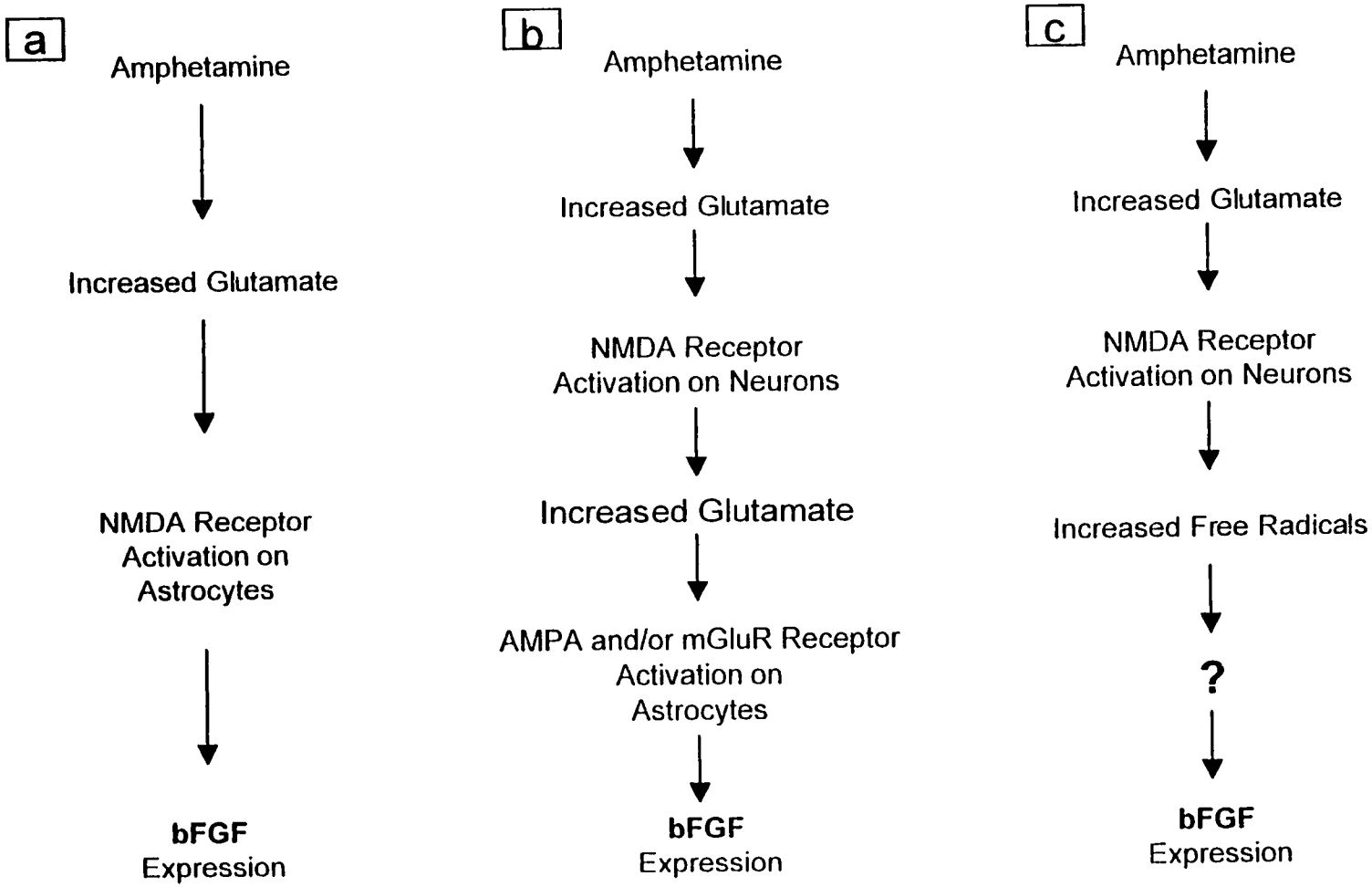
responding (Stewart, Deschamps, & Amir, 1994; Vezina, 1996). Because in the present study CPP was injected systemically, its effects on amphetamine induced locomotor activity may have resulted from actions of this drug on dopaminergic terminal regions. Intra-striatal, but not intra-VTA, injections of CPP have been shown to block the acute locomotor-activating effects of amphetamine (Cador et al., 1999; Wolf, 1998)

NMDA Receptor Activation and Amphetamine-Induced bFGF

One question that arises from these findings is how CPP pretreatment prevents the increases in bFGF. As mentioned earlier, systemic and intra VTA NMDA receptor antagonists, including CPP, block the development of sensitization to amphetamine (Cador et al., 1999; Vezina and Queen, 2000; Jeziorski et al., 1994; Karler et al., 1989; Karler et al., 1990; Stewart & Druhan, 1993; Wolf & Jeziorski, 1993; Wolf & Khansa, 1991). The effects of CPP on bFGF expression in the VTA and SNc are very likely to be mediated by blockade of local NMDA receptors.

If glutamate activation of NMDA receptors in midbrain dopaminergic regions mediates the effects of amphetamine on astrocytic bFGF, the question to be asked is: How does the activation of NMDA receptors in the VTA result in increased astrocytic bFGF expression. As shown in Fig. 3.7a, the most straight forward explanation is that the NMDA receptors responsible for this effect are located on astrocytes and that their activation stimulates bFGF synthesis. There is evidence showing that glutamate induces bFGF mRNA

Fig. 3.7. Schematic representation of some of the possible mechanism mediating the effects of NMDA receptor activation in the VTA on bFGF expression. **a**, NMDA receptors may be located on astrocytes and their activation may result in increased bFGF expression. **b**, Activation of NMDA receptors located on VTA neurons may induce further increases in extracellular glutamate which, in turn, could activate non-NMDA receptors expressed by astrocytes and lead to increased bFGF expression. **c**, Activation of NMDA receptors located on VTA neurons may induce further increases in extracellular glutamate which, in turn, may results in increased production of free radicals. These molecules could then induce, by unknown mechanisms, bFGF expression.


a
Amphetamine
Increased Glutamate
NMDA Receptor Activation on Astrocytes
bFGF Expression
b
Amphetamine
Increased Glutamate
NMDA Receptor Activation on Neurons
Increased Glutamate
AMPA and/or mGluR Receptor Activation on Astrocytes
bFGF Expression
c
Amphetamine
Increased Glutamate
NMDA Receptor Activation on Neurons
Increased Free Radicals
?
bFGF Expression

expression in cultured astrocytes (Pechan et al., 1993), and, furthermore, NMDA receptors have been visualized on astrocytes in vivo in adult rat hippocampus and cortex (Conti, DeBiasi, Minelli, & Melone, 1996; Gottlieb & Matute, 1997). However, results from electrophysiological studies have raised concerns about whether astrocytic NMDA receptors are functional (for discussion see Conti, Minelli, DeBiasi, & Melone, 1997; Steinhauser & Gallo, 1996).

Alternatively, activation of NMDA receptors located on VTA neurons may indirectly induce bFGF expression in astrocytes (see Fig. 3.7b). Recent evidence showing that amphetamine-induced VTA glutamate efflux is prevented by coinjection of an NMDA antagonist (Wolf & Xue, 1999) suggests that NMDA activation increases VTA glutamatergic release in a feed-forward manner. In addition, astrocytes are known to express AMPA and mGluR and, in contrast to NMDA receptors, their activation has been clearly shown to have functional consequences, including increases in astrocytic intracellular Ca^{++} concentration (Bezzi et al., 1998; Steinhauser & Gallo, 1996). Finally, astrocytes express glutamate transporters that are known to be responsible for most synaptic glutamate uptake (Bergles & Jahr, 1998; Duan et al., 1999). It is possible that amphetamine-induced extracellular glutamate in the VTA stimulates bFGF synthesis in astrocytes via activation of non-NMDA receptors, but that in the presence of NMDA antagonists this effect is attenuated because the rise in extracellular glutamate is blocked.

Finally, NMDA receptor activation in the VTA may induce astrocytic bFGF expression through production of free radicals (see Fig. 3.7c). Acute amphetamine injections cause increases in glutamate release in the VTA which is known to promote the production of reactive oxygen species (for review see Simonian & Coyle, 1996). Interestingly, free radicals have been shown to induce bFGF gene expression in cultured astrocytes (Pechan et al., 1992). Thus, because NMDA antagonists blocked amphetamine-induced glutamate release in the VTA (Wolf & Xue, 1999), these drugs are likely to prevent increases in free radicals, and as a consequence, bFGF expression. The effects of antioxidant agents on amphetamine-induced increases in astrocytic bFGF expression should be explored.

Amphetamine-Induced bFGF expression: Injury-Like Effect?

In the context of possible mechanisms underlying the effects of amphetamine on bFGF expression, the results of this study support the idea that increased glutamatergic activity elicited by amphetamine may place excessive demands on the functioning of dopaminergic neurons and, in turn, recruit regulatory and neuroprotective processes such as increased expression of bFGF. In adult animals, bFGF expression is increased after injury to midbrain dopamine neurons (Chadi et al., 1994) and appears to participate in survival and sprouting of injured neurons (Chadi et al., 1993; Kawamata et al., 1997). Although, amphetamine treatment has not been associated with significant reductions of dopamine and metabolites in terminal regions seen

after partial lesions within the dopaminergic system (Robinson, Jurson, Bennett, & Bentgen, 1988) repeated administration of stimulant drugs can induce injury-like changes in the VTA such as increased GFAP immunoreactivity, decreased expression of neurofilament proteins, and decreased dopaminergic cell size (Beitner-Johnson et al., 1992; Beitner-Johnson et al., 1993; Sklair-Tavron et al., 1996).

Interestingly, recent work from Jane Stewart's laboratory has shown that the behavioral and neurochemical recovery seen two to four weeks after partial 6-OHDA lesions of nigrostriatal dopamine neurons (Castañeda, Whishaw, & Robinson, 1990; Robinson, Mocsary, Camp, & Whishaw, 1994; Robinson & Whishaw, 1988) can be blocked by daily injections of NMDA antagonists given during the first week after injury (Emmi, Rajabi, & Stewart, 1996). These findings suggested that the compensatory changes in the remaining dopamine neurons seen after partial lesions are stimulated, at least in part, by glutamatergic activity. These results led to the proposal that common mechanisms might underlie the development of sensitization and the development of compensatory changes in dopaminergic neurons remaining after the lesions. This idea is made more compelling by the fact that after both 6-OHDA lesions and repeated exposure to amphetamine there is a sustained increase in astrocytic bFGF immunoreactivity in the SNc and VTA.

In summary, the current study shows that repeated administration of amphetamine results in increased expression of astrocytic bFGF in

dopaminergic cell body regions and that this effect is dependent on activation of NMDA receptors. The effects of amphetamine on bFGF induction are observed in animals that exhibit behavioral sensitization and a high positive correlation exists between bFGF expression and the magnitude of sensitization. Both effects are prevented by CPP treatment.

CHAPTER IV

REQUIREMENT OF ENDOGENOUS bFGF FOR SENSITIZATION TO AMPHETAMINE

4.1. Effects of bFGF Immunoneutralization in the VTA on the Development of Behavioral Sensitization

4.1.1. Introduction

In the previous chapters I have presented evidence suggesting that astrocytic bFGF in dopaminergic cell body regions is implicated in the mechanisms underlying the development of sensitization to the effects of amphetamine. I have shown that repeated administration of amphetamine results in long-lasting increases in astrocytic expression of bFGF in cell body regions of midbrain dopaminergic cells, but not in terminal zones. I have shown results that demonstrate that the number of bFGF-positive astrocytes in the VTA and SNc not only is increased in animals that exhibit behavioral sensitization, but is also significantly and positively correlated with the magnitude of sensitization induced. Finally, I have shown that both the development of sensitization to amphetamine and the enhanced expression of bFGF are dependent on activation of NMDA receptors, suggesting that glutamate may be playing a role in the induction of long-lasting changes in the responding to stimulant drugs via bFGF effects. What remains to be proved, therefore, is whether bFGF actions in the cell body region of midbrain dopaminergic cells are necessary for sensitization to develop.

To directly test for an obligatory action of bFGF in the development of sensitization to amphetamine, it was decided to block the activity of bFGF in the VTA using the same antibody that has been used to detect changes in bFGF immunoreactivity in all the experiments presented in this thesis. This antibody, which recognizes native protein, has been shown to specifically block endogenous bFGF activity *in vitro* (Matsuzaki et al., 1989) and *in vivo* (Tao, Black, & DiCicco-Bloom, 1997). Bilateral intra-VTA microinfusions of the neutralizing antibody to bFGF were made during the induction phase of sensitization to amphetamine. The consequences of these infusions on the development of behavioral sensitization to amphetamine were examined in tests made on the expression of sensitization given one and two weeks after the last drug injection. Because the bFGF antibody is produced in mice and belongs to the class of immunoglobulin G (IgG), as a control, a solution of mouse IgG proteins was microinfused.

The results from this study have been published (Flores et al., 2000).

4.1.2. Materials and Methods

4.1.2.1. Subjects

Male Wistar rats (Charles River, Quebec; 325-350g), housed in a colony room on a normal light-dark schedule with free access to food and water, served as subjects.

4.1.2.2. Drugs and Antibodies

The bFGF antibody used in this study was a gift from Dr. K. Nishikawa at Kanazawa Medical University, Japan, and is the same antibody used in all the experiments described in this thesis. This mouse monoclonal antibody specifically recognizes the biologically active conformation of bFGF and has been shown to be an effective immunoneutralization reagent both *in vitro* and *in vivo* (Matsuzaki et al., 1989; Tao et al., 1997). Mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) was used as control. Antibodies were administered to unrestrained rats (0.5 mg/ml in 0.9% saline, 0.5 µl/side, over 60 sec). The dose used was based on pilot work, on previous in vivo studies with bFGF antibodies (Tao et al., 1997) and on the advice of Dr. Nishikawa. No obvious behavioral alterations were detected after intra-VTA infusions with either of the protein solutions, nor were there any effects on body weight. D-amphetamine sulphate (SmithKline Beecham Pharma) was dissolved in saline and injected i.p.

4.1.2.3. Surgery

Rats were anesthetized with sodium pentobarbital (65 mg/kg i.p.) after being given subcutaneous atropine (0.25 mg/kg,) to reduce bronchial secretions and were fixed in a stereotaxic apparatus. With stereotaxic arms angled at 15^{0} off the sagittal plane, 22-gauge guide cannulae were bilaterally implanted into the VTA, 5.3 mm posterior from bregma, 2.8 mm lateral from the mid-sagittal sinus, and 6 mm below the dura (Paxinos & Watson, 1997).

Stainless-steel obturators (28-gauge) were inserted into the guide cannulae, extending 2 mm beyond the tip. The guide cannulae were held to the skull with jeweler's screws and dental acrylic cement. At the end of the surgery, all animals were injected intramuscularly with 0.1 ml of antibiotic (Penlong Rogar/STB). Rats were allowed to recover for two weeks before experiments began.

4.1.2.4. Microinfusions

Intra-VTA microinfusions were performed by inserting, into the guide cannulae, 28-gauge injector cannulae that extended 2 mm beyond the tip. Injector cannulae were held in place in the guide cannulae by a brass screw cuff and were connected via polyethylene tubing to 1-µl Hamilton syringes. Antibodies were administered in unrestrained rats in a volume of 0.5 µl/side over 60 sec. The injectors remained in place for 120 sec before and after antibody infusions. Obturators were immediately replaced after removal of the injectors. Injector cannulae and obturators were wiped with 70% ethanol and dried immediately before being inserted into the guide cannulae.

4.1.2.5. Apparatus

The locomotor activity setup was as described in section 3.2.2.2.

4.1.2.6. Immunohistochemistry

For detection of intracranially administered antibodies in brain tissue, some animals were perfused transcardially in exactly the same manner as

described in section 2.1.3.3. When the perfusion was completed, the brains were removed and placed overnight in the fixative solution at 4 ^{0}C. Coronal sections, 50 µm thick, were cut on a vibratome and were rinsed 3 times (5 min each) in PB. Free-floating tissue sections were process for immunohistochemistry using the ABC method (Hsu et al., 1981) in the same manner as described before, but omitting incubation with primary antibody (which had already been microinjected into the VTA). For control purposes, some tissue sections were incubated in the avidin-horseradish peroxidase complex, but not in the secondary antibody solution, and no labeling was obtained. Processed sections were mounted onto gelatin-coated slides and were coverslipped with Permount in the same manner as described (section 2.1.2.4).

4.1.2.7. Histology

After completion of the experiment, rats were injected with a lethal dose of sodium pentobarbital and were perfused with 0.9% saline followed by 10% formalin. Brains were removed and stored in 10% formalin. After formalin fixation, and a 24 h incubation period in 20% sucrose formalin, 30 µm coronal sections were cut on a cryostat and mounted on gelatin-coated slides. Sections stained with 0.1% cresyl violet to reveal anatomical landmarks. The location of injector tips were identified using Paxinos and Watson (1997) stereotaxic atlas.

4.2. Statistical Analysis

Data were analyzed by 1- and 2-way ANOVAs as required. Post hoc comparisons were made using 1-way ANOVAs or Fisher's protected LSD test ($p \leq .05$)

4.3 Procedures

Before the start of the induction phase of sensitization, a pretest was done to ensure that the treatment groups did not differ in spontaneous activity levels. All rats were taken to the activity room for the first time and placed in the activity boxes where their activity was monitored for 2 h. Activity counts were obtained by recording the number of interruptions of horizontal photocells beams. Animals were then assigned to matched groups on the basis of these original scores (four different groups: antibFGF-amphetamine, antibFGF -saline, IgG-amphetamine, and IgG-saline).

Induction Phase:

On day 1 of the induction phase, in order to assess any possible adverse effects of the antibodies, rats received bilateral VTA microinfusions of the bFGF antibody or mouse IgG, in the colony room and were placed back in their home cages. On days 3, 5, 7 and 9, rats were given similar intra-VTA infusions of either bFGF antibody or mouse IgG and, 1 h later, were taken to the activity monitoring room where they were injected i.p. with either saline

or amphetamine (1.5 mg/kg) and placed immediately in the activity boxes for 2 h.

Test:

To determine whether pretreatment with neutralizing antibody to bFGF blocked the development of sensitization to amphetamine, tests were conducted one and two weeks after the last day of the induction phase. For these tests all animals, whether exposed previously to amphetamine or saline, were given a single i.p. injection of 0.75 mg/kg amphetamine and were placed immediately in activity boxes for 2 h. For these tests no infusions of antibodies were given. The dose of amphetamine given during the induction phase is one that increases locomotion and, after repeated administration, stereotypy. For the test phase, therefore, the dose was halved to reveal primarily locomotion.

Antibody Detection

To rule out the possibility that the effect on the test day would result from residual antibody in the VTA the presence of antibody in the brain was detected using immunohistochemistry. Groups of animals (n=2/group) were infused with anti-bFGF antibody or with mouse IgG either 1 h or one week before perfusion. Their brains were processed for immunoreactivity.

4.4. Results

Sensitization Test:

Immunoneutralization of bFGF during the induction phase completely blocked the development of sensitization to amphetamine (Fig. 4.1, right panel). On the test day, animals that had been exposed to amphetamine in the presence of VTA infusions of the bFGF antibody during the induction phase responded to amphetamine challenge in a manner similar to that of animals previously exposed to saline. In contrast, animals that, during the induction phase, had been exposed to amphetamine, in the presence of the control infusions of mouse IgG during the induction phase, showed sensitized responding on the test day (Fig. 4.1, left panel). Locomotor activity in these animals, in response to the single injection of amphetamine, was significantly greater than that seen in animals given amphetamine for the first time. Similar effects were seen in a second test given one week later.

To rule out the possibility that the lack of sensitization observed on the test days resulted from residual antibody in the VTA at the time of test, immunohistochemistry was used to determine whether the bFGF antibody was present in the VTA of animals that had been infused either 1 h or one week before perfusion. As shown in Fig. 4.2, immunoreactivity for the bFGF antibody was evident and localized in the VTA 1 h after infusion, but was undetectable in animals that had received infusions one week earlier; similar results were found after IgG infusions.

Fig. 4.1. Test for sensitization: All animals received 0.75 mg/kg amphetamine, i.p. before being placed in the activity boxes. Left panel: mean ± SEM activity counts in animals exposed during the induction phase to amphetamine or saline in the presence of control intra-VTA infusions of mouse IgG. Right panel: activity counts in animals exposed during induction to amphetamine or saline in the presence of the bFGF antibody. Two-way ANOVA for pretreatment (bFGF antibody vs. IgG) by drug (amphetamine vs. saline) revealed significant main effect of antibody ($F(1,16) = 6.0$, $p = .02$), significant main effect of drug ($F(1,16) = 26.5$, $p = .0001$) and a significant interaction ($F(1, 16) = 8.9$, $p = .008$). One-way ANOVAs revealed significant differences between amphetamine and saline groups that were pretreated with mouse IgG during the induction phase ($F(1, 8) = 41.6$, $p = .0002$) and between the two groups previously exposed to amphetamine ($F(1, 6) = 11.3$, $p = .01$). The two saline groups did not differ ($F(1,10) = .20$, ns). Amphetamine: n= 4/group; saline: n = 6/group.

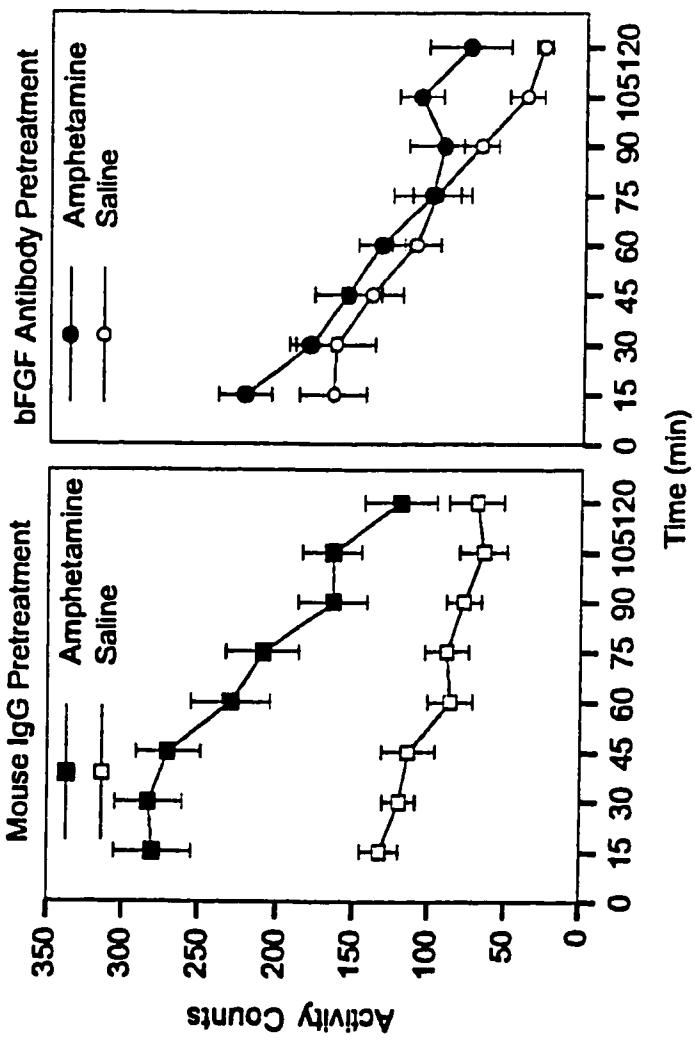

Mouse IgG Pretreatment
bFGF Antibody Pretreatment
Amphetamine
Saline
Activity Counts
Time (min)
0
50
100
150
200
250
300
350
0 15 30 45 60 75 90 105120

Fig. 4.2. Digitized images showing bFGF-immunoreactivity in the VTA of animals that received bilateral intra-VTA microinjections of the neutralizing antibody for bFGF, 1 h or 1 week before perfusion.

1 week
1h

Cresyl violet staining confirmed that all microinfusions were made into the VTA (Fig. 4.3) and no detectable differences were found in tissue damage (glial scar) between brain sections of rats given mouse IgG or anti-bFGF antibody.

Induction Phase:

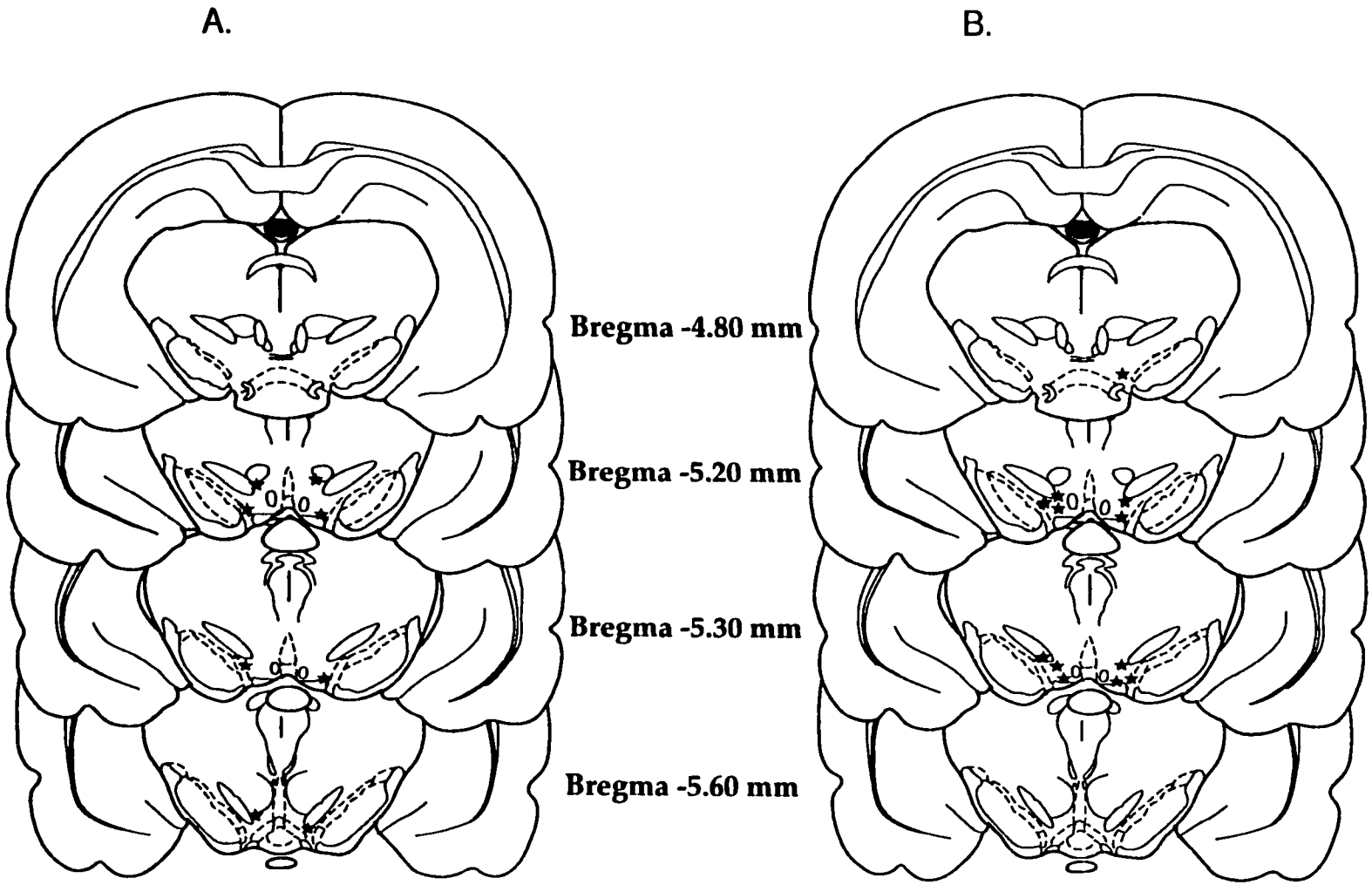
Fig. 4.4 shows the total activity counts recorded in the test chambers during each of 5 days of the induction phase. A clear locomotor-activating effect of each amphetamine injection can be seen in animals that had been microinfused with either bFGF antibody or IgG 1 h before. However, the effects of amphetamine on locomotor activity were significantly decreased by the bFGF antibody.

4.5. Discussion

4.5.1. bFGF Actions in the VTA are Necessary for the Development of Sensitization to Amphetamine

Blockade of bFGF activity in the VTA during the period of repeated exposure to amphetamine (the induction phase) was sufficient to prevent the development of sensitized responding to amphetamine. On the tests for sensitization, given one and two weeks following the induction phase, animals previously exposed to amphetamine in the presence of the bFGF antibody, showed no evidence of sensitized responding to amphetamine. These findings show that bFGF in the VTA is directly involved the

Fig. 4.3. Placements of injector tips for animals with VTA cannulae implants reconstructed onto tracings of coronal plates from Paxinos and Watson (1997). **A**, animals from bFGF-antibody-amphetamine group; **B**, animals from bFGF-antibody-saline group; **C**, animals from mouse IgG-amphetamine group; **D**, animals from mouse IgG-saline group.


A.
B.
Bregma -4.80 mm
Bregma -5.20 mm
Bregma -5.30 mm
Bregma -5.60 mm

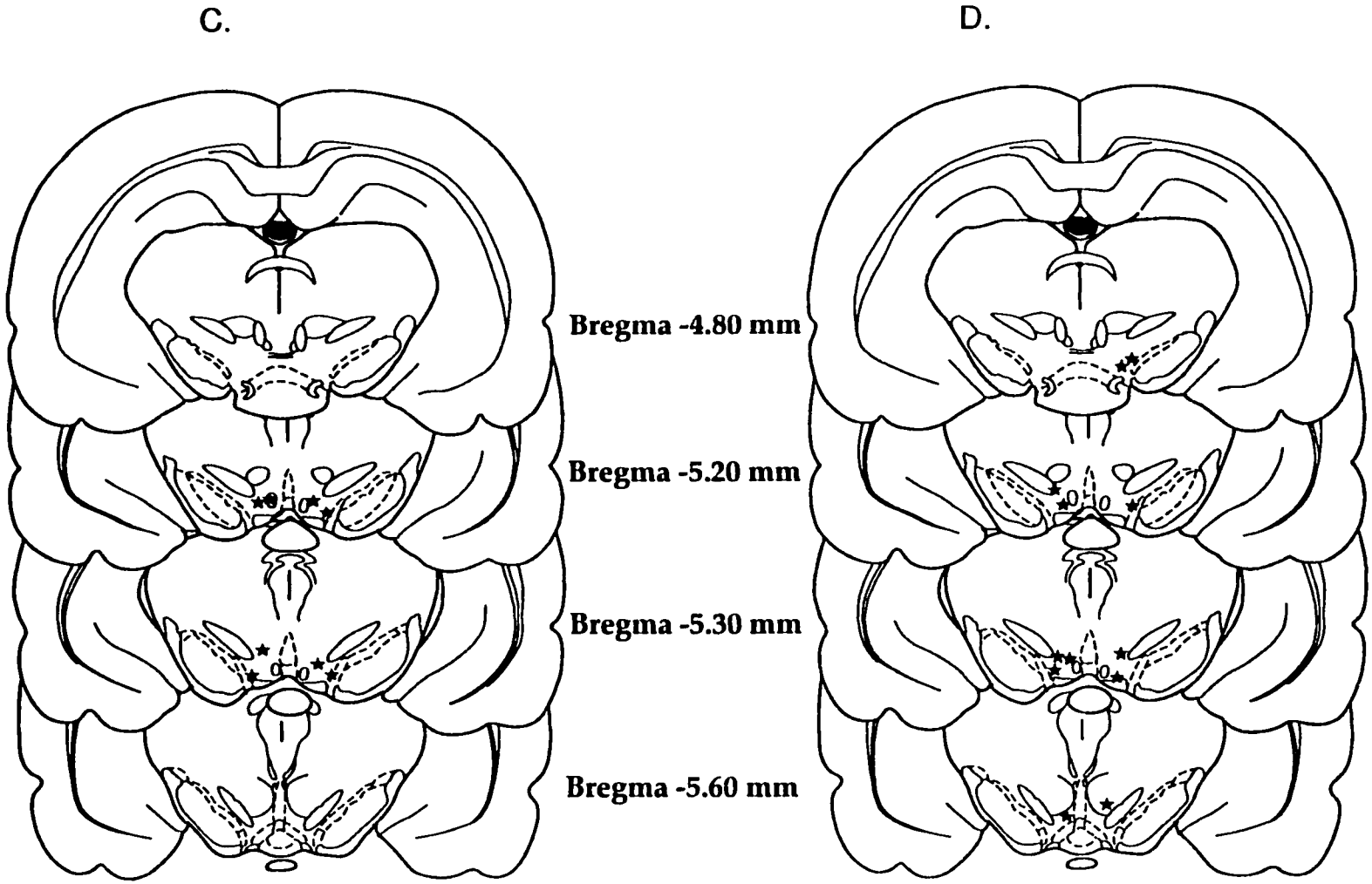
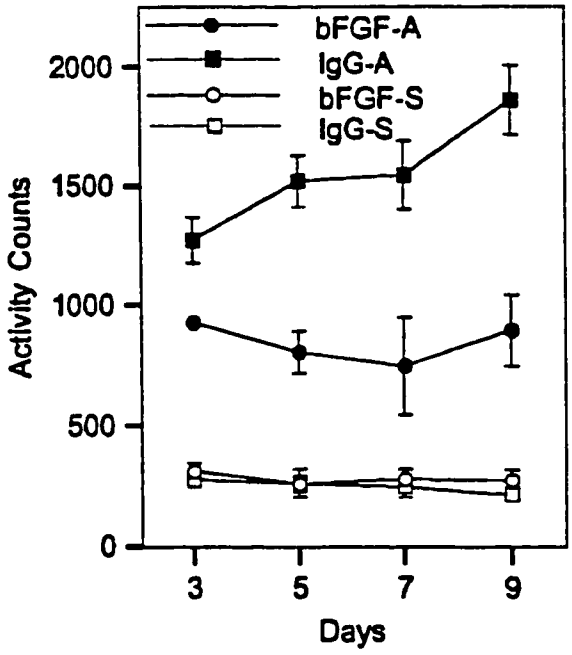

C.
D.
Bregma -4.80 mm
Bregma -5.20 mm
Bregma -5.30 mm
Bregma -5.60 mm

Fig. 4.4. Mean ± SEM activity counts during the induction phase. Animals were infused intra-VTA with bFGF antibody or mouse IgG before each amphetamine (A, n= 4/group) or saline (S, n=6/group) injection. A mixed-design 3-way ANOVA (antibody and amphetamine as between factors; days as repeated measure) revealed: significant amphetamine treatment $F(1, 48) = 287.6$, $p = .0001$); significant amphetamine by antibody interaction ($F(1, 48) = 45.1$, $p = .0001$).


bFGF-A
IgG-A
bFGF-S
IgG-S
2000
1500
1000
500
0
Activity Counts
3
5
7
9
Days

development of sensitization to amphetamine. This is the first demonstration that an endogenous neurotrophic factor is required for the development of sensitization to stimulant drugs and implies that the development of the enduring changes in neurochemistry, neuron morphology, and behavior that result from repeated exposure to stimulants may arise from the actions of neurotrophic factors induced by these drugs.

As shown in Fig. 4.5, the effects of the bFGF antibody on the development of sensitization were strikingly similar to those observed in the CPP experiment described in Chapter 3 . Because NMDA receptor activation is required for amphetamine-induced bFGF immunoreactivity, the present results make it likely that the effects of glutamate on the development of sensitization are mediated by enhancement of bFGF expression in the VTA. This idea receives support from evidence showing that repeated injections of amphetamine into the VTA are sufficient to induce sensitization (Bjijou et al., 1996; Cador et al., 1999; Cador et al., 1995; Hooks et al., 1993; Kalivas & Weber, 1988; Vezina, 1993; Vezina, 1996; Vezina & Stewart, 1990); that systemic and intra-VTA amphetamine increase glutamate release in the VTA (Wolf & Xue, 1998; Wolf & Xue, 1999; Xue et al., 1996); and that intra-VTA injections of NMDA antagonists block the development of sensitization to amphetamine (Cador et al., 1999; Vezina and Queen, 2000).

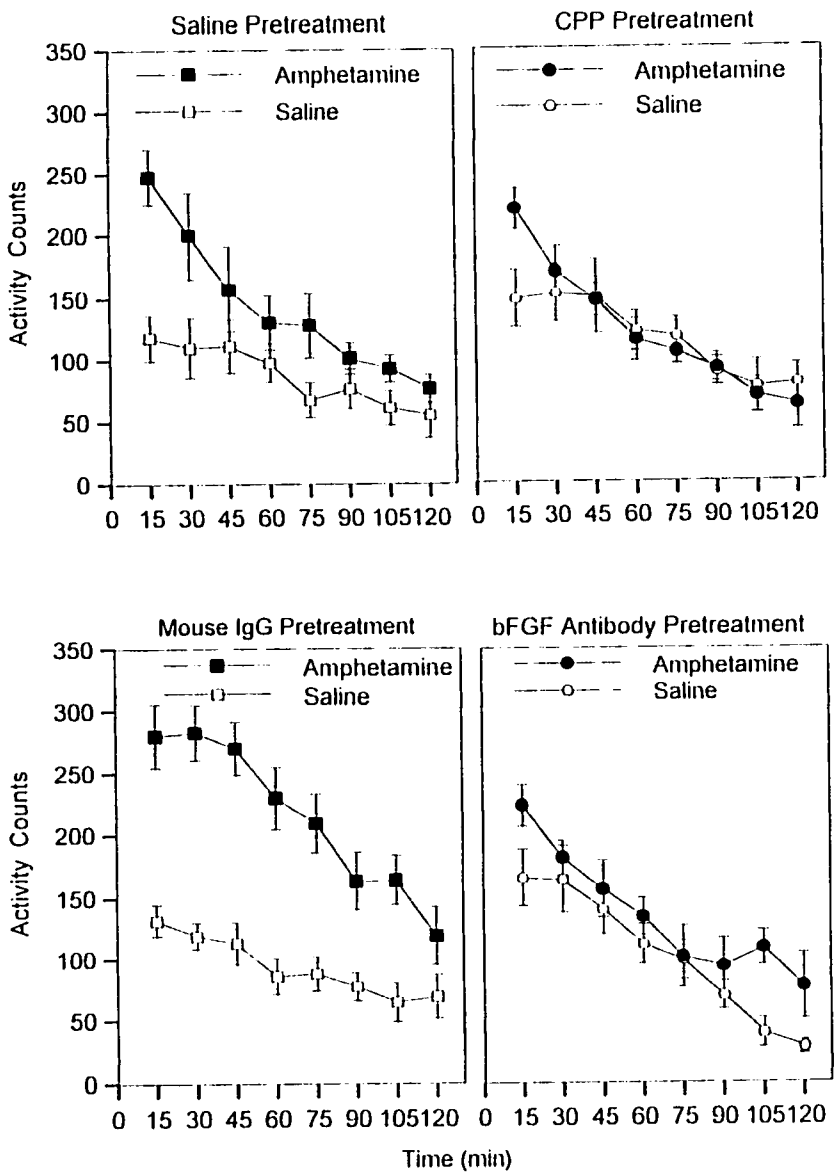
The finding that bFGF actions in the VTA are necessary for the development of sensitization to amphetamine may be related to the finding reported by Pierce et al. (1999) that intra-VTA microinfusions of an inhibitor

of the Ras-MAP signal transduction pathway given just before three daily injections of cocaine prevent the development of sensitized responding to subsequent cocaine challenge. bFGF acts on specific receptor tyrosine kinases, stimulation of which has been shown to activate the Ras-MAP pathway (Chew et al., 1997; Hill & Treisman, 1995; Thomas, 1993). Its effects on the development of sensitization may be mediated, at least in part, via this pathway.

As mentioned in Chapter 1, the bFGF molecule lacks a secretory signal (short amino acid sequence that determines that the eventual location of a protein is a secretory vesicle). Although, bFGF has been shown to be released *in vitro* (Araujo and Cotman, 1992b; Florkiewicz et al., 1995), whether it is released from cells *in vivo* remains a debatable issue (see Thomas, 1993). Because IgGs do not readily gain access to the cytoplasm and, if they do, they are directly stored in peroxisomes and degraded (Alberts et al., 1994), the magnitude and specificity of the effects observed in the present study argue for the extracellular presence of bFGF and for its ability to be released.

There is, in fact, evidence that bFGF can be released from cells by an unconventional mechanism (Araujo & Cotman, 1992a; Florkiewicz et al., 1995). Furthermore, *in vitro* and *in vivo* studies have shown that the same bFGF antibody used in the present study neutralizes actions of endogenous bFGF that are assumed to be mediated by interactions with the transmembrane tyrosine kinase receptor. In cultures of cells extracted from bovine brain cortex (bovine capillary endothelial cells, BCE), basal growth or

Fig. 4.5. Similarities between the effects of infusions of bFGF antibody and systemic injections of CPP on the development of sensitization to the behavioral effects of amphetamine. See captions for Figs. 3.3 and 4.1.


Saline Pretreatment
CPP Pretreatment
Mouse IgG Pretreatment
bFGF Antibody Pretreatment
Amphetamine
Saline
Activity Counts
Time (min)
0
50
100
150
200
250
300
350
0 15 30 45 60 75 90 105120

that stimulated by exogenous bFGF, is inhibited, in a dose-dependent manner, by adding neutralizing antibody to bFGF (Matsuzaki et al., 1989). These results show that bFGF can be released from cells and that the particular amino acid sequence of the bFGF molecule recognized by the bFGF antibody overlaps with that one responsible for receptor-binding. In another study, Tao et al., (1997) showed that *in vitro* and *in vivo* administration of bFGF neutralizing antibody inhibits neuroblast proliferation, but that this effect is not observed if the antibody is heat inactivated before infusion. These findings, in agreement with ours, indicate that bFGF is secreted. Moreover, they suggest that the effects of the neutralizing bFGF antibody are mediated by blockade of bFGF-FGFR1 interactions.

As to why the presence of bFGF in the extracellular space has not been observed in any of the immunohistochemical studies presented, the most parsimonious explanation is that the perfusion and processing of the tissue does not allow for bFGF detection outside cells. It is known that when bFGF attaches to its low affinity receptor HSPG, which is found in the extracellular matrix (see Chapter 1), the FGF-HSPG complex that is formed, has a proteolytic cleavage sequence adjacent to the plasma membrane that, when cleaved, renders the FGF-HSPG a hydrophilic molecule free to diffuse (Thomas, 1993). Finally, it has been suggested that bFGF could be released following cell death or disruption of the integrity of the plasma membrane (see Thomas, 1993). The idea that the amphetamine regimen used in these studies would induce cell death seems far fetched (Robinson et al., 1988), but

perhaps transient alterations in the plasma membrane, that would allow the release of bFGF, do occur.

The results of this study show that during the induction phase, intra-VTA infusions of bFGF antibody diminished the acute effect of amphetamine on locomotor activity (Fig. 4.3). It is improbable that the lack of sensitization observed on the test days was caused by this effect, however. As discussed earlier, a substantial amount of evidence shows that increased locomotor activity in response to amphetamine injections during the induction phase is not required for the development of sensitization. For example, intra-VTA, injections of amphetamine induce sensitization, but do not have acute effects on locomotor activity (Cador et al., 1995; Hooks et al., 1993; Kalivas & Weber, 1988; Vezina, 1993; Vezina, 1996; Vezina & Stewart, 1990). Furthermore, blockade of the acute effects of amphetamine do not prevent the development of sensitized responding (Stewart et al., 1994; Vezina, 1996). How blockade of bFGF activity in the VTA alters the acute effect of amphetamine remains to be elucidated.

It is shown here, that an endogenous neurotrophic factor, bFGF, known to promote growth and survival of midbrain dopaminergic cells (Hou et al., 1997; Takayama et al., 1995) is directly involved in the development of sensitization of the locomotor effects of amphetamine. It is proposed that in response to amphetamine, increased extracellular glutamate activates astrocytic bFGF which, in turn, acts directly on neurons, or indirectly though

astrocytes (see below) to initiate long-lasting changes in sensitivity and connectivity.

In summary, it has been found that the endogenous astrocytic neurotrophic factor, bFGF, acting in dopaminergic cell body regions, plays a crucial role in the development of enduring behavioral changes that follow repeated amphetamine treatment. These findings provide new insight into the basis of the long-lasting consequences of repeated exposure to stimulant drugs and point to the similarities between the mechanisms underlying this and other examples of experience-dependent plasticity.

4.5.2. Similarities Between Sensitization to the Effects of Stimulant Drugs and Long-Term Potentiation

The results of this study provide evidence that the processes involved in the development of sensitization to stimulant drugs share many features with those events implicated in the induction of a compelling form of experience-dependent synaptic plasticity called long term potentiation (LTP). LTP, probably the most widely studied form of experience dependent plasticity in the mammalian brain, refers to the long-lasting, activity-dependent changes in the efficacy of synaptic communication. LTP was first discovered in the hippocampus when it was shown that repetitive brief high frequency stimulation of excitatory synapses caused an increased in synaptic strength that could last for hours or even days (Bliss & Lømo, 1973; Jeffrey, Abraham, Dragununow, & Mason, 1990; Lømo, 1966). This remarkable form of synaptic

plasticity has been observed in several brain regions including amygdala and visual cortex (Kirkwood, Rioult, & Bear, 1996; Rogan, Staubli, & LeDoux, 1997), has been observed in brain slices and *in vivo*, and may provide important clues about the cellular and molecular mechanisms whereby experience can induce changes in synaptic strength and perhaps changes in brain function.

The increased sensitivity to the effects of stimulant drugs that results from repeated exposure shares several features with LTP. The induction of both phenomena requires NMDA receptor activation (for reviews see Madison, Malenka, & Nicoll, 1991; Wolf, 1998) and Ca^{++} entry (Karler, Turkanis, Partlow, & Calder, 1991b; Lynch, Larson, Kelso, Barrionuevo, & Schottler, 1983; Malenka, Kauer, Zucker, & Nicoll, 1988; Yang, Tang, & Zucker, 1999); the development of sensitization and long-lasting LTP (days) require protein synthesis (Frey, Frey, Schollmeier, & Krug, 1996; Karler et al., 1993; Krug, Lossner, & Ott, 1984; Linden, 1996; Madison et al., 1991; Robinson, 1991; Sorg & Ulibarri, 1995); and both LTP and sensitization are associated with increases in dendritic spine density (Engert & Bonhoeffer, 1999; Robinson & Kolb, 1997; Robinson & Kolb, 1999). As mentioned before, GluR1 expression in the VTA is elevated after repeated stimulant drug treatment (Fitzgerald et al., 1996) and its overexpression results in sensitized responses to morphine (Carlezon et al., 1997). Similarly, increasing evidence shows elevated responsiveness of post-synaptic AMPA receptors following the

induction of LTP presumably due to alterations in GluR1 subunit (for recent reviews see Malenka & Nicoll, 1999; Nicoll & Malenka, 1999).

Because of the similarities between sensitization and LTP, it has been thought that the increased sensitivity to the effects of stimulant drugs may be brought about by changes in synaptic plasticity at excitatory synapses on VTA dopaminergic cells induced by repeated exposure to these drugs (see Wolf, 1998). Two studies have now shown that LTP can be induced at excitatory synapses on dopaminergic cells in the VTA and SNc. In a study conducted on adult rat sagittal slices of the midbrain, Clark and colleagues reported that LTP can be generated on excitatory synapses on dopaminergic cells in the SNc by applying tetanic stimulation to the subthalamic nucleus. This enhancement in synaptic strength was found to be dependent on NMDA receptor activation (Overton, Richards, Berry, & Clark, 1999). In another study, Bonci and Malenka examined excitatory synapses on VTA dopaminergic and non-dopaminergic neurons in rat VTA slices and found that excitatory synapses on dopaminergic, but not on nondopaminergic neurons can express NMDA receptor-dependent LTP (Bonci & Malenka, 1999). Because this experiment was conducted on horizontal slices and repeated stimulation was applied to excitatory efferents directly in the VTA, it was not possible to identify the source of excitatory efferents that were stimulated. However, one would presume that the majority of these efferents originate in the PFC because, as discussed previously (Chapter 1), the VTA is densely innervated by excitatory PFC terminals (Christie et al., 1985; Sesack & Pickel, 1992) whose integrity has

been shown to be a requirement for the development of sensitization to stimulant drugs (Cador et al., 1999; Wolf et al., 1995).

LTP-like changes have been observed after repeated administration of stimulant drugs. In vivo electrophysiological recordings have shown that iontophoretic administration of glutamate into the VTA increases the firing rate of midbrain dopaminergic neurons (e.g., White et al., 1995) and similar effects are seen with electrical stimulation of the PFC (e.g., Tong et al., 1996a; Tong, Overton, & Clark, 1996b; Tong et al., 1995). Following repeated cocaine or amphetamine treatments, VTA dopaminergic neurons become hyper-responsive to glutamate (White et al., 1995) and to PFC stimulation (Tong et al., 1995) because of increased sensitivity to AMPA receptor activation (Zhang et al., 1997c).

Interestingly, bFGF has been shown to increase the magnitude of LTP induced in hippocampal CA1 neurons when added to brain slices of adult rats during tetanic stimulation (Terlau & Seifert, 1990). Similarly, when infused intracranially to anaesthetized rats, bFGF has been found to facilitate the generation of LTP on hippocampal dentate gyrus neurons by subthreshold tetanic stimulation of the perforant path (Ishiyama et al., 1991). Thus, the findings showing that bFGF is expressed in the VTA after amphetamine treatment and is required for the development of sensitized responding to amphetamine, suggest that bFGF may be involved in the development and maintenance of LTP-like processes underlying sensitization. Our results provide further support for the idea that increased sensitivity to the effects of

stimulant drugs may be brought about by changes in synaptic plasticity at excitatory synapses on VTA dopaminergic cells induced by repeated exposure to these drugs. Whether immunoneutralization of bFGF prevents the induction of LTP at excitatory synapses on dopaminergic cells in the VTA remains to be determined.

4.5.3. bFGF and the Effects of Contextual Stimuli on Sensitization

Drug-induced bFGF expression may participate in the processes underlying the effects of contextual stimuli on sensitization. A large body of evidence has shown that the expression of behavioral sensitization readily comes under the control of the contextual stimuli with which the drug injections are explicitly paired. If, for example, one group of animals is given amphetamine injections in a distinctive environment, and saline injections in a different environment, the sensitized response to amphetamine will be seen only in the environment with which the drug was previously paired (for reviews see Stewart, 1992; Stewart & Vezina, 1988).

In the studies with bFGF, increased bFGF expression has been observed after amphetamine treatment under conditions in which animals received all drug injections in the home-cage environment and were killed in another environment after days or weeks, and when amphetamine injections were given in a distinctive environment and animals were killed immediately after being tested in that environment. Thus bFGF appears to be a direct effect of amphetamine, and not an effect that is dependent on the environment where the drug was originally given. Nonetheless, the induction of bFGF

expression by stimulant drugs, such as amphetamine, might help explain the fact that the expression of behavioral sensitization can become context dependent. Drug-induced bFGF expression may provide the conditions for the facilitation of the synaptic changes induced by the explicit pairing of the drug effects with the contextual stimuli.

CHAPTER V

CHANGES IN ASTROCYTIC bFGF EXPRESSION DURING AND AFTER PROLONGED EXPOSURE TO ESCALATING DOSES OF AMPHETAMINE

5.1. Introduction

The results from the study presented in the previous chapter show that endogenous bFGF in the VTA plays a critical role in the development of sensitization to amphetamine. However, the mechanisms whereby bFGF brings about long-lasting changes in the sensitivity to the effects of amphetamine are presently unknown. The long-lasting nature of the changes in dopaminergic function and behavior induced by repeated exposure to stimulant drugs suggest enduring and perhaps permanent neuroadaptations. Morphological changes in neurons and their consequent alterations in neuronal wiring could potentially mediate such long-term alterations in brain function. One could speculate, therefore, that bFGF participates in the processes underlying sensitization by inducing neurite outgrowth and, as a consequence, changes in neuronal connectivity.

In this last chapter, I present results of two experiments that were conducted to explore the idea that bFGF participates in the development of sensitization to amphetamine by modifying the morphology of neurons in dopaminergic regions. Extensive evidence has shown that bFGF has trophic actions on adult neurons. For instance, striatal GABAergic neurons that are cultured in the presence of bFGF show significant increases in axonal and

dendritic outgrowth compared to untreated cells (Zhou & Difiglia, 1993). Cholinergic sprouting in the denervated hippocampus is stimulated by bFGF (Fagan et al., 1997). In addition, there is evidence that the effects of bFGF on neuronal morphology have behavioral repercussions because both sprouting of cortical pyramidal neurons and recovery of motor function seen after lesion of the motor cortex are prevented by immunoneutralization of bFGF (Rowntree & Kolb, 1997).

In a series of recent studies Robinson and Kolb have found increases in the dendritic arbor and spine density of neurons in dopaminergic terminal regions in NAcc and PFC after repeated exposure to relatively high-dose and prolonged-treatment regimens of amphetamine and cocaine (Robinson & Kolb, 1997; Robinson & Kolb, 1999). As a first step toward investigating the idea that bFGF may mediate amphetamine-induced long-term neuroadaptations by promoting morphological plasticity, I examined whether the temporal and spatial changes in neuronal structure observed by Robinson and Kolb would parallel changes in bFGF expression. Thus, the effects of prolonged treatment with high doses of amphetamine on bFGF expression in dopaminergic cell body regions in the VTA and SNc, and in dopaminergic terminal regions in the NAcc core and shell, STR, and PFC, were determined.

bFGF immunoreactivity was assessed following an escalating-dose amphetamine regimen. This regimen mimics the pattern of drug exposure typically observed in addicts (Ellinwood, 1972; Kramer, Fischman, &

Littlefield, 1967) and produces robust and persistent changes in behavioral and neurochemical responses to subsequent injections of amphetamine in both rats and monkeys (Castner & Goldman-Rakic, 1999; Paulson et al., 1991; Paulson & Robinson, 1995). More importantly, this regimen has been associated with structural modifications in neurons in dopaminergic terminal regions observed one month after treatment termination (Robinson & Kolb, 1997). Because there is evidence that increased expression of neurotrophic factors in response to lesion is finished before complete neuronal sprouting and reactive synaptogenesis occurs, bFGF immunohistochemistry was examined during and after the escalating-dose amphetamine treatment (Nieto-Sampedro & Cotman, 1985).

The results of this study have been published (Flores & Stewart, 2000a).

5.2. Materials and Methods

Drugs and antibodies used in this experiment and procedures for immunohistochemistry and image analysis have been described in section 2.1.3.

5.2.1. Subjects

Male Wistar rats, weighing 300-350 g at the start of the experiment, were housed individually in standard stainless steel hanging boxes and were maintained on a 12 h light/dark cycle. Food and water were provided ad lib.

5.2.2. Image Analysis

In this experiment sections from medial prefrontal cortex (PFC) layers V and VI of the pregenual cingulate cortex area 2, and occipital cortex area 2 mediolateral (Oc2) layers V and VI were also digitized and analyzed. Boundaries of cortical structures were defined using as guide Zilles (1985). Sampling areas Oc2 were taken from sections corresponding to plates 38 and 39; sampling areas of PFC were taken from sections corresponding to plate 11 of Paxinos and Watson (1997) atlas. Images of each structure were taken from three different sections from each brain with the exception of the PFC from which images were taken from two different sections.

5.3. Statistical Analysis

All analyses were done on the raw data using number of bFGF-immunoreactive cells per square mm. The data were analyzed using *t*-tests for independent samples. All data in the figures are presented as percent of the saline control groups.

5.4. Procedures

Fig. 5.1. outlines the timing of the treatment and experimental manipulations in Experiments 1 and 2.

bFGF Expression During Escalating Dose Treatment (Experiment 1):

This experiment was conducted to assess changes in bFGF immunoreactivity in cell body (VTA and SNc) and projection regions (NAcc shell and core, STR, and layers V and VI of the PFC) of midbrain dopaminergic neurons one week after termination of a 2-week amphetamine treatment. The escalating-dose regimen used was similar to that described by Paulson et al., (1991) and is shown in Table 5.1. For comparison, bFGF immunoreactivity in layers V and VI of the Oc2 was also measured. The occipital cortex is not a dopaminergic terminal field (Fallon & Loughlin, 1987; Fallon & Moore, 1978) but after repeated treatment with escalating doses of amphetamine, morphological changes opposite to those observed in dopaminergic terminal regions have been found in this area (Robinson & Kolb, 1997).

Rats received two daily intraperitoneal injection of D-amphetamine sulfate in the colony room, 7-8 h apart, 5 days a week for two 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 groups received 0.9% saline (1 ml/kg). Groups of amphetamine-treated (n=6) and saline-treated (n=7) rats were killed and perfused one week after the last injection and their brains

Fig. 5.1 Diagram outlining the timing of the treatment and experimental manipulations conducted in Experiments 1 and 2.

1
DRUG
WEEK 1 2 3
Perfusion 3
2
DRUG
WEEK 1 2 3 4 5 6
Perfusion 6
Perfusion 9

Table 5.1. Amphetamine treatment .

The numbers represent mg/kg of amphetamine per injection on each day. All animals received two injections per day.

	DAY						
Week	MON	TUE	WED	THU	FRI	SAT	SUN
one	1	1	2	2	2	-	-
two	3	4	4	4	4	-	-
three	4	5	5	5	5	-	-
four	6	7	7	7	7	-	-
five	8	9	9	9	9	-	-

were processed for bFGF immunoreactivity. The time period between the last injection and perfusion was chosen on the basis of the previous results showing substantial and reliable increases in bFGF immunoreactivity one week after amphetamine treatment termination.

bFGF Expression after Escalating Dose Treatment (Experiment 2):

This experiment was conducted to assess changes in bFGF immunoreactivity in the same midbrain and forebrain regions as examined in Experiment 1, one week and one month after termination of a 5-week amphetamine treatment period. Rats received 2 daily intraperitoneal injections of amphetamine in the colony room, 7-8 h apart, five days a week for five weeks. The dose of amphetamine began with 1 mg/kg and escalated to 9 mg/kg for the last four days of treatment (see Table 5.1). Control groups received saline (1 ml/kg). Groups of amphetamine-treated and saline-treated rats were killed and perfused one week (n=4/group) or one month (n=8/group) after the last injection and their brains were processed for bFGF immunoreactivity. The one week time period was based on the previous findings. The one month period was chosen because of the prolonged treatment regimen and because structural modifications have been observed in NAcc and PFC neurons one month after the same 5-week escalating dose amphetamine treatment (Robinson & Kolb, 1997).

5.5. Results

Qualitative analysis revealed that, in all areas examined, bFGF immunoreactivity was confined to the nuclei of small cells, presumably glia. These observations are in agreement with the previous findings showing, by means of double-label immunohistochemistry, that the antibody used in this study detects bFGF immunoreactivity in astrocytic nuclei but not in dopaminergic neurons.

Changes in bFGF Immunoreactivity in Dopaminergic Cell Body Regions

Exposure to the escalating dose regimen for two weeks (1 mg/kg to 4 mg/kg amphetamine, twice a day, 5 days a week) resulted in an increase in bFGF immunoreactivity in cell body regions of dopaminergic cells over that seen in saline treated rats. As shown in Fig. 5.2, significant increases in bFGF immunoreactivity in the VTA (44 ± 5%) and SNc (42 ± 9%) were observed one week after the last drug injection.

When the escalating dose amphetamine treatment was prolonged to 5 weeks (1 mg/kg to 9 mg/kg, twice a day, five days a week), bFGF immunoreactive levels in the VTA and SNc were not different from those in saline-treated rats perfused one week after the end of treatment (Fig. 5.3, top panel). In fact there appeared to be a slight, but non-significant reduction in bFGF expression in the SNc (20 ± 15%). As shown in Fig. 5.3 (bottom panel), however, when animals were perfused one month after the 5-week escalating-dose treatment, bFGF expression in the VTA (41 ± 10%) and SNc

Fig. 5. 2 Short-term escalating dose treatment. bFGF immunoreactivity in the VTA and SNc following two weeks of exposure to two daily intraperitoneal injections of saline (S, 1 ml/kg) or of escalating doses of amphetamine (A, 1 mg/kg to 4 mg/kg), for five consecutive days followed by two drug-free days. All animals were perfused one week after the last injection. Values are expressed as the mean (± SEM) percent of the saline control group. * Student's t-test conducted on the actual counts showed a significant difference between amphetamine and saline groups (VTA, $t(11) = 14.18$, $p = .003$; SNc, $t(11) = 8.5$, $p = .01$).

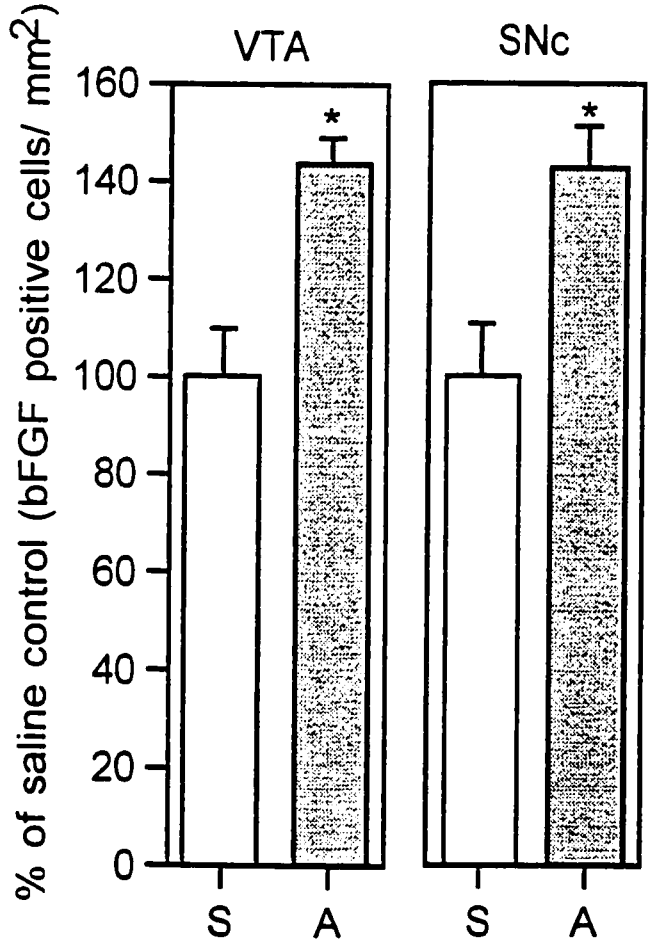
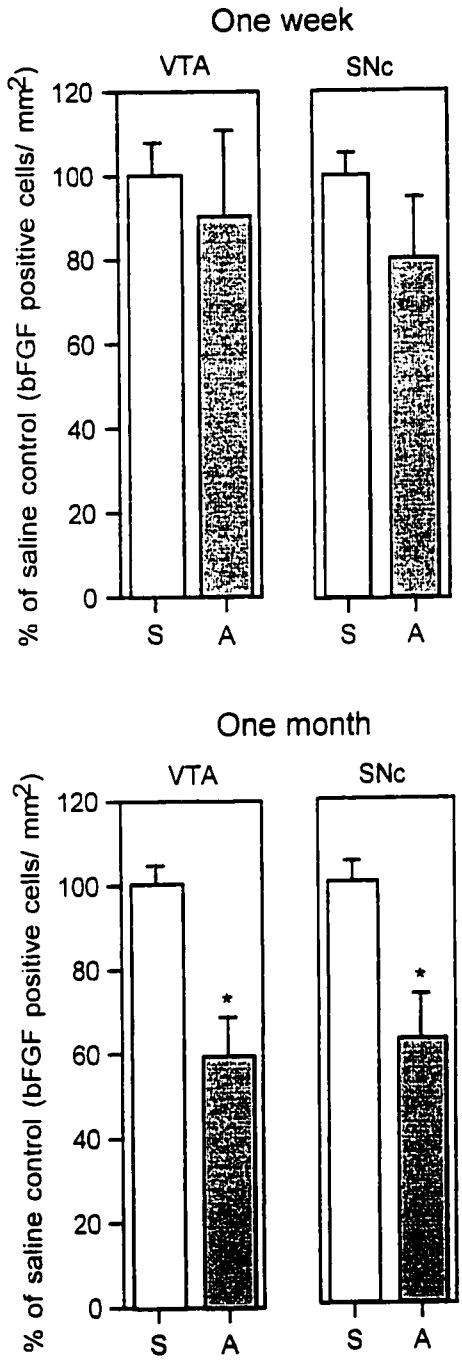

VTA
SNc
% of saline control (bFGF positive cells/ mm2)
160
140
120
100
80
60
40
20
0
*
*
S
A
S
A

Fig. 5.3 Prolonged escalating dose treatment. bFGF immunoreactivity in the VTA and SNc following five weeks of exposure to two daily intraperitoneal injections of saline (S, 1 ml/kg) or of escalating doses of amphetamine (A, 1 mg/kg to 9 mg/kg), for five consecutive days followed by two drug-free days. Animals were perfused one week after the last injection. Values are expressed as the mean (± SEM) percent of the saline control group.


One week
VTA
SNc
% of saline control (bFGF positive cells/ mm2)
120
100
80
60
40
20
0
S
A
S
A
One month
VTA
SNc
% of saline control (bFGF positive cells/ mm2)
120
100
80
60
40
20
0
*
*
S
A
S
A

(38 ± 11%) was significantly decreased compared to that found in saline-treated rats. Importantly, decreased expression of bFGF in the VTA and SNc seen after one month did not result from increased bFGF expression within these regions in saline-treated rats killed at the same withdrawal time. bFGF immunoreactive levels in the VTA and SNc of saline-treated rats perfused either one week or one month after the last injection, and whose brains were processed in parallel, were not statistically different.

Changes in bFGF Immunoreactivity in Dopaminergic Terminal Regions

Exposure to the escalating dose treatment for two weeks resulted in elevated bFGF immunoreactive levels in dopaminergic terminal regions. As shown in Fig. 5.4, there were significant increases in bFGF immunoreactivity in both the NAcc shell (20 ± 6%) and NAcc core (29 ± 5%), one week after the last amphetamine injection. In addition, increased numbers of bFGF immunoreactive cells were found in the STR (10 ± 4) and PFC (24 ± 6) of amphetamine-treated rats, but these changes did not reach statistical significance (p=.06 and p=.2, respectively). These results contrast with those found in the previous study in which the brains of animals given only three injections of amphetamine (3 mg/kg once every other day) exhibited no increases in bFGF expression in terminal regions (see Fig. 2.6).

When the escalating-dose regimen was prolonged to 5 weeks and animals were perfused either one week or one month later, no differences in bFGF immunoreactivity in the NAcc shell or PFC were observed between

Fig. 5.4. Short-term escalating dose treatment. Basic fibroblast immunoreactivity (bFGF) in the NAcc shell, NAcc core, dorsal striatum (STR), and medial prefrontal cortex (PFC) following two weeks of exposure to two daily intraperitoneal injections of saline (S, 1 ml/kg) or of escalating doses of amphetamine (A, 1 mg/kg to 4 mg/kg), for five consecutive days followed by two drug-free days. All animals were perfused one week after the last injection. Values are expressed as the mean (± SEM) percent of the saline control group. * Student's t-test conducted on the actual counts showed a significant difference between amphetamine and saline groups (Shell, $t(10) = 6.41$, $p < .05$; Core, $t(10) = 33.61$, $p < .001$; STR, $t(10) = 4.22$, $p = .06$)

% of saline control (bFGF positive cells/ mm²)
140
120
100
80
60
40
20
0
NAcc Shell
NAcc Core
STR
PFC
*
*
S
A

amphetamine-treated animals and those given saline injections (see Fig. 5.5). Small, non-significant decreases in bFGF expression in the NAcc core and STR were observed one week and one month after amphetamine treatment termination.

Changes in bFGF Immunoreactivity in Occipital Cortex

As shown in Fig. 5.6, treatment with escalating doses of amphetamine for two weeks resulted in significant decreases in bFGF expression in layers V and VI of Oc2 (40 ± 8%). This effect, however, was not observed when the treatment was prolonged for 5 weeks and the animals were killed either one week or one month later.

5.6. Discussion

The primary purpose of this study was to examine changes in bFGF expression in cell body and terminal regions of midbrain dopaminergic cells following an amphetamine regimen known to produce persistent increases in dendritic arbor of medium spiny neurons in the NAcc and of pyramidal cells in the PFC (Robinson & Kolb, 1997). This regimen resembles the pattern of drug taking typically observed in amphetamine addicts and induces long-lasting changes in the behavioral and neurochemical effects of amphetamine (Castner & Goldman-Rakic, 1999; Paulson et al., 1991; Paulson & Robinson, 1995). bFGF immunoreactivity was assessed in cell body and terminal regions of midbrain dopaminergic cells during and after treatment with escalating

Fig. 5.5. Prolonged escalating dose treatment. bFGF immunoreactivity in the NAcc shell, NAcc core, STR, and PFC following five weeks of exposure to two daily intraperitoneal injections of saline (S, 1 ml/kg) or of escalating doses of amphetamine (A, 1 mg/kg to 9 mg/kg), for five consecutive days followed by two drug-free days. Animals were perfused either one week or one month after the last injection. Values are expressed as the mean (± SEM) percent of the saline control group.

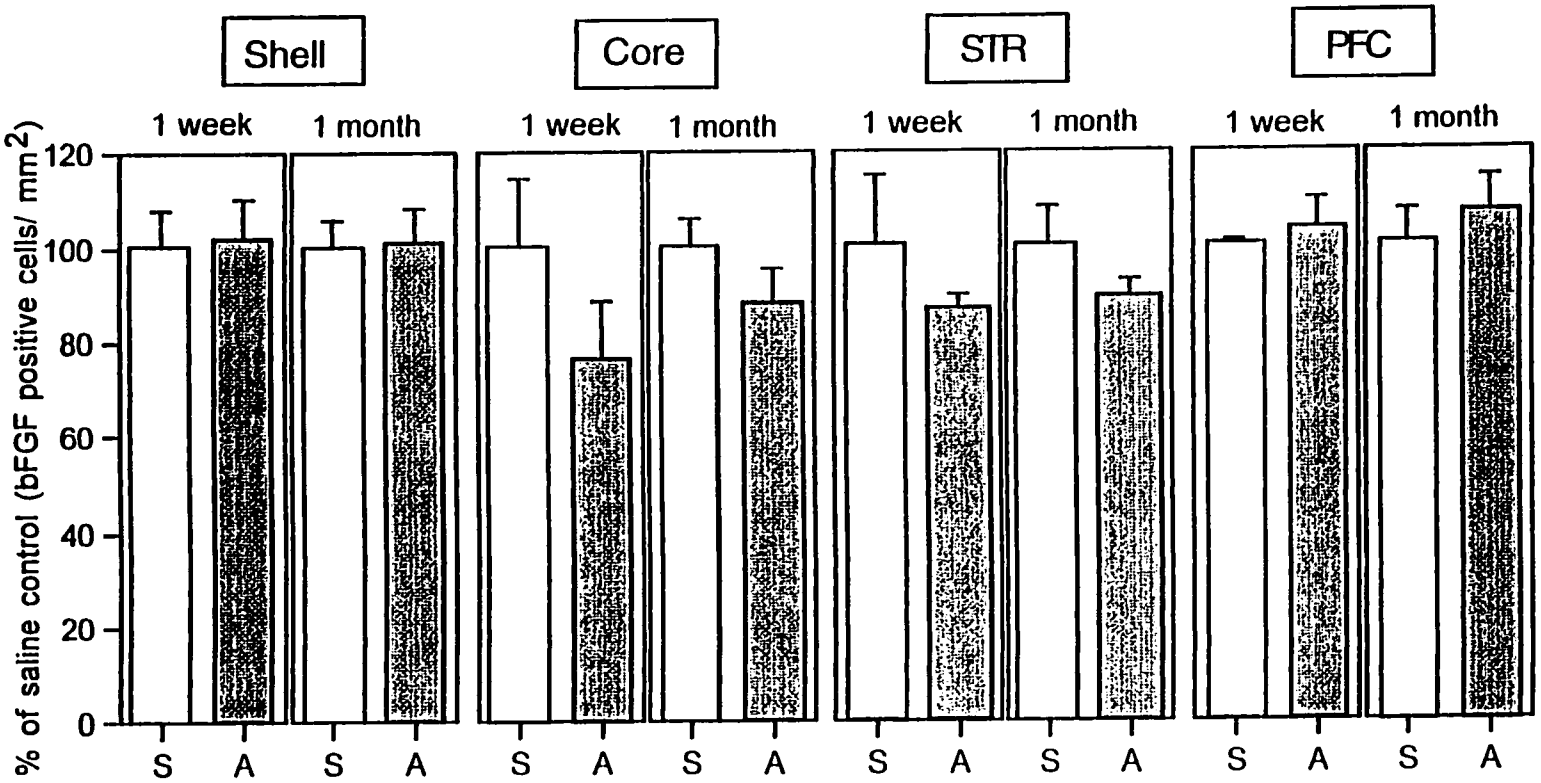
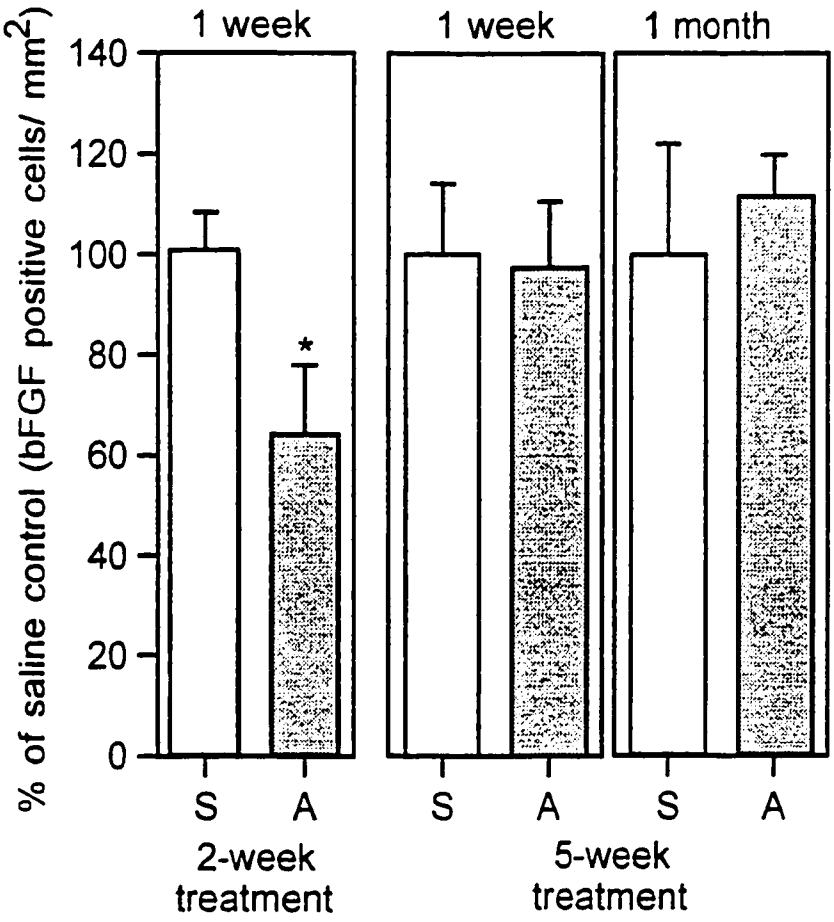

Shell
Core
STR
PFC
1 week
1 month
1 week
1 month
1 week
1 month
1 week
1 month
% of saline control (bFGF positive cells/ mm2)
120
100
80
60
40
20
0
S
A
S
A
S
A
S
A
S
A
S
A
S
A
S
A

Fig. 5.6. bFGF in the occipital cortex area 2 following two or five weeks of exposure to two daily intraperitoneal injections of saline (S, 1 ml/kg) or of escalating doses of amphetamine (A, short treatment: from 1 mg/kg to 4 mg/kg; prolonged treatment: from 1 mg/kg to 9 mg/kg) for five consecutive days followed by two drug-free days. After the short treatment, animals were perfused one week after the last injection. After the prolonged treatment, animals were perfused either one week or one month after the last injection. Values are expressed as the mean (± SEM) percent of the saline control group. * Student's t-test conducted on the actual counts showed a significant difference between amphetamine and saline groups (2-week treatment, $t(9) = 4.67$, $p = .05$).


Occipital Cortex
1 week
1 week
1 month
% of saline control (bFGF positive cells/ mm2)
140
120
100
80
60
40
20
0
*
S
A
S
A
S
A
2-week treatment
5-week treatment

doses of amphetamine. It was found that two weeks of treatment with escalating doses of amphetamine resulted in increased bFGF expression in both dopaminergic cell body and terminal regions, one week after the last injection. When the treatment with escalating doses of amphetamine was prolonged to 5 weeks, however, bFGF expression within these regions either did not differ from that observed in saline-treated rats or was decreased.

Effects in Dopaminergic Cell Body Regions

The finding that bFGF expression in the VTA and SNc is increased following a 2-week escalating-dose regimen confirms and extends the previous report that three intermittent injections of amphetamine induced increases in bFGF expression in these regions one week after the last injection. Because the effects of bFGF in VTA and SNc are blocked by coadministration of either the non-selective glutamate receptor antagonist, kynurenic acid, or the NMDA antagonist, CPP, it is proposed that these changes in bFGF are induced by amphetamine-induced glutamate release in the VTA. Extracellular glutamate in dopaminergic cell body regions is elevated during or after injections of amphetamine or D_1 receptor agonists (Kalivas & Duffy, 1995a; Kalivas & Duffy, 1998; Wolf & Xue, 1998; Wolf & Xue, 1999; Xue et al., 1996) and glutamate has been shown to induce bFGF expression in cultured astrocytes (Pechan et al., 1992).

In contrast to the increases in bFGF expression observed after the 2-week escalating treatment, no changes in bFGF expression were observed in

either the VTA or SNc when the escalating regimen was extended for 5 weeks. In fact, bFGF immunoreactivity in these regions was actually *decreased* one month after this prolonged treatment. The initial increase in bFGF expression followed by the decrease as this escalating regimen was prolonged is intriguing. Several studies have shown that following injury there is an initial increase in bFGF expression that returns to basal levels after a period of time. Chadi and colleagues examined changes in expression of bFGF mRNA and protein in adult rats after a 6-OHDA injection. They found strong increases in astrocytic bFGF mRNA in SNc and VTA as early as 2 h after the injection, peaking at 24 h, and returning to basal levels two weeks later. bFGF immunoreactivity was also increased in astroglial cells in the VTA and SNc as early as 2 h after the injection, peaked around one week, and began to decrease two weeks after (Chadi et al., 1994). Similar results have been observed after different types of brain insults. For instance, Rowtree and Kolb (1997) reported that suction lesion to the motor cortex causes increased expression of astrocytic bFGF in tissue surrounding the lesion that peaks at 7 days after injury and begins to decline thereafter. A transient enhancement (10 h) in bFGF mRNA in entorhinal cortex and hippocampus has also been shown after induction of seizures in rats (Follesa, Gale, & Mocchetti, 1994).

In addition, in a detailed analysis of the time course of events that take place following brain injury, Nieto-Sampedro and Cotman showed that increased activity of neurotrophic factors precedes the formation of reactive and regenerative neuronal sprouts by a few days, and that this response is

over before neurite outgrowth is completed (Nieto-Sampedro & Cotman, 1985). These observations were confirmed in the study conducted by Rowtree and Kolb (1997) because the increased expression of bFGF observed preceded neuronal sprouting of pyramidal neurons.

The fact that bFGF expression in the VTA and SNc was below basal levels one month after the last amphetamine injection suggests not only that the initial signals that induced bFGF expression are no longer present, but that the bFGF response is spent or is actually being inhibited. I can only speculate about the mechanisms underlying this effect. If it is the case that increased bFGF expression is an initial response to metabolic stress and injury, it may be that its expression is inhibited by the consequences of its effects. As it has been seen, it takes very little exposure to amphetamine to induce increases in bFGF expression in these regions, and it is only after severe exposure that decreases are evident. By this time many subsequent changes may have occurred including changes in receptor configuration (Fitzgerald et al., 1996), in the density of astrocytic processes (Beitner-Johnson et al., 1993), in expression of other neurotrophic factors (Krieglstein et al., 1998), and in neuronal morphology.

In vivo and in cultured astrocytes, bFGF has been shown to induce the expression of its own gene and protein (Flott-Rahmel et al., 1992; Gomez-Pinilla et al., 1995; Moffett, Kratz, Florkiewicz, & Stachowiak, 1996; Moffett et al., 1998). Thus, it appears that under conditions of metabolic stress or minor injury, initial factors, such as increased glutamate, induce bFGF expression

which, in turn, activates transcription of its own gene. This effect, however, seems to last only until a new steady state is attained. Interestingly, in low density cell cultures bFGF mRNA is increased, whereas in high density cultures it is decreased. It appears that increased cell contact inhibits the bFGF gene promoter (Moffett et al., 1996). Furthermore, cell contact prevents bFGF induction by bFGF, itself, by other neurotrophic factors, and by stimulation of cyclic AMP or protein kinase C signaling pathways (Moffett et al., 1998). Because during intense and prolonged amphetamine treatment, increases in bFGF are initiated relatively early, it may be, that by the end of the treatment, increases in astrocytic processes and neuronal sprouting have taken place. These, in turn, may inhibit the transcription of the bFGF gene. To date, however, there are no studies of the effects of amphetamine treatment on neuronal sprouting within the VTA and SNc.

It is important to mention that during the 2-week treatment regimen the dose of amphetamine was increased from 1 to 4 mg/kg, whereas during the 5-week treatment it was increased to 9 mg/kg. Thus, it is not clear whether the effects on bFGF expression resulted from the 'amount' of drug administered or from the 'duration' of drug exposure. It would be interesting to examine changes in both bFGF immunoreactivity and neuronal morphology following a 2-week treatment with escalating doses of amphetamine that begin at 1 mg/kg and escalate to 9 mg/kg, similar to the one used by Camp, DeJonghe, and Robinson (1997).

Effects in Dopaminergic Terminal Regions

An important finding of this study is that after two weeks of the severe amphetamine regimen, there were increases in bFGF expression in the NAcc shell, NAcc core, STR, and PFC in addition to those seen in dopaminergic cell body regions. These findings are particularly interesting in light of the recent reports that intensive and prolonged treatments with amphetamine lead to morphological changes in NAcc medium spiny neurons and PFC pyramidal cells (Robinson & Kolb, 1997; Robinson & Kolb, 1999). As mentioned before, a large body of evidence indicates that bFGF induces neuronal sprouting in various neuronal populations, including striatal medium spiny neurons and cortical pyramidal cells (Rowntree & Kolb, 1997; Zhou & Difiglia, 1993). As was seen previously for the cell body regions, the number of cells expressing bFGF in terminal regions following the 5-week escalating-dose amphetamine treatment was not different from that seen in saline-treated animals.

The mechanisms whereby amphetamine induces bFGF expression in dopaminergic terminal regions are not known. There is, however, evidence showing that acute amphetamine injections increase extracellular glutamate not only in the VTA but also in the NAcc and PFC (Reid, Hsu, & Berger, 1997; Xue et al., 1996), suggesting that glutamate could mediate the effects of amphetamine on bFGF expression in these regions as well. Another possibility is that following repeated injections of high doses of amphetamine bFGF expression is induced by increased concentration of free radicals. Acute amphetamine injections cause massive increases in dopamine release in

terminal, as well as cell body regions and dopamine autoxidizes *in vivo* to form free radicals (Fornstedt, Pileblad, & Carlsson, 1990). It is likely, therefore, that the concentration of free radicals in these tissues is elevated following the numerous amphetamine injections given during the 2-week escalating-dose treatment. It has been shown that, *in vitro*, free radicals induce astrocytic bFGF gene expression (Pechan et al., 1992).

Effects in Occipital Cortex

Unlike the dopaminergic terminal regions studied, in Oc2 *decreases* in bFGF expression were observed after the 2-week amphetamine treatment. In their study, Robinson and Kolb (1997) also selected the occipital cortex as a region unlikely to be affected by dopaminergic stimulation. In contrast to their findings in the PFC, they observed *decreases* in dendritic spine density in occipital pyramidal cells after the prolonged and severe amphetamine regimen. Although I cannot offer an explanation for the decreases in bFGF in this region and for their functional relevance, it is remarkable that changes in bFGF parallel those in morphology.

Conclusions

Sensitization to the effects of amphetamine develops gradually and is long-lasting suggesting that changes in neuronal function including structural alterations may be necessary. bFGF has the necessary properties to initiate changes in neuronal connectivity and, as shown before, its actions in the VTA are required for the development of sensitization to amphetamine.

In this study it has been found that the pattern of changes in bFGF expression following escalating doses of amphetamine corresponds to the pattern of changes seen in neuronal structure after a similar drug treatment. These results suggest that bFGF may participate in the development of sensitization to the behavioral and neurochemical effects of amphetamine by altering neuronal morphology.

5.6.1. Alternative Mechanisms Mediating the Effects of bFGF on the Development of Sensitization.

Alternatively, or in addition, to modifications in neuronal structure, bFGF may participate in the development of sensitization by inducing alterations in the sensitivity of dopaminergic neurons to glutamatergic activation, by protecting dopaminergic neurons from potential injury, and/or by activating other neurotrophic factors. In this section I examine each of these possibilities in more detail.

Alteration in Glutamate Receptor Subunits

Exposure of cultured rat hippocampal neurons to bFGF has been shown to selectively increase GluR1 protein levels in a concentration-dependent manner and to produce more than a twofold enhancement in Ca^{++} entry in response to AMPA receptor activation (Cheng et al., 1995). As mentioned before, GluR1 immunoreactivity in the VTA is increased following repeated exposure to stimulant drugs (Fitzgerald et al., 1996).

Moreover, dopamine cells become more responsive to AMPA receptor activation following treatment with amphetamine (Zhang et al., 1997c). Because up-regulation of GluR1 increases the sensitivity to the stimulant effects of morphine (Carlezon et al., 1997), bFGF may exert its effects on the development of sensitization by increasing levels of GluR1 in VTA dopaminergic neurons. Interestingly, both stimulant drugs and bFGF appear to increase GluR1 via post-transcriptional mechanisms because they do not alter GluR1 mRNA levels (Ghasemzadeh, Nelson, Lu, & Kalivas, 1999; Mattson, Kumar, Cheng, Wang, & Michaelis, 1993; Wolf, 1998).

Selective increases in GluR1 expression and associated increases in Ca^{++} entry may trigger changes in the functioning of dopamine neurons through alterations in gene and protein expression that eventually may result in enduring enhancement of neuronal function. It is intriguing that repeated amphetamine treatment has been associated with increased responsiveness of dopamine cells to AMPA, but not NMDA receptor activation. While large increases in Ca^{++} entry through NMDA receptors have been widely associated with neuronal injury and death, increased Ca^{++} influx through AMPA-receptor channels is accompanied by enhanced synaptic transmission and neuronal plasticity (see Cheng et al., 1995).

The effects of amphetamine on increased bFGF expression are blocked by NMDA antagonists suggesting that NMDA receptor activation is a necessary event in the development of sensitization because it activates bFGF expression in the VTA. AMPA antagonists have been shown to prevent the

development of sensitization to amphetamine (Li et al., 1997), but their effects on amphetamine-induced bFGF expression have not yet been examined. Experiments aimed at addressing this question would provide information about the sequence of events that take place during the induction of sensitization to amphetamine (see Fig. 6). If AMPA antagonists given during the induction phase of sensitization to amphetamine were found to prevent the development of behavioral sensitization, but not the expression of bFGF, it could be concluded that AMPA antagonists prevent the development of sensitization by acting downstream from bFGF. That is, AMPA antagonists would be blocking one of the effects of increased bFGF expression in the VTA namely increased Ca^{++} entry through homomeric GluR1 AMPA receptors located on dopaminergic cells.

Neuroprotective Effects

In addition to its neurotrophic effects, bFGF is a neuroprotective agent. Indirect evidence supports the idea that bFGF participates in the development of sensitization by protecting dopaminergic neurons from potential injury. bFGF has been shown to decrease the vulnerability of cultured hippocampal neurons to NMDA neurotoxicity by suppressing the immunoreactivity and mRNA expression of a 71 kDa NMDA receptor protein (NMDARP-71) that is part of the NMDA receptor complex (Mattson et al., 1993). The presence of NMDARP-71 is strongly associated with increased vulnerability to glutamate toxicity (Mattson, Wang, & Michaelis, 1991). Thus, it appears that bFGF may

produce changes in dopaminergic function responsible for sensitized responding to stimulant drugs by increasing synaptic transmission through AMPA receptors and by simultaneously decreasing glutamatergic toxicity through NMDA receptors. This dual function of bFGF renders this protein a very suitable candidate for mediating the changes that take place during the induction of sensitization to stimulant drugs because it allows glutamate-dependent (most probably Ca^{++}-mediated) neuroadaptations, but prevents neurotoxicity.

bFGF has also been found to protect cells from oxidative stress by increasing the cellular defense system. Oxidative stress refers to the cellular consequences of imbalance between the production of free radicals (reactive oxygen species) and the ability of the cell to defend against them. Built-up reactive oxygen species can cause neuronal dysfunction and under certain conditions, neuronal death (for review see Simonian & Coyle, 1996). Most cellular H_2O_2 (a predominant source of free radicals) is removed by glutathione peroxidase which uses it to oxidize reduced glutathione (GSH) to glutathione disulfide (GSSG). Thus, cellular levels of GSH and activity of GSH peroxidase and GSH reductase significantly influence the metabolic state of cells.

6-OHDA-induced dopaminergic damage is believed to result from accumulation of highly toxic reactive oxygen species which are generated during its autoxidation (Lotharius, Dugan, & O'Malley, 1999). Interestingly, Hou et al. (1997) have shown that damage to dopaminergic cell cultures

caused by adding 6-OHDA to the preparation is prevented by bFGF. This neuroprotective action of bFGF appeared to be mediated by increases in GSH levels because dopaminergic cultures treated with bFGF had higher levels of GSH which increase even further after 6-OHDA exposure. In fact, when GSH synthesis was blocked, the neuroprotective effects of bFGF were decreased (Hou et al., 1997). bFGF has also been shown to increase the activity of GSH reductase in cultures of hippocampal neurons that are exposed to high concentrations of NMDA. This effect is mediated by bFGF-FGFR1 interactions because it is prevented by inhibition of tyrosine kinase activity (Mattson, Lovell, Furukawa, & Markesbery, 1995).

In adult animals, bFGF expression is increased after injury to midbrain dopamine cells (Chadi et al., 1994) and appears to participate in the survival of these neurons. When added intracerebroventricularly after an MPTP injection (which increases reactive oxygen species), bFGF has been shown to attenuate dopaminergic cell loss and promote behavioral recovery (Chadi et al., 1993; Lotharius et al., 1999; Otto et al., 1990). Moderate doses of amphetamine or cocaine cause substantial dopamine and glutamate release in cell body regions and they have been associated with increased production of hydroxyl radicals in the VTA (Wolf, 1998). Thus, bFGF may participate in the development of sensitization by increasing the concentration and activity of antioxidative agents, protecting dopaminergic cells from oxidative stress.

Alterations in Expression of Other Neurotrophic Factors

bFGF might play its role in the development of sensitization by altering the expression of other neurotrophic factors. The results from the experiments presented in this thesis, together with the results obtained from studies with BDNF, NT-3, and CNTF (reviewed in Chapter 1), suggest that there might be interactions between these proteins. bFGF has been shown to induce the expression of transforming growth factor β (TGF-β) in neonatal rat astrocytes cultured from midbrain, striatum, and cortex (Krieglstein et al., 1998). TGF-β has been shown to mediate the neuroprotective and neurotrophic effects of bFGF on dopaminergic cells because antibodies against it abolish bFGF-induced survival of dopaminergic neurons *in vitro* (Krieglstein et al., 1998). Interestingly, TGF-β plays a crucial role in the development of long-term synaptic facilitation in *Aplysia* (Zhang, Endo, Cleary, Eskin, & Byrne, 1997b). In addition, interleukin-1B, epidermal growth factor (EGF), and platelet derived growth factor (PDGF) all have been found to stimulate bFGF expression in astrocytes by activating the promoter of the bFGF gene (Moffett et al., 1998). To my knowledge, the interactions between bFGF and CNTF or between bFGF and the neurotrophins, BDNF and NT-3, have not been investigated.

Role of Astrocytes

bFGF has significant effects on astroglial function and many of its actions on neuronal function may be mediated by astrocytes. In astrocytic cultures and in adult rats, bFGF has been shown to up-regulate the expression of neurotrophic factors such as NGF and TGF-β, GFAP immunoreactivity, its own gene transcription, and FGF-R1 immunoreactivity (Flott-Rahmel et al., 1992; Gomez-Pinilla, Vu, & Cotman, 1995; Unsicker et al., 1993). In addition, the antioxidative effects of bFGF appear to be mediated by its effects on astrocytes. When cultured dopaminergic cells are treated with bFGF, the rise in GSH before and after 6-OHDA exposure is abolished if astrocytic proliferation is blocked (Hou et al., 1997). Several studies have shown that astrocytes have higher GSH content than neuronal cells (Makar et al., 1994; Pileblad & Eriksson, 1991; Sagara, Maura, & Bannai, 1993) and that astroglial cells influence neuronal GSH levels in culture by effectively transporting cystin (the disulfide-linked dimer of the amino acid cystein) from the medium and converting it to cysteine. Cysteine is then released in the medium, taken up by neurons, and used in the synthesis of GSH (Griffith & Meister, 1979). Thus, bFGF may stimulate directly GSH production in astrocytes and, indirectly, in neurons.

The neuroprotective and neurotrophic actions of bFGF during stimulant drug treatment, therefore, may be mediated by alterations in astrocytic function. In fact, repeated treatment with morphine and cocaine

have been shown to produce increases in GFAP immunoreactivity and activity, respectively, in the VTA (Beitner-Johnson et al., 1993). Furthermore, glutamate has been shown to induce bFGF and FGFR1 gene transcription in astrocytes, suggesting that increases in extracellular concentration of the excitatory amino acid leads to bFGF autocrine effects.

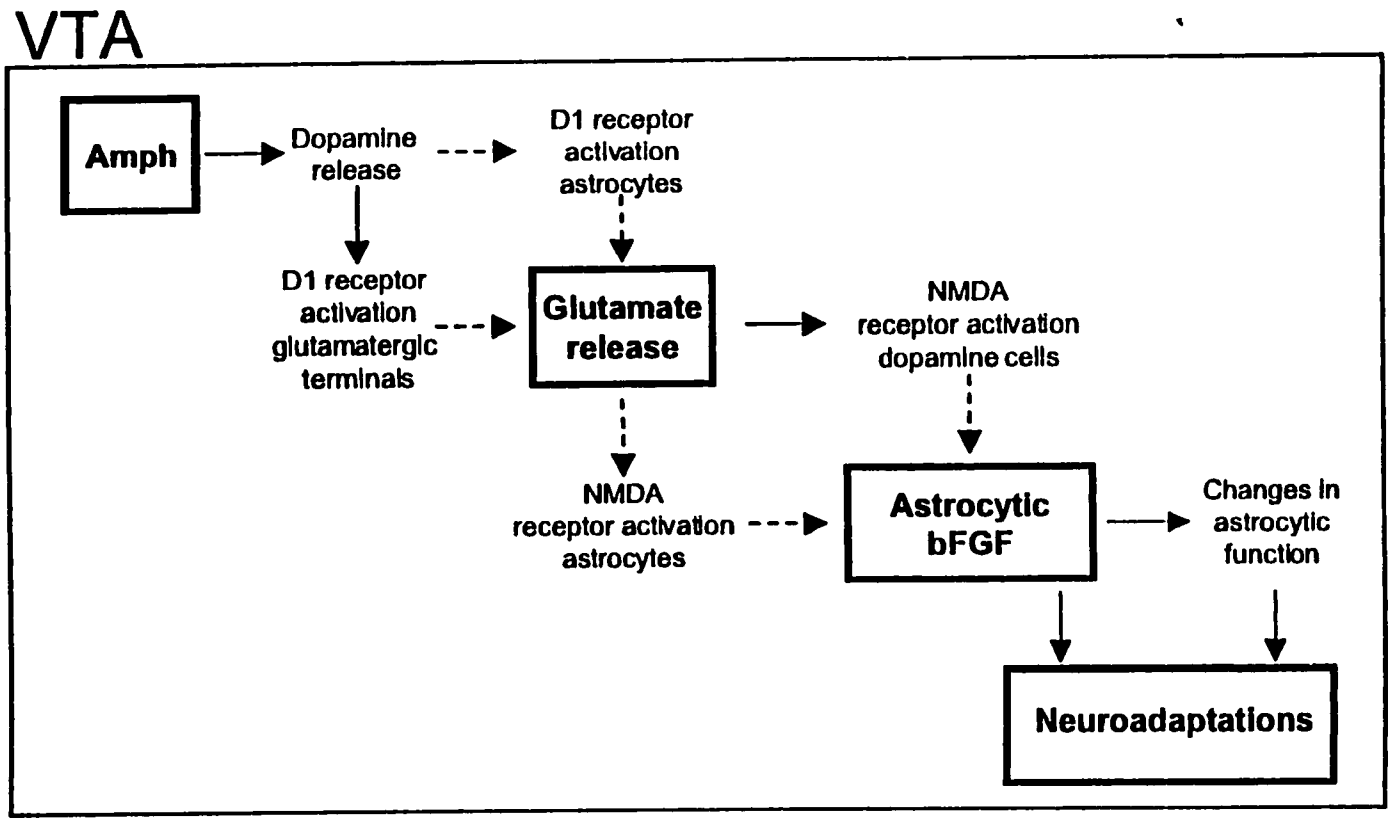
SUMMARY AND CONCLUSIONS

Exposure to stimulant drugs has enduring consequences for brain function and behavior. The evidence presented in this thesis strongly suggests that bFGF expressed by astrocytes in the midbrain dopaminergic cell body region plays a crucial role in the development of the long-lasting behavioral sensitization that follows repeated amphetamine treatment. In addition, these findings point to bFGF as being a mediator of the glutamatergic-dopaminergic interaction that initiates persistent changes in neurochemistry, neuronal morphology, and behavior brought about by repeated stimulant drug use.

Repeated injections of amphetamine result in long-lasting increases in the protein expression of bFGF in the cell body region of midbrain dopaminergic neurons. These increases are positively correlated with the level of behavioral sensitization that develops. Both behavioral sensitization and increased bFGF expression are prevented when amphetamine injections during the induction phase are preceded by injections of NMDA receptor antagonists. Finally and most importantly, blockade of bFGF during the induction phase of sensitization prevents the increased locomotor activation elicited by subsequent amphetamine challenge.

As summarized in Fig. 6, it is proposed that in response to amphetamine, increased extracellular glutamate activates bFGF which, in turn, acts directly on neurons, or indirectly though astrocytes, to initiate long-lasting changes in sensitivity and connectivity that underlie increased

Fig. 6. A schematic representation of the possible steps involved in the amphetamine-induced, glutamate-mediated increases in bFGF expression in the VTA. The boxes show events that are known to occur. Straight arrows represent well established pathways, whereas dashed arrows illustrate possible ones. Amphetamine increases extracellular dopamine levels. Extracellular dopamine activates D_1 receptors located on glutamatergic terminals, stimulating the release of glutamate. Alternatively. or in addition, dopamine may also activate D_1 receptors located in astrocytes and this may lead to glutamate release. Increased extracellular glutamate activates NMDA receptors on dopaminergic cells and possibly astrocytes resulting in increased expression of astrocytic bFGF. 4) Actions of bFGF, in turn, lead, directly, or indirectly through alterations in astrocytic function, to neuroadaptations ultimately responsible for the persistent behavioral, neurochemical, and morphological changes induced by repeated amphetamine exposure.


VTA
Amph
Dopamine release
D1 receptor activation astrocytes
D1 receptor activation glutamatergic terminals
Glutamate release
NMDA receptor activation dopamine cells
NMDA receptor activation astrocytes
Astrocytic bFGF
Changes in astrocytic function
Neuroadaptations

dopaminergic function characteristic of sensitization to amphetamine. Modifications in neuronal structure appear to be implicated, but the exact mechanisms whereby bFGF brings about these changes are yet to be explored (see Flores and Stewart 2000b).

Previous exposure to systemic injections of cocaine, amphetamine or morphine potentiates acquisition and expression of place preferences and the incentive to self-administer these drugs (Horger et al., 1992; Horger et al., 1990; Lett, 1989; Lorrain et al., 2000; Mendrek et al., 1998; Piazza et al., 1989; Shippenberg & Bals-Kubik, 1995; Valadez & Schenk, 1994; Vezina et al., 1999b; Woolverton et al., 1984). Increased motivation to self-administer amphetamine has been observed in rats pre-exposed to intra-VTA injections of this drug, and has been correlated with sensitized locomotor responding and dopaminergic release in NAcc (Lorrain, Austin, Arnold, & Vezina, 1999; Vezina, Arnold, & Lorrain, 1999a; Vezina et al., 1999b). These findings indicate that prior exposure to drugs of abuse may predispose individuals to addictive behavior (Robinson & Berridge, 1993).

In addition, exposure to stimulant drugs has been shown to alter subsequent responses to motivationally-significant stimuli. Repeated exposure to morphine or amphetamine increases responding to sex-relevant objects. Male rats have been shown to exhibit facilitated sexual behavior when presented with a receptive female in an environment that was paired with repeated morphine injections days or weeks before (Mitchell & Stewart, 1990). Similar effects have been observed in rats previously treated with

amphetamine injections. Increased behavioral responding to sexual stimuli was correlated with locomotor sensitization and increased dopaminergic release in NAcc. Amphetamine-induced facilitation of sexual behavior was observed in context-dependent and independent conditions (Fiorino & Phillips, 1999).

Feeding behavior also becomes sensitized after repeated amphetamine treatment. Rats that receive repeated amphetamine injections and are tested for intake of palatable food two weeks later, consume significantly more food than saline-pre-treated rats. This effect is long-lasting because it is evident for at least ten days. Feeding-induced behavior in response to stress has also been found to be potentiated after pre-exposure to amphetamine injections (Moroz, 1997).

Finally, repeated administration of stimulant drugs enhances responding to stressors. Fibiger and colleagues have shown that behavioral and neurochemical responses to footshock are significantly greater in rats pre-exposed to repeated injections of amphetamine one week earlier (Hamamura & Fibiger, 1993). In addition, they have reported that amphetamine treatment enhances regional *c-fos* (an immediate early gene) expression produced by conditioned fear (Hamamura & Ichimaru, 1997).

The findings presented in this thesis, therefore, not only provide novel mechanisms mediating the development of long-lasting consequences of repeated drug use, but they may also have implications for the understanding of the diverse behavioral changes that result from exposure to drugs.

Furthermore, information about brain processes involved in amphetamine-induced plasticity may contribute to our understanding of the neurobiological basis of goal-directed behavior.

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