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Dopamine Receptor-Dependence of the Amphetamine Induction
of the Immediate Early Gene *C-Fos*

by

David Alex Jaeger

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

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Abstract

Dopamine Receptor-Dependence of the Amphetamine Induction
of the Immediate Early Gene *C-Fos*

by

David Alex Jaeger

Adviser: Dr. Stuart Sealfon

Amphetamine is a psychostimulant whose behavioral effects have been directly attributed to increased subcortical dopaminergic neurotransmission. The goal of this thesis was to identify the relationship between signal transduction in neuronal pathways subserving the behavioral effects of amphetamine and dopamine receptor activation. This was accomplished by administering dopamine receptor-selective antagonists prior to treatment with amphetamine, and measuring the effects these drugs have on the induction of the immediate early gene *c-fos* in the striatum and primary motor cortex. Fos represents a signal transduction mechanism which has previously been shown to play a direct role in the locomotor effects of amphetamine. Positive Fos-immunolabeling of digitized images was determined utilizing an algorithm for selection of intensity thresholds, as this method was determined to be as accurate and more precise than subjective assessments.

D1-like dopamine receptor antagonism was less effective in reducing the amphetamine induction of *c-fos* in the primary motor

cortex than D2-like dopamine receptor antagonism, despite the ability of D1-like dopamine receptor antagonism to eliminate the amphetamine induction of *c-fos* in the striatum. We suggest that the induction of *c-fos* in layer V pyramidal neurons of the primary motor cortex, like the striatum, is dependent upon dopamine receptor activation. The pattern of dopamine receptor-dependent gene induction likely reflects dopamine-receptor specific modulation of pathways communicating with the motor cortex. These studies contribute to an understanding of the molecular mechanisms through which dopaminergic neurotransmission, particularly that which is produced by the administration of drugs of abuse such as amphetamine, influences the neuronal circuits which subserve locomotion.

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love and support have been my stability

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1. Background

1.1. Amphetamines: Behavioral Effects

Amphetamine is a psychostimulant, a member of a class of drugs which have been used throughout history and in many cultures for their central effects (Hoffman and Lefkowitz, 1990). Other agents include ma-huang (ephedrine) from China and cocaine from South America (Robinson and Becker, 1986; Hoffman and Lefkowitz, 1990; Gold *et al.*, 1992; King and Ellinwood, 1992). Shortly after amphetamine had been synthesized by Edeleano in 1887, its central stimulant properties were recognized (Edeleano, 1887; Stern, 1889; Airila, 1913). These properties include feelings of increased energy, euphoria, decreased appetite and insomnia (Hoffman and Lefkowitz, 1990; Gold *et al.*, 1992; King and Ellinwood, 1992). In addition, amphetamine abuse has also been shown to lead to altered individual and social behaviors such as repetitive, aimless movements, perseverative speech, social withdrawal and paranoia (Ellinwood *et al.*, 1973; Schiørring, 1977).

The administration of psychostimulants to rodents has proven to be an important model system for investigating the biochemical and neuroanatomical basis of psychostimulant-induced behaviors. As discussed below, amphetamine produces motor behaviors in rodents which are similar to many of the central effects of drug administration to humans. Although individual variability in the behavioral response of rodents to single doses of amphetamine has been documented (Segal and Kuczenski, 1987a,b) in general, administration of less than 1.5 mg/kg of amphetamine produces increased horizontal movements, doses between 1.5 mg/kg and 2.5 mg/kg produce horizontal movements interspersed by brief episodes of stereotyped behaviors, while doses above 2.5 mg/kg lead

to a multiphasic response pattern. This multiphasic response pattern involves a duration of continuous, intense stereotypy, preceded and followed by periods of increased horizontal locomotion (Segal and Schuckit, 1983; Segal and Kuczenski, 1994).

There are two stereoisomers of amphetamine, *d*- amphetamine sulfate and *l*- amphetamine sulfate, and these differ in the behavioral effects which they elicit in rodents. The relationship between dose and behavioral effect, referred to as the cumulative locomotion relationship (CL), has been used to compare the behavioral potencies of both isomers. At equal CL values, *d*- amphetamine produces increased locomotion earlier than *l*- amphetamine, and subsequently, rats enter the "stereotypy" phase earlier with *d*- amphetamine than with *l*- amphetamine. However, the duration of the stereotypy phase is identical for both drugs given at equal values for CL (Segal, 1975). References to amphetamine in this thesis refer to the *d*- isomer.

1.2. Amphetamines: Neurochemical Effects

Evidence from experiments employing pharmacological techniques and neurotoxic lesioning, in conjunction with behavioral studies, all support a role for dopamine as a crucial neurotransmitter in the production of amphetamine-mediated behaviors. Amphetamine produces increased extracellular dopamine in the striatum and prefrontal cortex of rodents (Sharp *et al.*, 1987; Kuczenski *et al.*, 1991; Guix *et al.*, 1992). Unilateral injection of the catecholamine-specific neurotoxin 6-hydroxydopamine (6-OHDA) into the dopaminergic mesencephalon, followed by dopamine receptor agonism, has been demonstrated to produce directional movement which reflects imbalanced dopaminergic

neurotransmission (Paul *et al.*, 1992). Amphetamine administration to unilaterally dopamine-depleted rats leads to ipsiversive rotation (towards the lesioned hemisphere), due to comparatively greater dopamine efflux contralateral to the lesion, which can be antagonized by dopamine receptor antagonists (Pijnenburg *et al.*, 1975a,b; Arnt and Hyttel, 1984; Yurek and Hipkens, 1993). Lastly, studies in which basal ganglia dopaminergic terminals are bilaterally destroyed have demonstrated that dopaminergic neurotransmission in the nucleus accumbens and striatum are essential for the production of horizontal locomotion and stereotypy following amphetamine administration (Kelly *et al.*, 1975; Kelly and Iversen, 1976).

Amphetamine administration also influences the activity of non-dopaminergic systems, an effect which may also contribute to the motor behaviors associated with the drug. Amphetamine causes a dose-dependent increase in extracellular norepinephrine in rodent cortex (Arnold *et al.*, 1977; Kuczenski and Segal, 1992). Amphetamine has been shown to be a potent catecholamine uptake inhibitor (Raiteri *et al.*, 1974). However, the role of noradrenergic neurotransmission in the behavioral effects of amphetamine in rodents is controversial. It is unlikely that blockade of reuptake of norepinephrine is responsible for the behavioral effects of amphetamine, as amphetamine also reduces the firing rate of locus coeruleus noradrenergic neurons (Graham and Aghajanian, 1971; Engberg and Svensson, 1979; Ramirez and Wang, 1986), which would act to reduce the basal concentration of norepinephrine. One study has shown that the locomotor behavior which follows direct bilateral administration of amphetamine into the rat nucleus accumbens is attenuated through blockade of cortical alpha-adrenergic receptors (Blanc

et al., 1994). On the other hand, central lesioning of noradrenergic neurons has been shown to have only minimal effects on amphetamine-mediated locomotion (Archer *et al.*, 1986).

Amphetamine's effect on serotonergic systems appears to contribute only modestly to its behavioral effects. Electrolytic lesions of the dorsal and median raphé nuclei were found to potentiate the locomotor effects of amphetamine (up to 3 mg/kg, i.p.) (Neill *et al.*, 1972). Although in one study serotonin depletion with *p*-chlorophenylalanine was shown to attenuate certain amphetamine-induced stereotypies, the doses of amphetamine used were extremely high (up to 80 mg/kg), and several animals in the experiment died of apparent acute amphetamine toxicity (Sloviter *et al.*, 1978). The precise roles norepinephrine and serotonin play in the behaviors associated with amphetamine administration can only be established by further research.

1.3. Immediate Early Gene Induction

Experimental evidence clearly supports a role for increased extracellular dopamine in the production of many of the behavioral effects of amphetamine in rodents. The question which arises is that of how to investigate the molecular mechanisms by which specific neuronal pathways are influenced by an amphetamine-mediated dopamine efflux. One way to study the effects of a specific neurochemical change involves the detection of a neuron-specific protein (or its mRNA). For example, a large percent of the neurons which project from the striatum to the globus pallidus contain the neuropeptide enkephalin, which has been demonstrated to be upregulated following dopamine depletion (Kubota *et al.*, 1986; Gerfen *et al.*, 1991; Ingham *et al.*, 1991; Kowalski and Giraud,

1993). However, it cannot be assumed that all stimuli which modulate the striatopallidal neuron affect enkephalin transcription. Furthermore, since enkephalin is strictly localized to a subpopulation of neurons (Le Moine *et al.*, 1990a), it cannot be used to map neuronal pathways.

On the other hand, the direct detection of a substance which is common to many neuronal populations and whose production has been directly correlated with dopamine receptor subtype activation would provide information concerning the patterns of neurons which respond to the stimulus. It is for this reason that the present investigation has focused on amphetamine-mediated induction of the immediate early gene *c-fos*, and its dependence on dopamine receptor subtype activation.

Immediate early genes are transiently expressed following certain stimuli in neuronal and nonneuronal cells, and may represent a rapid cellular response to that stimulus. The immediate early gene *c-fos* encodes a protein, Fos, which is a transcriptional activator (Sheng and Greenberg, 1990). This protein can combine with the Jun protein via a leucine zipper motif to activate specific elements on the DNA directly, at AP-1 (activator protein-1) sites in the control regions of many genes (Chiu *et al.*, 1988; Curran and Franza, 1988; Kouzarides and Ziff, 1988; Sassone-Corsi *et al.*, 1988). For example, the expression of both proenkephalin mRNA and neurotensin/neuromedin N are increased by the induction of the immediate early gene *c-fos* in rodents (Sonnenberg *et al.*, 1989; Robertson *et al.*, 1995).

Many stimuli have been shown to induce the immediate early gene *c-fos* in rodents, including seizures, kindling simulation, peripheral stimulation, nerve injury, ischemia, nociceptive stimulation and osmotic stress (Dragunow and Robertson, 1987a,b; Hunt *et al.*, 1987; Morgan *et al.*,

1987; Bullitt, 1989; Onodera *et al.*, 1989; Sharp *et al.*, 1989; Bullitt, 1990; Popovici *et al.*, 1990; Williams *et al.*, 1990; Anton *et al.*, 1991; Jones and Evinger, 1991; Sharp *et al.*, 1991; Uemura *et al.*, 1991). This induction occurs in distinct neuronal populations depending upon the stimulus, and reflects the activation of different neurotransmitter systems in specific neuroanatomical pathways.

The pattern of induction of *c-fos* in medium-sized, densely spiny neurons of the rodent striatum following the administration of indirect and direct dopaminergic ligands has been well characterized. In order to understand the relationships between dopamine receptor activation in the striatum and induction of *c-fos*, it is first necessary to review the heterogeneity of dopamine receptor subtypes and their known effects on cellular physiology.

1.4. Dopaminergic Effector Mechanisms

Since 1958, when it was first reported that dopamine could act as a neurotransmitter in the brain, there has been an intensive investigation into the mechanisms by which cells recognize and respond to extracellular dopamine (Carlsson *et al.*, 1958). Even before the cloning of dopamine receptor subtypes, the regulation of adenylate cyclase by dopamine had been observed (Kebabian, 1978). Five dopamine receptors (D1 - D5) belonging to a large family of G-protein coupled receptors (GPCR's) have been cloned to date. Through G-protein association, ligand binding modulates the production of second messengers, such as cyclic AMP, inositol triphosphate and diacylglycerol (Probst *et al.*, 1992).

The D2 receptor was the first dopamine receptor to be cloned, using a probe generated from the beta-adrenoceptor (Bunzow *et al.*, 1988). Using

nonradioactive *in situ* hybridization histochemistry, Brouwer *et al.* (1992) documented the presence of D2 dopamine receptor mRNA in the rat caudate-putamen, ventral striatum (olfactory tubercle and nucleus accumbens) and deep layers of the frontal cortex where dopaminergic projections from the ventral tegmental area are known to terminate. This is consistent with hybridization results using radiolabeled probes (Fox *et al.*, 1993; Gaspar *et al.*, 1995). Although *in situ* hybridization only indicates that these regions transcribe the gene encoding the D2 receptor, these results are in accord with ligand binding data using [³H]quinpirole, which binds to striatal D2 receptors (and D3 and D4 receptors) with nanomolar affinity (Mansour *et al.*, 1990; Levant *et al.*, 1992; Seeman *et al.*, 1992).

Within the striatum, the D2 receptor is expressed in enkephalinergic, striatopallidal, medium-sized, densely-spiny neurons (Gerfen *et al.*, 1990; Le Moine *et al.*, 1990a), though their presence on D1-containing striatonigral neurons may be significant. This is supported by studies which demonstrate colocalization of mRNAs for the D1 and D2 receptors using radiolabeled oligonucleotide probes (Lester *et al.*, 1993) as well as quantifiable protein as demonstrated by D2 antibody immunohistochemistry combined with retrograde labeling (Ariano *et al.*, 1992). In the dopaminergic mesencephalon, many D2 receptors are found on TH-immunolabeled dendrites, while in the striatum, D2 receptors are localized to striatal dendrites in contact with TH-immunoreactive terminals, and, occasionally, on those terminals themselves (Sesack *et al.*, 1994).

The D2-like subfamily of dopamine receptors have been postulated as having an inhibitory effect on the neurons which express them. D2-

like receptor agonism increases potassium conductance resulting in a net hyperpolarization (Lacey *et al.*, 1987; Freedman and Weight, 1988; Zoltay and Cooper, 1994). Hyperpolarization decreases cellular calcium influx via voltage-gated Ca^{2+} channels, and hence decreases Ca^{2+} -dependent neurotransmitter release (Kelly, 1993). A component of the dopaminergic inhibition of neurotransmitter release may also be due to closure of voltage-independent calcium channels (Merritt and Brown, 1984; Memo *et al.*, 1985; Memo *et al.*, 1986). Lastly, the D2 receptor has been shown to inhibit adenylate cyclase in synaptosomal preparations of brain tissue and in *in vitro* transfection systems, through association with an inhibitory G-protein (Battaglia *et al.*, 1985; Onali *et al.*, 1985; Cooper *et al.*, 1986; Pizzi *et al.*, 1987; Senogles *et al.*, 1987; Senogles *et al.*, 1990; Senogles, 1994).

The exact role that the D3 receptor subtype plays in dopaminergic transmission is not certain, in part due to a lack of selective pharmacological agents (Levesque *et al.*, 1992; Large and Stubbs, 1994). Furthermore, there is conflicting information concerning the distribution of this receptor. The expression of D3 mRNA has suggested that this receptor may be localized to "limbic" regions of brain in rat and human, in particular the nucleus accumbens and the Islands of Calleja of the olfactory tubercle (Landwehrmeyer *et al.*, 1993; Le Moine and Bloch, 1996). This expression in the Islands of Calleja is supported with a binding study utilizing [3H] 7-OH-DPAT as a D3-specific ligand (Levesque *et al.*, 1992). However, investigation of the distribution of the D3 receptor protein suggests a more widespread distribution (Ariano and Sibley, 1994).

The D4 dopamine receptor is the other member of the D2-like subfamily of dopamine receptors. The purported role of the D4 receptor has been hypothesized based upon the localization of its mRNA and the

unique characteristics of the antipsychotic drug clozapine, a D4 receptor-selective antagonist ($K_i = 21$ nM) (Seeman and Van Tol, 1994). In both the rat and monkey brain, the distribution of D4 receptor mRNA is predominantly localized to limbic and cortical regions, with significantly lower concentrations in the basal ganglia (Van Tol *et al.*, 1991; O'Malley *et al.*, 1992; Strange, 1994). The latter is supported by data showing immunostaining with D4-selective antisera (Mrzljak *et al.*, 1996).

Tissue localization by *in situ* hybridization shows that the caudate-putamen, nucleus accumbens and olfactory tubercle represent areas of high D1 receptor expression, although this receptor can also be found in the cerebral cortex, hypothalamus, and other regions of the limbic system (Dearry *et al.*, 1990; Monsma *et al.*, 1990; Fremeau *et al.*, 1991; Weiner *et al.*, 1991). Within the striatum, most of the neurons expressing the D1 receptor subtype belong to the striatonigral projection system (Gerfen *et al.*, 1990).

The D1 dopamine receptor is coupled to the stimulatory G protein (G_s), and in a variety of tissue preparations, dopamine or the D1 dopamine receptor agonist SKF38393 has been demonstrated to produce a dose-dependent increase in adenylate cyclase activity (Stoof and Kebabian, 1981; Onali *et al.*, 1985). With regard to regulation of ion channels, indomethacin has been shown to block the potassium efflux due to dopaminergic agonism, suggesting that cyclooxygenases may be an intracellular messenger through which dopaminergic neurotransmission modulates potassium channels (Zoltay and Cooper, 1994). This is supported by evidence in smooth muscle cells that arachidonic acid can activate potassium channels (Ordway *et al.*, 1989).

Lastly it should be noted that the D5 receptor has been cloned from a human genomic library and was found to be D1-like in pharmacology. Although it has been shown to exhibit a greater affinity for dopamine than the D1 receptor subtype, its extremely low level of expression (predominantly in limbic regions of brain) suggests that this receptor may play only a minor function in dopaminergic transmission as compared to the D1 receptor subtype (Grandy *et al.*, 1991; Sunahara *et al.*, 1991).

1.5. Dopamine Receptor-Dependent Induction of the Immediate Early Gene *C-Fos*

The dopamine-mediated induction of *c-fos* in the neurons of the rat striatum has been characterized through a combination of pharmacological tools, immunocytochemistry for the Fos protein and retrograde labeling from two regions in which striatal neurons terminate. The amphetamine induction of *c-fos* in the striatum; (1) occurs preferentially in neurons of the patch compartment which likely project to the substantia nigra, (2) is dependent upon D1 dopamine receptor activation and (3) is dependent upon D2 dopamine receptor activation (Gerfen, 1985; Gerfen and Young, 1988; Graybiel *et al.*, 1990; Berretta *et al.*, 1992; Ruskin and Marshall, 1994). The D1 dopamine receptor dependence of *c-fos* is consistent with experimental evidence involving *in situ* hybridization demonstrating that D1 dopamine receptors are preferentially localized to striatonigral neurons (Le Moine *et al.*, 1991). However, D1 dopamine receptor activation is necessary but insufficient for the induction of *c-fos* in striatonigral neurons (Robertson *et al.*, 1989a,b). The induction of *c-fos* in striatonigral neurons requires an additional dopaminergic stimulus such as D2 dopamine receptor

activation, or even the creation of a heightened state of sensitivity as produced by dopamine-depletion (Robertson *et al.*, 1992; Ruskin and Marshall, 1994).

Evidence from studies investigating the electrophysiological effects of amphetamine on striatonigral neurons does not exclude the possibility that the induction of *c-fos* coexists with neuronal activation. For example, amphetamine has been shown to increase the firing rate of some striatal neurons, while decreasing the firing rate of other striatal neurons (Gardiner *et al.*, 1988; Haracz *et al.*, 1989; Ryan *et al.*, 1989; Rosa-Kenig *et al.*, 1993). Since the striatopallidal neuron predominantly expresses the D2 dopamine receptor subtype (Le Moine *et al.*, 1990a) which has been discussed to have an inhibitory effect on neuronal firing rate (Lacey *et al.*, 1987; Freedman and Weight, 1988; Zoltay and Cooper, 1994), it is likely that the amphetamine-mediated excitation of striatal neurons occurs in the population projecting to the substantia nigra. Just as D1 or D2 dopamine receptor antagonism has been shown to block the amphetamine-mediated striatal induction of *c-fos* (Berretta *et al.*, 1992; Ruskin and Marshall, 1994), D1 or D2 dopamine receptor antagonism blocks the amphetamine-mediated increase in the firing rate of striatal neurons (Rosa-Kenig *et al.*, 1993). There is also evidence that the effects of dopamine receptor activation on the activity of striatal neurons may be a modulatory rather than a stimulatory influence, as the increased firing rate produced by dopamine is dependent upon glutamatergic cortical input (Tschanz *et al.*, 1991) and the induction of *c-fos* by dopaminergic agents is dependent upon glutamatergic stimulation (Dragunow *et al.*, 1991; Ziolkowska and Holtt, 1993).

There is some evidence that the Fos protein may be a necessary component for psychostimulant-induced locomotion. Elimination of the Fos protein through intrastriatal or intraaccumbens injections of antisense oligodeoxynucleotides against *c-fos* mRNA has been demonstrated to interfere with the motor effects of amphetamine and cocaine (Heilig *et al.*, 1993; Hooper *et al.*, 1994). In these experiments it is important to note that Fos antisense was injected many hours prior to the administration of amphetamine or cocaine, and it is possible that mechanisms are induced to compensate for the lack of striatal Fos which may interfere with movement. For example, an important factor which has been demonstrated to influence the rodent behavioral response to amphetamine, particularly the locomotor sensitization that is associated with repeated drug administration, is the state of stress of the animal. This relationship has been studied through disruption of the hypothalamo-pituitary-adrenal (HPA) axis (a neuroanatomical circuit integral to the mammalian stress response) and by the implementation of various stressors such as food deprivation or foot shocks (Campbell and Fibiger, 1971; Carroll *et al.*, 1979; Antelman *et al.*, 1980; De La Garza *et al.*, 1981; Piazza *et al.*, 1990; Cador *et al.*, 1993). It is possible that removal of the Fos protein may interfere with the normal role of the HPA axis in psychostimulant-induced movement.

1.6. Amphetamines: Mechanism of Action

Amphetamine administration to rodents produces neurochemical changes which modulate the anatomical pathways subserving movement. One event which has been discussed to play an important part in amphetamine-mediated motor behaviors is subcortical and

cortical dopamine efflux. Understanding the molecular mechanisms by which amphetamine produces efflux of dopamine provides insight into physiological factors which affect amphetamine-mediated behavior. An important issue is how amphetamine is taken up by neurons. Active uptake of amphetamine via a transporter protein implies that sequestration of the drug in neuronal terminals may be restricted by the expression of this protein and dependent upon the ionic milieu and the state of energy of the cell. Once in the cell, how is dopamine released? Amphetamine-stimulated exocytosis would deplete stored dopamine, while a non-exocytotic mechanism might be dependent upon ongoing dopamine synthesis. Elucidating the nature of the post-synaptic effects of amphetamine-mediated dopamine efflux clearly involves characterizing the mechanisms by which amphetamine produces increased extracellular dopamine.

Much insight concerning how amphetamine (and dopamine) is taken up from the extracellular milieu began with the cloning of the dopamine transporter (Giros *et al.*, 1991; Kilty *et al.*, 1991; Shimada *et al.*, 1991). In situ hybridization evidence supports the role of this protein as a dopamine transporter, as its mRNA is found in the tyrosine hydroxylase (TH)-expressing cells of A9 and A10, regions defined by Dahlström and Fuxe as dopaminergic (Augood *et al.*, 1993; Cerruti *et al.*, 1993; Blanchard *et al.*, 1994). Dopamine uptake by the plasma membrane dopamine transporter is highly dependent upon the ionic milieu. Reducing the extracellular concentration of sodium through addition of lithium causes a dose-dependent decrease in the uptake of [³H]dopamine (Giros *et al.*, 1991; Giros and Caron, 1993), while inhibition of the Na⁺/K⁺ ATPase with ouabain reverses the direction of the sodium flux and produces a

carrier-mediated release of dopamine (Sirinathsinghji *et al.*, 1988; Hurd and Ungerstedt, 1989a). Similarly, the process of sequestering amphetamine in the dopaminergic neuron terminal is an active process requiring the dopamine transporter and the Na⁺/K⁺ ATPase. This has been determined largely through the creation and use of synaptosomal preparations of brain tissue. Pharmacological blockade of the dopamine transporter has been shown to interfere with the uptake of radiolabeled amphetamine in rat striatal synaptosomes (Zaczek *et al.*, 1991a,b) or tissue slices (Liang and Rutledge, 1982). Amphetamine uptake by striatal synaptosomes is saturable and sodium dependent, further supporting this hypothesis that amphetamine uptake occurs via a plasma membrane catecholamine transporter using the Na⁺/K⁺ ATPase as a source of energy (Liang and Rutledge, 1982; Zaczek *et al.*, 1990; Zaczek *et al.*, 1991a,b).

Several lines of evidence support a non-exocytotic mechanism for amphetamine-mediated catecholamine release. Normal vesicle degranulation (exocytosis) is a calcium-dependent process (Kelly, 1993), while amphetamine-induced dopamine release is calcium-independent (Hurd and Ungerstedt, 1989b). Unlike other psychostimulants such as cocaine, the central effects of amphetamine are resistant to the catecholamine-depleting drug reserpine, suggesting that amphetamine-induced dopamine efflux may be dependent upon newly synthesized dopamine (Bertler, 1961; Callaway *et al.*, 1989). The latter is supported by evidence which shows that inhibition of the synthesis of TH attenuates the central effects of amphetamine (Joensson *et al.*, 1969).

Exocytotic neurotransmitter release is dependent upon the number of filled vesicles which may fuse with the plasma membrane and release their contents, while non-exocytotic release of neurotransmitter is

dependent upon the cytosolic concentration of neurotransmitter (Kelly, 1993). As the concentration of amphetamine in the cytoplasm rises, dopamine is displaced from cytosolic binding sites and therefore the amount of releasable, free, cytosolic dopamine is increased (Liang and Rutledge, 1982). This “exchange diffusion model” was first proposed by Fischer and Cho in 1979. One explanation for how amphetamine may produce increased cytosolic levels of catecholamines is the weak base hypothesis. The weak base hypothesis suggests that amphetamine, a weak base, reduces the pH gradient between the synaptic vesicles and the cytoplasm, disrupting catecholamine packaging. The resulting increased cytoplasmic catecholamine levels could exit the neuron terminal through a reversal of the transporter molecule. Evidence that other weak bases which reduce the pH gradient between synaptic vesicles and the cytoplasm also produce catecholamine release support the weak base hypothesis (Sulzer *et al.*, 1993). However, these other weak bases differ from amphetamine in that they lead to the production of a large concentration of the dopamine metabolite DOPAC, suggesting that these compounds may be acting differently from amphetamine (Sulzer *et al.*, 1993).

Lastly, the finding that mice which have been genetically engineered to lack the dopamine transporter show no locomotion or dopamine release following administration of amphetamine supports a role for this protein in the mechanism of amphetamine’s action (Giros *et al.*, 1996). Two possible explanations for this lack of responsiveness to amphetamine in dopamine transporter-deficient mice are a decreased ability of amphetamine to enter dopaminergic nerve terminals and an

inability of an amphetamine-mediated increase in cytoplasmic dopamine to be released.

1.7. Cortical-Subcortical Motor Circuits

Investigations into the mechanisms of action of amphetamine have focused attention on mesostriatal dopamine efflux. The rodent caudate-putamen contains a high concentration of dopaminergic terminals and dopamine receptors (Arluison *et al.*, 1984; Freund *et al.*, 1984; Hökfelt *et al.*, 1984; Dearry *et al.*, 1990; Gerfen *et al.*, 1990; Le Moine *et al.*, 1990a; Monsma *et al.*, 1990; Fremeau *et al.*, 1991; Weiner *et al.*, 1991), a region in which *in vivo* microdialysis techniques have demonstrated an amphetamine-mediated catecholamine efflux (Sharp *et al.*, 1987; Kuczenski *et al.*, 1991; Guix *et al.*, 1992). Furthermore, lesioning the dopamine projection to the striatum leads to profound motor dysfunction (Creese and Iversen, 1972; Creese and Iversen, 1973; Kelly *et al.*, 1975; Kelly and Iversen, 1976). However, in order for the amphetamine-induced striatal dopamine efflux to translate into locomotion or stereotyped behaviors, as discussed earlier in this section, the modulation of striatal neurons must be communicated to the cortex. The pathways through which amphetamine-mediated dopaminergic neurotransmission influence cortical regions subserving motor behavior is the subject of this section.

Motor information is processed through anatomical circuits which transmit information from the cortex to the basal ganglia, to the motor thalamus and ultimately back to the cortex, influenced by modulatory information from the cerebellum and brainstem, by corticocortical connections, and by information from the periphery (Alexander *et al.*,

1986; Alexander and Crutcher, 1990; Groenewegen *et al.*, 1991; Parent and Hazrati, 1995). In order to understand how an amphetamine-mediated dopamine efflux may affect the activity of given neuronal circuits, these pathways must be defined both anatomically and neurochemically.

The innervation of the rodent basal ganglia by the cortex represents a site of tremendous convergence (Gerfen, 1984; McGeorge and Faull, 1987). One way to understand the organization of this diverse projection system is within the context of the intrinsic arrangement of the striatal medium-sized, densely spiny neurons; the efferent cells of the striatum. Anatomical compartmentalization of the striatum exists in the adult rodent and is present early in development with the emerging dopamine system. Dopaminergic innervation of the developing striatum begins with the appearance of microscopic "islands" which have a high level of expression of TH and acetylcholinesterase (Olson *et al.*, 1972; Graybiel, 1984). As the animal matures, these "islands" gradually lose acetylcholinesterase activity and two immunohistochemically distinct compartments emerge. The striosomal compartment lacks acetylcholinesterase immunohistochemical labeling, while the matrix compartment shows labeling for acetylcholinesterase (Graybiel and Ragsdale, 1978). It should be noted that the patch striatal compartment was also defined immunohistochemically as having μ -opiate receptors (Graybiel *et al.*, 1981; Herkenham and Pert, 1981) while lacking the calcium-binding protein calbindin (Gerfen *et al.*, 1985), and exactly coincides with the striosomal compartment. As techniques for labeling neurons and neuronal compartments have evolved, it has become increasingly clear that these two compartments are fundamentally

different, both on the anatomical and the molecular level, and that the functional implication of these differences may be profound.

In order to understand how the widespread cortical input to the striatum is channeled to the basal ganglia output nuclei, several features of this projection system must be addressed. These include the cortical layers from which the corticostriatal neurons originate, the striatal region and the immunohistochemically-distinct compartment to which each neuronal subset projects. In addition, it is important to understand whether the projection system is unilateral or bilateral, and whether it represents the one terminal field of the neuron or an axon collateral. Studies which have addressed these issues were performed through discreet injections of anterograde tracer substances in cortical regions of interest, often in combination with injections of retrograde tracer substances in the striatum. However, if retrograde tracer molecules injected into the striatum are also taken up by axons traversing the striatum, it may be difficult to determine whether the cell bodies which contain the labeled material project to the striatum, or travel through the striatum to terminate in other brain regions. Therefore, because it is possible for these compounds to be taken up by "fibers of passage," these studies must be interpreted with caution.

In general, prelimbic cortex projects throughout the caudate-putamen while the sensory cortex projects primarily to the body and tail of the striatum (Donoghue and Herkenham, 1986). By combining the injection of anterograde tracers into cortical regions whose somatic region was defined by microstimulation, the striatal terminus of the motor cortex has been studied. Limb areas of motor cortex project to more dorsolateral regions of the striatum, as compared with regions which

subserve the face and mouth of the rodent, which are more ventrolaterally located. The vibrissae motor-frontal eye field projects more dorsomedially (Ebrahimi *et al.*, 1992).

By combining anterograde labeling with immunohistochemical delineation of the striatal compartments, compartment-selective projection systems have been defined. In one study, motor and somatosensory cortical regions project predominantly to the matrix compartment, while prelimbic cortical regions project primarily to the patch compartment. Of note is that, of these regions, all project bilaterally with the exception of the somatosensory cortex, which projects predominantly ipsilaterally (Donoghue and Herkenham, 1986). Analysis of another study suggests that it is not the region but the cortical lamina which predicts which striatal compartment will be the site of termination. Corticostriatal neurons from deep layer V and superficial layer VI were found to project predominantly to the patch compartment, while corticostriatal neurons from more superficial regions of cortex project predominantly to the matrix. The exact origin of compartment-selective corticostriatal projections is therefore still a matter of debate.

In general, the corticostriatal projection originates from superficial layer V in the cortex, regardless of whether this is the frontal, cingulate, parietal, insular or occipital cortex (Veening *et al.*, 1980). Furthermore, cortical cells which could be antidromically stimulated from both striata were labeled with HRP and found to project bilaterally. In other words, the bilateral nature of a cortical projection system might not only be due to the presence of some cells which project ipsilaterally and the presence of other cells which project contralaterally- a subpopulation of individual cortical layer V pyramidal neurons project bilaterally (Wilson, 1987).

The relationship between the activation of striatal neurons and the activation of thalamic neurons is complicated due to the many connections within the subcortical regions. Which subpopulations of neurons are activated or suppressed will affect how the thalamus is stimulated. Activation of striatal neurons can lead to release of the inhibitory neurotransmitter GABA on the output nuclei of the basal ganglia; the entopeduncular nucleus (EPN) and the substantia nigra pars reticulata (SNpr), through direct projections to these nuclei (Williams and Faull, 1985; Bolam and Smith, 1992). Alternatively, activation of striatal neurons may enhance GABAergic neurotransmission in the globus pallidus (GP) via striatopallidal neurons (Nagy *et al.*, 1978; Jones and Mogenson, 1980; Scarnati *et al.*, 1983; Smith and Bolam, 1990). Due to the known connectivity between the GP, the basal ganglia output nuclei and the subthalamic nucleus (STN), activation of the GP can release GABA on the output nuclei of the basal ganglia (von Krosigk *et al.*, 1992) or in the STN (Smith *et al.*, 1990). Lastly, the STN sends a glutamatergic (excitatory) input to the basal ganglia output nuclei (Nakanishi *et al.*, 1987; Smith and Parent, 1988; Robledo and Feger, 1990); hence attenuation of GABAergic tone on STN neurons would act to suppress an excitatory tone on the output nuclei.

Activation of the thalamus is dependent upon suppression of the basal ganglia output nuclei, as these neurons send a GABAergic input to the thalamus (Ueki *et al.*, 1977; Di Chiara *et al.*, 1979; Kilpatrick *et al.*, 1980; MacLeod *et al.*, 1980; Parent and Hazrati, 1995). Therefore, activation of GABAergic neurons which project directly to the output nuclei (striatonigral or striatoentopeduncular neurons) would lead to a net thalamic excitation. Alternatively, activation of neurons which project to

the globus pallidus would lead to a net inhibition of the thalamus, potentiated through the subthalamic nucleus.

The thalamic regions which receive basal ganglia input have been characterized through a variety of labeling techniques, including silver impregnation of damaged neurons, and the application of anterograde and retrograde tracer molecules. Based on these techniques, the substantia nigra pars reticulata/entopeduncular nucleus have been demonstrated to terminate in four thalamic regions: the ventromedial nucleus, the parafascicular nuclei, the lateral portion of the mediodorsal nucleus and the rostromedial portion of the ventrolateral nucleus (Di Chiara et al., 1979; Herkenham, 1979; Deniau *et al.*, 1992).

Studying the relationships between dopamine receptor pharmacology and the striatal induction of the immediate early gene *c-fos* has provided much information concerning the modulation of medium spiny neuron activity in the parallel pathways through which motor information is processed. Investigation of this relationship can be extended into the cortical areas subserving integrated locomotion and stereotyped behaviors in the rat, namely the primary motor and somatosensory cortices. Of particular interest, for the reasons discussed below, are the layer V pyramidal cells of the primary motor cortex.

The rat primary motor cortex (MI) can be defined anatomically based upon morphological, anatomical and electrophysiological parameters. MI in rat is defined as that region of the frontal or parietal lobes in which electrical excitation induces movement of any part of the rat's body (Donoghue and Wise, 1982). Known as an agranular region (as opposed to the somatosensory cortex which contains a granular layer IV), it is divided into medial and lateral compartments (Hall and Lindholm,

1974; Donoghue and Wise, 1982; Donoghue and Parham, 1983). The primary motor cortex (MI) receives much of its afferent input from the ventrolateral (VL) nucleus of the thalamus, with a restricted topographical distribution, inverted in the rostrocaudal axis (Cicirata *et al.*, 1986). It also receives input from the dysgranular field of the primary somatosensory cortex (SI), deep layers of the granular SI and the second somatic sensory area (SII) (Donoghue and Parham, 1983). Experiments using injections of the retrograde tracer horseradish peroxidase (HRP) into the spinal cord at the level of the cervical enlargement have shown definitively that the pyramidal cells of layer V represent the efferent component of the motor cortex (Donoghue and Wise, 1982).

Studies in which cortical ablations in rodents are correlated with behavioral deficits have helped to identify the role of the sensorimotor cortex in motor tasks, and the capability of the rodent brain to compensate for motor deficits. The rodent develops the ability to walk and run at about postnatal day 17. Unilateral lesions of the motor cortex immediately following birth, or bilateral lesions in the adult rodent, result in a loss of tactile placement during this transitional period (tactile placement is measured by observing rats maneuver across two narrow dowels). However, bilateral ablations in the newborn do not lead to deficits in tactile placement (although they do cause other locomotor deficits). This suggests that the motor cortex has an influence on the development of tactile placing in the normal rodent. Furthermore, only if this facilitatory input is entirely removed can other mechanisms be recruited to serve this function (Hicks and D'Amato, 1970; Hicks and D'Amato, 1975). Finally, deletion of the motor cortex in the mature rat, even bilaterally, has minimal effects on horizontal locomotion. However, unilateral

cortical lesions do produce a decrease in the force and rate of responding of contralateral limbs in tasks requiring fractioned, complex movements (Price and Fowler, 1981). The precise role of the motor cortex in integrated movement planning and execution is still unclear.

1.8. Central Aim of the Thesis

The presence of multiple motor pathways and dopamine receptor subtypes raise fundamental questions about what the molecular events are that enable amphetamine to influence the neuroanatomical circuitry subserving motor function. For example, if amphetamine induces *c-fos* in the striatum in a dopamine receptor subtype-specific manner, is this induction present in other regions of the motor circuit, namely the primary motor cortex? Is activation of both the D1 and the D2 receptor subtype necessary for the induction of *c-fos* in the cortex? In this thesis, pharmacological agents and molecular biological techniques were employed in order to answer these questions.

2.0. A Method for Reliable Quantitative Digital Analysis of Immunohistochemical Tissue Utilizing an Algorithm for Automated Threshold Selection

2.1. Abstract

Computer-based image analysis systems convert immunohistochemical staining into digital data. While this transformation holds the promise of facilitating the objective analysis of data, the discrimination between immunopositive signals and background in digital images is still usually dependent on subjective delineation. The approaches which have been proposed to eliminate subjectivity in threshold assignment for digital images have proven to be inappropriate for this application, principally because of the gradient in labeling intensity and the section-to-section variability typically encountered. In order to improve threshold selection in digitized images of immunohistochemically-stained tissue sections, an algorithm which minimizes observer-bias in assigning positive labeling has been developed and tested. The algorithm, which derives the threshold level from the intensity of representative background signal intensity, was designed to minimize the variability associated with quantification of immunohistochemical tissue with differing degrees of background staining or illumination. This approach was evaluated using an Apple MacIntosh / Adobe Photoshop-based image analysis system that has specific advantages in the study of tissue stained for multiple immunohistochemical markers. A comparison of the threshold for positive immunolabeling determined using the algorithm with that selected subjectively by skilled observers demonstrates that the algorithm is equally accurate and less variable than subjective assessment.

2.2. Introduction

Digital image processing represents an important advance in the analysis of tissue stained with immunohistochemical techniques. Conversion of a microscopic field into digital data allows accurate quantification as well as archival preservation of images from histological material. This quantification is often performed by image analysis software packages which provide pixel-by-pixel brightness information in the form of a gray scale histogram (Mize, 1994). The digital images that result can then be analyzed by applications which assess morphological features. Measurements can be made to determine the number of immunolabeled objects within a given area of a tissue section (Fuxe *et al.*, 1991), or the intensity of immunolabeling within a field (Enestrom and Kniola, 1990). With regard to staining preservation, since a single exposure of a tissue specimen is sufficient for image production, changes in the condition of the sample over time, such as quenching of immunofluorescence or altered optical density of chromophore, are irrelevant to the analysis.

Typical image analysis systems includes a light microscope equipped with a camera or photometric scanning device, a control processor unit with the necessary frame grabbing and image analysis software, peripheral monitors and a data storage device (Walter and Berns, 1986). The immunohistochemical marker is illuminated with an appropriate bright field or fluorescent light source, and a portion of the microscopic field is displayed on a monitor. Once this image has been converted to digital information and stored, each unit area, or pixel, is attributed a number that reflects the amount of immunoreactive product.

These intensity levels can be used to generate a gray scale histogram that serves as the basis for subsequent analysis.

A critical step in analysis of digitized images is the selection of a global intensity threshold value to distinguish positive from non-specific labeling. Each pixel of the digital image corresponds to a specific area on the tissue and has a specific gray scale value (Vennari, 1994). Each pixel can be judged as labeled or unlabeled by comparison with a specified intensity level, the threshold level (Tieman, 1989). Typically, threshold selection is based upon a visual assessment of the image. Unfortunately, such a subjective procedure generates significant variability, both between and within observers (Jagoe *et al.*, 1991). This is especially true in images where the marker of interest is present at a range of intensity levels (the gray scale histogram lacks clear peaks and valleys).

One approach to automating analysis of immunohistochemical tissue relies on the morphological features of the objects of interest. Filters can be created to recognize specific shapes or patterns such that nonspecific labeling can be distinguished from the stained target (Agnati *et al.*, 1982). Also, image enhancing operators can be applied with a range of effects such as sharpening, edge enhancement or contour tracing (McClelland, 1993). However, unless an overall global threshold level is also utilized, lightly and heavily-stained objects are not distinguished.

There are two crucial criteria in assessing the validity of a system for determining threshold levels: precision and accuracy. Precision represents the consistency with which an image analysis system evaluates data over repeated trials (Petrusz, 1983). However, even if a specific thresholding technique has greater precision than subjective determinations, the resulting thresholds may be quite unsatisfactory

(Jagoe *et al.*, 1991). The precision of an approach is independent of consistent errors of any magnitude, and it is for this reason that the accuracy of the technique must also be assessed. In the absence of a known criterion for positive staining or object recognition, the standard for accuracy depends on the consensus of expert observers. Therefore, the success of any system to automate and standardize digitized images is represented by the accuracy and precision of the technique. As the standard for accuracy is subjective, an automated system can never exceed the subjective accuracy. The goal is thus to improve reproducibility without sacrificing accuracy.

In the analysis of immunohistochemical tissue, classical approaches for thresholding have been shown to be effective for images without a gradient of labeling intensity; in other words, images which produce clear peaks and valleys on a gray scale histogram (Weszka, 1978). However, tissue stained with immunohistochemical techniques often displays many intermediate intensity levels. To circumvent this problem, an approach for intensity threshold selection has been developed which relies on the observer selecting background regions within each section. Identifying a region of background labeling in a section proves to be a highly reproducible operation. An empirically-derived mathematical algorithm is then utilized to calculate the threshold level for that image. This approach was tested in evaluating the immunohistochemical detection of the immediate early gene product Fos in the rat striatum following acute treatment with either cocaine or amphetamine (Graybiel *et al.*, 1990; Young *et al.*, 1991; Cenci *et al.*, 1992).

2.3. Materials and Methods

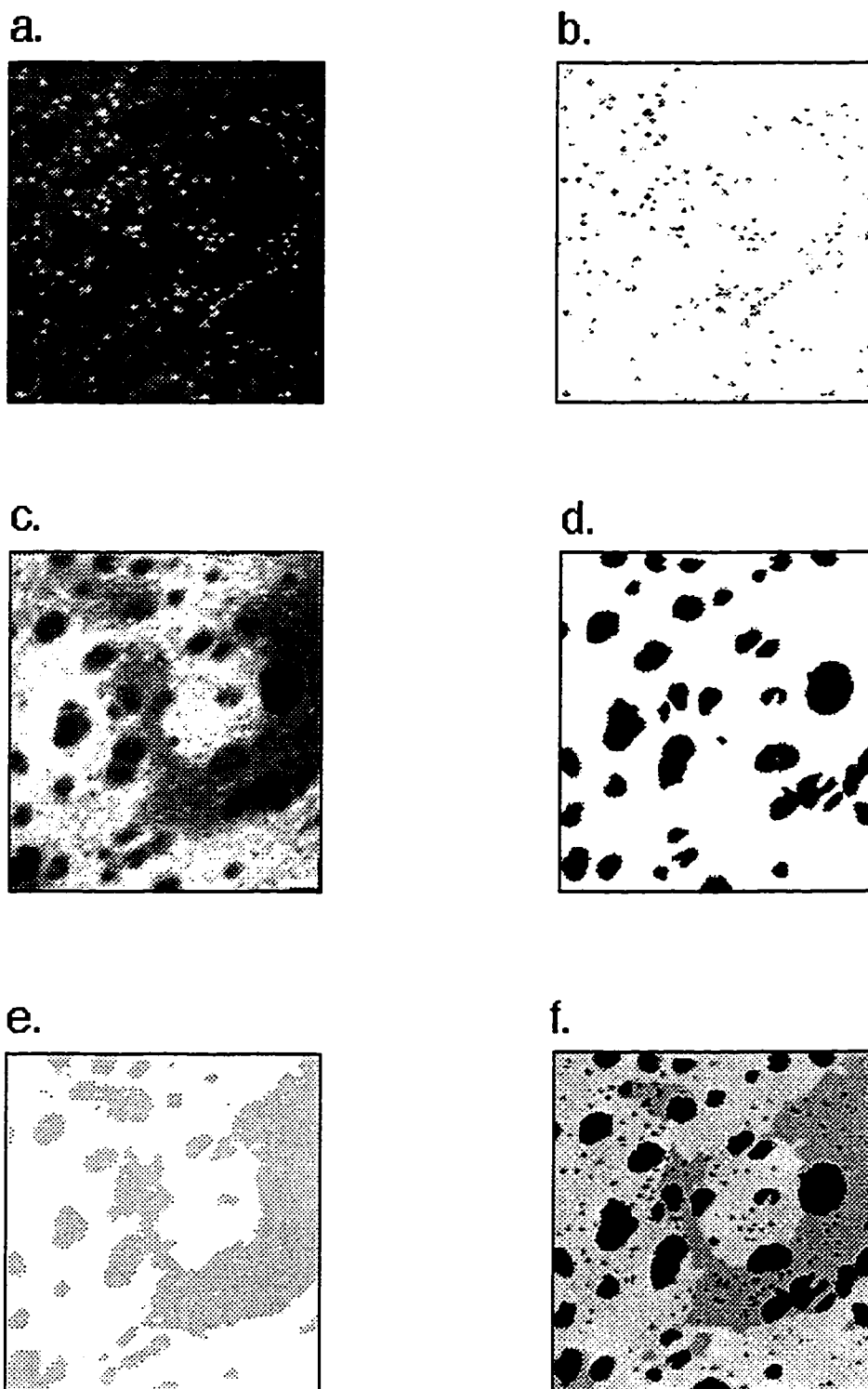
2.3.1. Experimental Design

The images used in this study represent Fos immunolabeling in the rat striatum or olfactory tubercle following treatment with the indirect dopamine agonists cocaine or amphetamine. Male Sprague Dawley rats (200-250 g) were treated with either S(+) -amphetamine sulfate (10 mg/kg or 20 mg/kg), cocaine hydrochloride (25 mg/kg) or saline 2 hours before transcardial perfusion with 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS, pH 7.4). Brains were removed and postfixed in 4% paraformaldehyde, cryoprotected in sucrose (15% and 30% in PBS) and frozen on dry ice. Tissue was cut into 20 μ m-thick sections on a sliding microtome, and stored in either storage solution (4.1 M glycerol, 5.4 M ethylene glycol, 0.02 M PBS) or 0.1 M PBS with 0.1% sodium azide (pH 7.4).

Free-floating sections were washed in 0.01 M PBS between incubations. The primary antibody solutions contain 0.1% Triton X-100 for cell permeabilization and bovine serum albumin (0.1 mg/kg) as a blocking agent. A rabbit polyclonal antibody to *c-fos* was used at a dilution of 1:500 (antibody cat # sc-52, Santa Cruz Biotechnology, Santa Cruz, CA), and a mouse monoclonal antibody to the calcium-binding protein calbindin (immunolabel shown in Figure 1C) was used at 1:1000 (Swant Laboratories, Bellinzona, Switzerland). Secondary antibodies were Texas Red-conjugated donkey antirabbit IgG and fluorescein-conjugated donkey antimouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), both used at dilutions of 1:200. Subsequent to immunolabeling, sections were mounted onto glass slides and

Figure 1: Generation of composition images of tissue stained with multiple immunohistochemical markers. This figure illustrates the process of converting digitized images of the same microscopic field into composite pictures in which each component is quantifiable. These images represent immunoreactivity for Fos and calbindin in the rat striatum. Image (a) shows Fos immunohistochemistry. Inversion of the image (such that the brightest fluorescence appears darkest) followed by thresholding results in image (b). Similarly, image (c) represents calbindin immunohistochemistry, and image (e) represents image (c) following the application of thresholding and smoothing filters. Fibers which course perpendicular to the plane of the tissue section were eliminated in image (d). Merging of images (b), (d), and (e) results in image (f), a composite image. Note that all four possible combinations of Fos and calbindin immunostaining are designated separate gray scale levels, as well as those regions to be eliminated from analysis, such that the pixel density of each component of the image can be quantified.

Figure 1



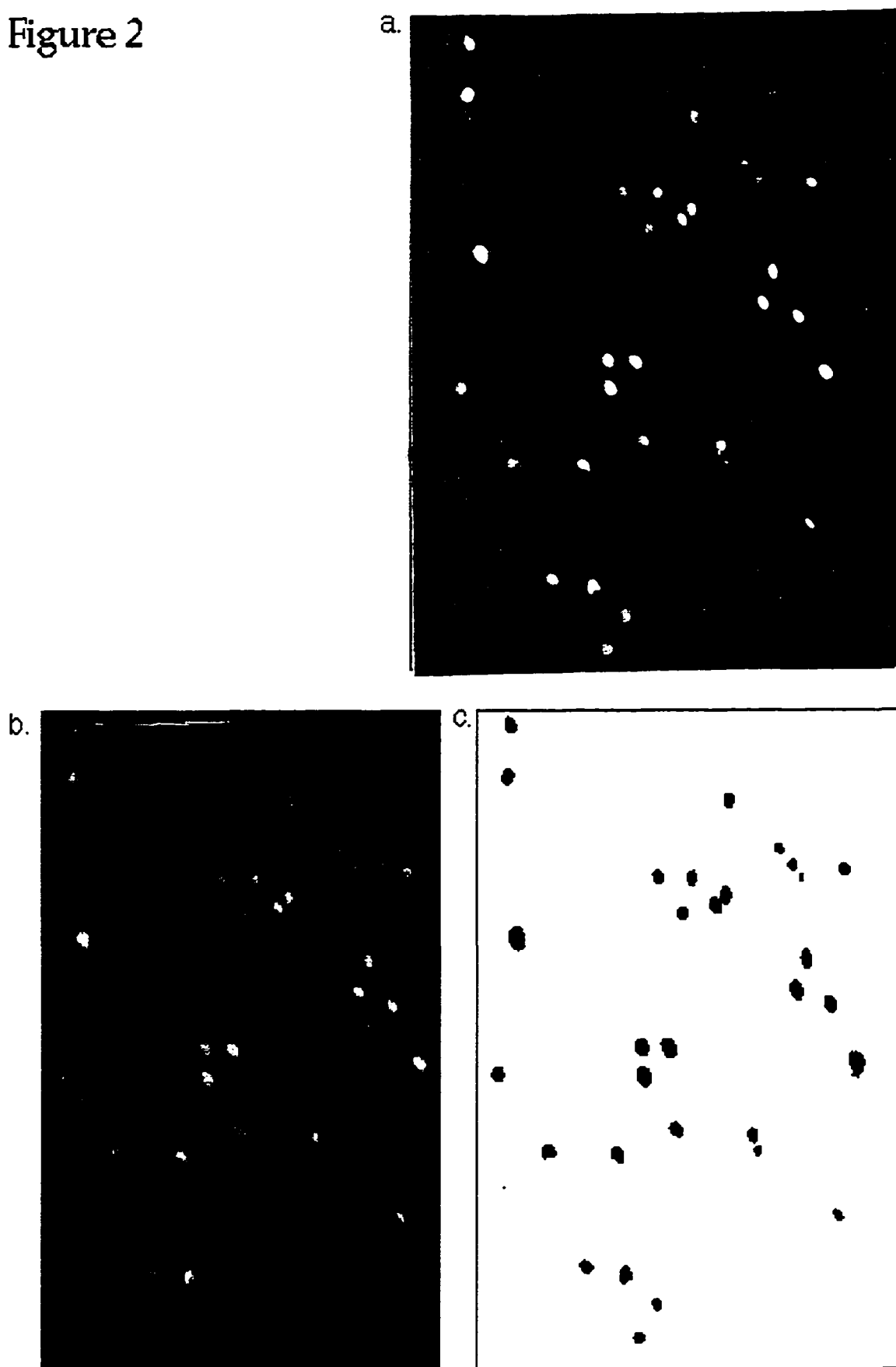
coverslipped with PermaFluor aqueous mounting media (Shandon Lipshaw, Pittsburgh, PA).

2.3.2. Digital Data Capture

Images from immunohistochemically-treated slides were captured using a Zeiss Axiophot photomicroscope equipped with a Zeiss MSP65 computer-controlled motorized stage (Zeiss, Oberkochen, Germany), a Zeiss ZVS-47E video camera (Zeiss, Thornwood, NY), a Sony Trinitron video monitor and a DEC 5000 workstation (Digital Equipment Corporation, Maynard, MA). Using a mercury bulb and specific filters, Texas Red- and FITC-labeled antibodies were visualized with illumination in the excitation ranges 510-560 nm and 450-490 nm respectively. The six images captured from the video monitor onto the computer had a resolution of 72 pixels per inch. The first image was digitized using a 20X Zeiss Plan Neofluar objective with a resolution of 0.6 μm /pixel in the x and y axes, while the remaining five images were digitized with a 10X Zeiss Plan Neofluar objective with a resolution of 1.5 μm /pixel in the x and y axes. Images were saved as PICT files and transported onto peripheral Macintosh computers. The files were opened as raw data into Adobe Photoshop 2.0.1. (Adobe Systems Inc., Mountain View, CA), an application designed for photographic manipulation. All image analysis and enhancement steps were performed with Photoshop. A comparison between a photograph and a digitized image of the identical field is presented in Figure 2, along with a map created from the digitized image.

Figure 2: Comparison of photograph, digitized image and map of fluorescent microscopic field. (a) Photograph of Fos immunohistochemistry from a rat treated with amphetamine (20 mg/kg, i.p.) at 200X magnification with Tmax 400 film (Eastman Kodak Company, Rochester, NY). (b) Identical microscopic field is digitized, and enhanced in Adobe Photoshop for contrast and brightness to best resemble microscopic image. (c) Map generated from image (b) using algorithm-derived threshold selection technique and application of a smoothing filter.

Figure 2



2.3.3. Application of Image Manipulation

Adobe Photoshop contains a wide variety of image enhancing features, and many other such "filters" are commercially available. Many of these features were made accessible with a single keystroke (Quickeys, CE Software Inc., West Des Moines, IA). Among the many filters useful for immunohistochemistry are "unsharp mask" which increases the resolution of edges, contrast and brightness adjustments, and noise reduction.

The ability to convert immunofluorescent tissue into thresholded, black-and-white (bitmap) images, and ultimately into color or gray scale representations is a major advantage of image analysis in Photoshop. In this way, multiple labeling can be clearly and efficiently presented and quantified. Positive and negative immunoreactivity (determined using the method described in Section 2.3.4) are designated different colors or gray scale levels both within an image and for different fluorophores or chromogens. The images can then be overlaid for comparative quantification, or for morphometric analysis (see Figure 1).

2.3.4. Determination of Threshold Setting

An algorithm was designed to correlate the selection of brightness threshold with the image background intensity. The user selects areas of background using any of the Adobe software palette tools for geometric shapes and chooses the histogram function. The mean intensity level of the selected area, as well as other relevant information such as the pixel density, is provided. The average mean intensity of several background selections is substituted for b in Equation 1, and the result, t , is the

threshold at which each image is to be set. Note that for this system, $C_2 = 100$ (see below).

Equation 1

$$t = C_1 \log b - CF$$

$C_1 = M/\log M$, where M = maximal grayscale level achieved by the display monitor.

CF (correction factor) = $C_2 - ((C_2/M)*b)$, where C_2 is an empirically-derived constant.

Immunohistochemical testing of this algorithm has demonstrated that this algorithm works well for cellular labeling such as calbindin staining in the rat olfactory tubercle, or nuclear labeling, such as the Fos immunohistochemistry shown in Figures 1 and 2. It is not designed for threshold selection of diffuse staining such as the calbindin immunoreactivity in rat striatum shown in Figure 1. Derivation of this equation and choice of C_2 are explained in the discussion.

2.3.5. Validation of Algorithm for Automated Threshold Selection

A study was performed to compare the accuracy and precision of the algorithm utilized for threshold determination with values selected subjectively. Four skilled observers were asked to view images three times at six-day intervals. The order of the images was randomized in each trial. For each image they were given two tasks: (1) select a threshold setting which best represents true immunolabeling, and (2) select two areas of background encompassing an area greater than 200 pixels.

The subjects were trained prior to the experiment about the nature of the immunoreactivity such that overt positively and negatively immunostained objects were clear. In order to generate the algorithm-derived threshold values, the mean intensity of the two background selections were averaged, and that value was used in the threshold selection algorithm. The resulting algorithm-derived threshold levels were compared with the threshold levels selected subjectively.

2.3.6. Statistical Analysis

A comparison of the subjective method versus the algorithm-derived threshold selection technique was performed for each image by estimating two measures of variability: the within-observer variability and the between-observer variability. The within-observer variability was estimated by the within mean square value (WMS) in equation 2:

Equation 2

$$WMS = \left(\sum_{i=1}^k \sum_{j=1}^n (y_{ij} - \bar{y}_i)^2 \right) / k(n-1)$$

k = total number of observers

n = number of trials for each observer (n is constant for all observers)

y_{ij} = threshold value for observer i, trial j

$\bar{y}_i$ = average threshold value for observer i

An F test was performed to compare the subjective and algorithm-derived values for each image. The degrees of freedom were set at 8 for both numerator and denominator ($k(n-1) = 8$).

The between-observer variability is strictly that variability due to multiple observers and thus cannot contain the variability which occurs with repeated trials for each observer. This is accomplished by subtracting the mean square for within-observer variability (explained in the preceding paragraph) from the mean square for between-observer variability (BMS), and dividing the resulting value by n , the number of trials. Negative values of BOV were considered to approximate zero and were given that value.

Equation 3

$$BMS = \left(\sum_{i=1}^k n \bar{y}_i^2 - \left(\left(\sum_{i=1}^k n \bar{y}_i \right)^2 / kn \right) \right) / k - 1$$

Equation 4

$$\text{Between observer variability (BOV)} = (BMS - WMS) / n$$

An F test was performed to compare the subjective and algorithm-derived values for each image, with the degrees of freedom set at 3 for both the numerator and the denominator ($k - 1$).

The accuracy of the algorithm-derived threshold selection technique was judged by creating a 95% confidence interval for the difference between values selected using the algorithm versus values subjectively selected. A confidence interval which is exclusively positive or negative would imply that the technique consistently over- or underestimates threshold values, respectively. For all of the images for each observer, the three trials were averaged. For each observer, the mean

for the algorithm-derived threshold selection technique for all six images was subtracted from the mean for subjective determinations and the standard error for the observed difference between the means was calculated. The 95% confidence interval is that range of values calculated from the difference between the means $\pm ((t_{0.025,10}) * (\text{standard error of the difference}))$.

2.4. Results

The algorithm developed for threshold selection (Figure 3) was tested by comparing the results generated using this approach with the threshold levels selected subjectively by skilled observers. Figure 4 demonstrates the within-observer variability. For four of the six images, algorithm-derived threshold selection showed a statistically significant decrease in the variability ($p < 0.01$). Note that the results are expressed in threshold units², as this is the measurement of variance.

Between-observer variability is shown in Figure 5, and represents only that variability introduced by multiple observers (within-observer variability is removed, as described in Section 2.3.6). For all six images tested, the algorithm-derived threshold selection method had decreased between observer variability, which was statistically significant for four of the six images ($p < 0.05$ for 1 image, $p < 0.01$ for 3 images). Hence for both sources of observer variability, the use of the algorithm produced highly consistent results.

Accuracy represents a measurement distinct from reliability, and equally important. Since Figures 4 and 5 established that the variability for the algorithm-derived threshold selection technique is acceptable and significantly improved from subjective determinations, accuracy in this

Figure 3

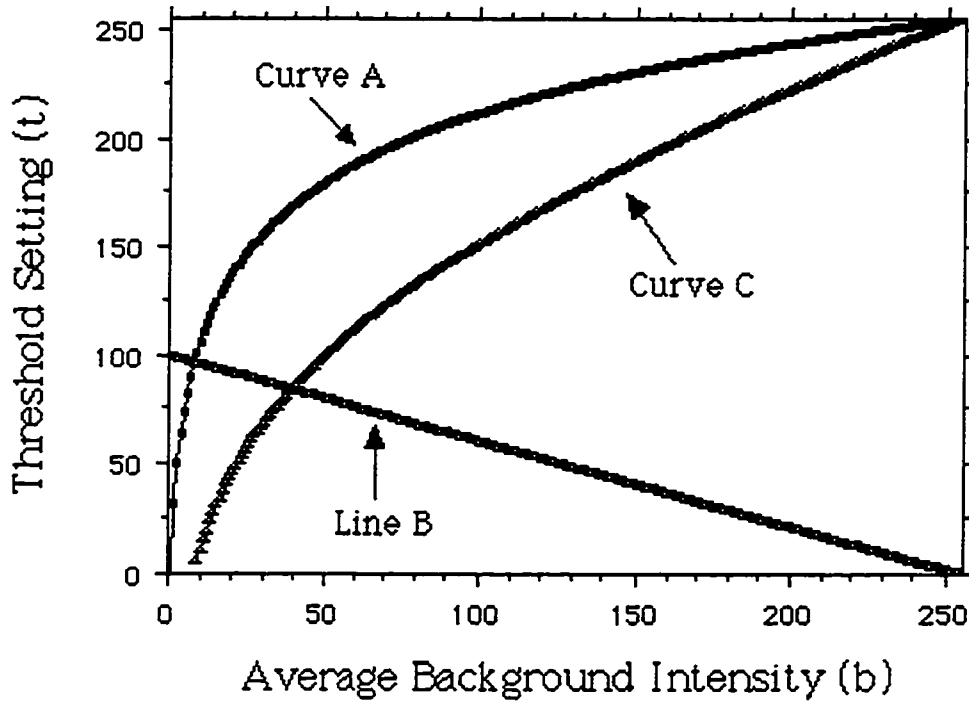


Figure 3: Algorithm-derived threshold selection. Areas determined to represent background on immunohistochemically-treated tissue are used to estimate the average background illumination intensity, b . Curve A shows the overall relationship between the average background intensity and the threshold setting for the image ($C_1 \cdot \log b$). Line B is a correction factor $CF = C_2 - ((C_2/M) \cdot b)$ which, when subtracted from Curve A, generates Curve C, the threshold algorithm: $t = C_1 \cdot \log b - CF$, where $C_1 = M/\log M$ and $C_2 = 100$.

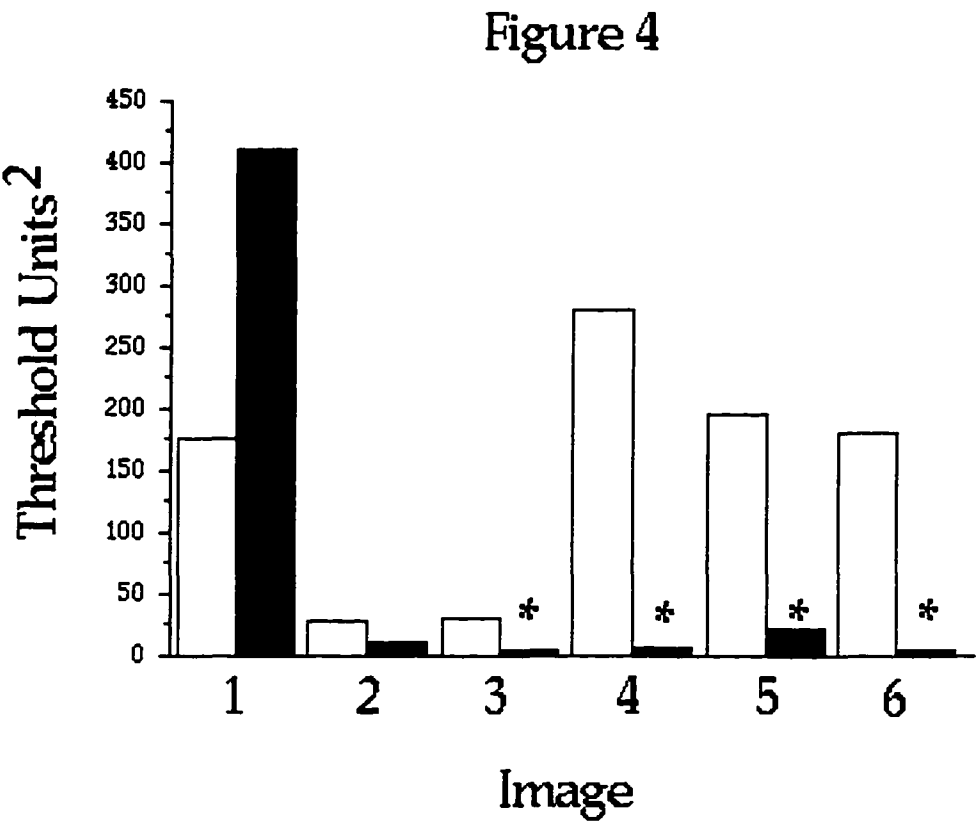


Figure 4: Within-observer variability for threshold selection techniques. Subjective threshold selection and algorithm-derived threshold selection are compared for the variability which occurs for each observer according to the formula presented in this chapter. The six images reflect Fos immunohistochemistry in the striatum or olfactory tubercle of rat. Each bar represents the within-observer variability for four observers, with three thresholding sessions per observer. Significant differences between the two techniques for each image was determined with an F test. White bars = subjective technique, black bars = algorithm-derived thresholding technique, * = $p < 0.01$.

Figure 5

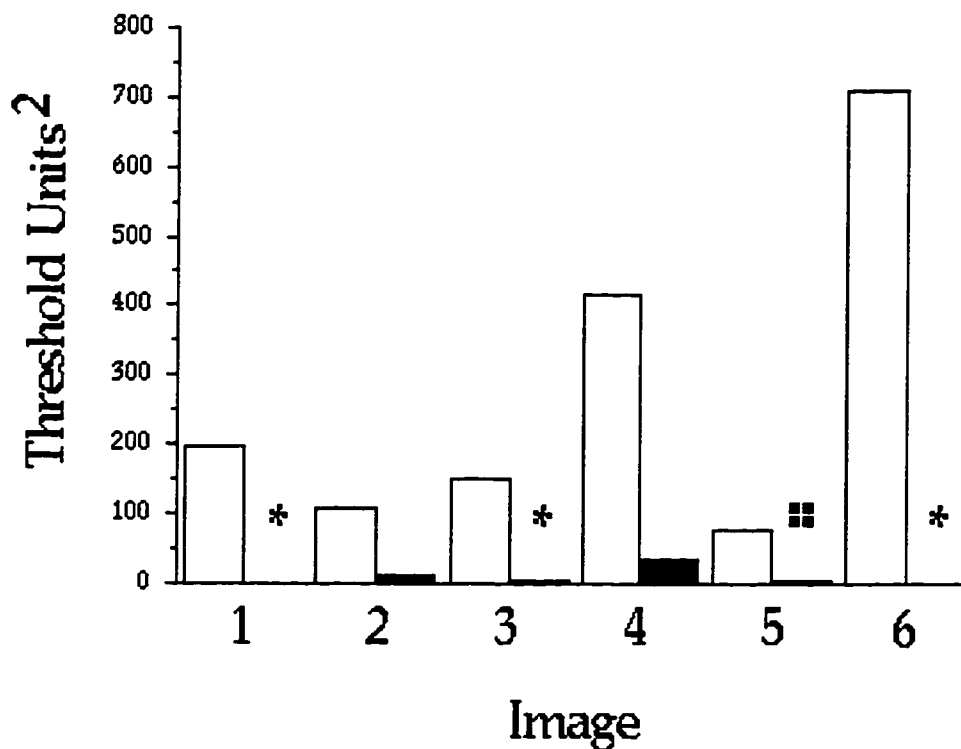


Figure 5: Between-observer variability for threshold selection techniques. Subjective threshold selection and algorithm-derived threshold selection are compared for the variability which occurs between observers for each of 6 images (same images as compared in Figure 6), with three thresholding sessions per image, according to the formula presented in this chapter. Each bar represents the variability specifically due to multiple observers, and is equal to the total variability - the within-observer variability. Significant differences between the two techniques for each image was determined with an F test. White bars = subjective technique, black bars = algorithm-derived thresholding technique, ** = $p < 0.05$, * = $p < 0.01$.

Figure 6

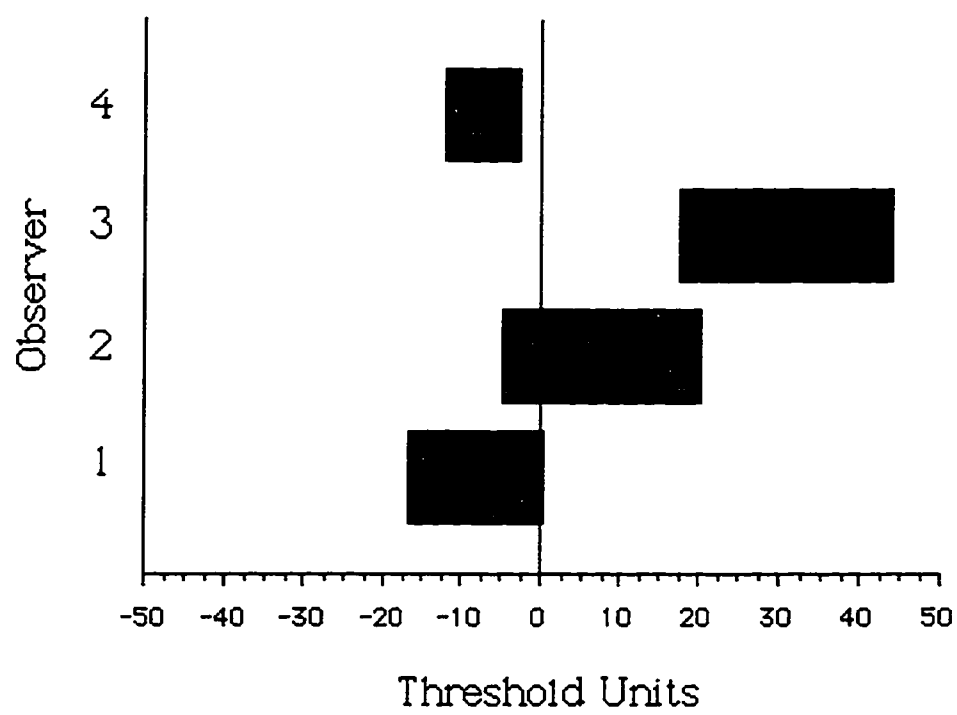


Figure 6: 95% confidence intervals for differences in mean threshold values. For each observer, the mean threshold level difference is determined for the six images by subtracting the mean algorithm-derived derived threshold value from the mean value of subjectively-determined data (the three trials are averaged for each image as described in this chapter). The difference and the standard error which results are used to generate a 95% confidence interval. Note that accuracy is reflected by whether the differences are consistently positive or negative.

scenario represents how close to the observer-defined values algorithm-based threshold selection method is. The differences between the two techniques for the four observers can be used to generate 95% confidence intervals, which is shown in Figure 6. Since these intervals include both positive and negative differences, these data suggest that the algorithm-derived threshold selection technique does not consistently over- or underestimate threshold levels as compared with observer-selected values. Hence, algorithm-derived threshold selection is accurate as well as less variable than subjective determinations.

2.5. Discussion

The utility of any approach to improve analysis of immunocytological data depends on the ability to define immunopositive labeling reliably. The determination of immunopositive objects in an image can be based upon the intensity of the immunosignal and/or the morphological features of the immunolabeled objects (Fritz *et al.*, 1992). Detailed quantification will often employ both approaches, although each type of analysis may be used individually in specific circumstances. For example, in studies in which there are no intermediate levels of staining, a satisfactory threshold for positive staining may be assigned subjectively. Similarly, studies in which the stained material represents a morphologically simple and easily identified structure, such as the nuclear labeling used to validate the algorithm in this study, may not require highly detailed morphometric analyses (Tieman, 1989). However, most immunohistochemical data does not fall into these categories and, because of this, automated or semiautomated techniques for discrimination of immunolabeling from

background have proven unreliable and/or inaccurate for most applications. Dissatisfaction with thresholding procedures has led most investigators to rely primarily on subjective assessment (Brown, 1990; Fuxe *et al.*, 1991; Colombi *et al.*, 1993; Auer, 1994). In this paper we provide an empirically accurate and reliable technique for recognizing positive immunolabeling that can be used for subsequent quantification of immunocytological material.

Several methods for algorithm-derived threshold selection have been proposed (Weszka, 1978), but none have proven acceptable for histological applications. Doyle described an approach in which the threshold level corresponds to a percentile of gray levels in the image (Doyle, 1962). This technique is inappropriate for most immunohistochemical analyses because the amount of immunopositive labeling cannot be determined beforehand. Another method for threshold selection, proposed by Prewitt and Mendelsohn, relies on the shape of the gray scale histogram. This technique is based on the assumption that the background and immunolabeled areas represent peaks of gray level subpopulations, while the edges, intermediate in intensity, represent valleys. This type of analysis is not appropriate for the common immunohistochemical staining patterns in which a continuous gradient of labeling intensity is observed.

The skilled microscopist uses two features, intensity of staining and morphological pattern, to identify immunolabeling. We reasoned that, while observers may differ considerably in their selection of a threshold that separates positive immunolabeling from background, the operation typically required for subjective analysis of digital images, different observers would have considerably less disparity in their selection of a

representative area of background in each slide. Accordingly, we attempted to develop an algorithm that correlates the selection of background regions in each image to an acceptable threshold selection, in order to minimize observer bias. This algorithm (see equation 1) relies on fundamental principles evident from immunohistochemical labeling. The relationship of the ideal threshold level to the background level varies according to the ratio of signal-to-noise of the sample. As the background level increases, the difference between signal and background decreases. Thus the relationship is most simply approximated by the logarithmic function: $t = \log b$, where t =threshold and b =background. In order to accommodate systems which display immunohistochemical labeling with different maximal grayscale levels, $\log b$ is multiplied by the constant C_1 . This constant is $M/\log M$, where M is equal to the maximal grayscale level which can be achieved in the system used. Hence $t = C_1 * \log b$, which is curve A in Figure 3.

The purpose of this algorithm is not to automate thresholding but instead to allow objective thresholding that is reliable from section to section and unbiased. Hence CF (line B on Figure 3) is a linear correction factor that improves the relationship between the threshold derived by algorithm and what the observer would select. $CF = C_2 - ((C_2/M)*b)$, where C_2 is an empirically-derived constant (100 in this system). While in our system CF is satisfactory over a reasonable experimental range, it is possible that other values for C_2 may be better if the signal/noise ratio varies dramatically. This can be determined empirically as the need arises. The resulting curve is $C_1 * \log b - CF$, which is represented by Curve C in Figure 3.

In an experiment designed to test the utility of this approach, we found that the application of this algorithm leads to acceptable accuracy and improved precision (between- and within-subject variability) in comparison to subjective threshold assignment (see Figures 4, 5, and 6).

The technique for algorithm-derived threshold selection presented in this paper is nested in the setting of a graphics program which can serve many functions for analysis of immunohistochemically-treated tissue. The primary function is to discriminate positive staining from background or nonspecific staining with the accuracy of expert observers but reducing the observer bias frequently associated with such studies. In addition to this function, analysis of digital images in Photoshop is particularly well-suited for the generation and quantification of composite maps from tissue stained for multiple markers. When the images of many immunolabels from one field of view are blended (as demonstrated in Figure 1), single labeling, multiple labeling and absence of labeling are designated distinct colors or gray scale values, which can be quantitated from a histogram. This adaptation can be used to determine the number of single or double-labeled cells or nuclei, or the number of labeled cells or nuclei with respect to immunohistochemically-defined boundaries. Indeed this procedure exploits the major advantage of analyzing captured, digitized images which is the ability to extract specific features of an image and subject them to analysis.

Accurate discrimination of immunopositive labeling is a necessary step for cytological studies, but depending upon the experimental design, may not represent a sufficient analysis. Random sampling of immunohistochemical tissue in discrete neuroanatomical regions is an efficient method for distinguishing overall differences between

experimental groups. However, random sampling of brain sections is an imprecise means to estimate the numbers of cells of specific morphology in a volume of tissue, due to the increased likelihood of larger cells to appear in sections (Bolender *et al.*, 1991). Techniques like stereology or serial section reconstruction, which convert two-dimensional images of tissue sections into three-dimensional data, can adjust for this effect (Auer, 1994; Mezin *et al.*, 1994). Although both experimental designs would utilize accurate and precise determination of positive labeling, cell counting requires morphometric analysis in addition to thresholding (Agnati and Fuxe, 1984). This is especially true for cells of complex structure such as the highly arborized medium spiny neurons of the striatum.

Image analysis systems can be configured in a variety of ways to minimize the error factors in the analysis of immunolabeled tissue. These error factors include uneven illumination in space, uneven illumination over time, and a factor known as the "densitometric transfer function" which is the accuracy with which the output, or digitized image, represents the input, or analog image (Mize, 1989). In order to correct for uneven illumination on an image, it is suggested that a "shading correction" (subtraction of a reference image from subsequent images) is performed (Willemse *et al.*, 1992; Vennari, 1994). On the other hand, uneven illumination over time is only a factor when one tissue sample has to be repeatedly examined and digitized, and this was not a factor in this experiment. Lastly, the densitometric transfer function is particularly affected by the precision of the camera (defined as the ability to reliably discriminate shades of gray). Therefore, an important factor for determining the type of camera or photometric scanning device to be

used should be the range of densities of the material to be studied (Ramm, 1994).

In summary, we feel that the method presented in this paper allows for more precise, reliable and efficient quantification of immunohistochemical data. In addition to reliable discrimination of positive labeling from nonspecific staining, multiple immunohistochemical markers may be visualized and counted, with histogram information of the amount of each immunolabel, and the amount of overlap. These techniques are valuable both for experiments designed for random sampling of tissue sections from experimental groups as well as in experiments in which cell counting of a discrete neuroanatomical area is performed. Combined with ease of use and comparatively modest cost, these methods of analysis provide a reliable and practical way to obtain data from immunohistochemically-treated issue.

3.0. The Dopamine Receptor Subtype-Specific Mechanisms by which Amphetamine Induces the Immediate Early Gene *c-fos*

3.1. Introduction

The psychomotor effects associated with amphetamine administration to rodents are dependent upon a dopamine efflux from mesencephalic dopaminergic neurons (Sharp *et al.*, 1987; Kuczenski *et al.*, 1991; Guix *et al.*, 1992). Supporting evidence comes from experiments in which bilateral lesioning of the rat dopaminergic projection to the nucleus accumbens or the caudate nucleus is produced by stereotactically-guided injections of the catecholamine-selective neurotoxin 6-hydroxydopamine (6-OHDA). These lesions interfere with the production of motor behaviors associated with amphetamine administration (Creese and Iversen, 1973; Kelly *et al.*, 1975; Kelly *et al.*, 1976). Further evidence suggesting a role for dopamine in the motor effects of amphetamine come from experiments in which unilateral lesioning of the rat dopaminergic system is followed by the administration of amphetamine. The subsequent ipsilateral rotation (turning towards the side of the lesion) has been postulated to be due to imbalanced dopamine release as it is inhibited by dopamine receptor antagonists (Arnt and Hyttel, 1984; Paul *et al.*, 1992; Yurek and Hipkens, 1993).

Regulation of transcription is an important mechanism by which neurons may produce a lasting response to changes in the synaptic concentration of dopamine. The relationships between specific dopamine receptor subtype activation and transcriptional plasticity have become important in addressing the molecular mechanisms by which neurons, and the anatomical pathways to which they belong, are modulated by dopamine. One such transcriptional factor which can be

immunohistochemically-detected is the Fos protein, encoded by the immediate early gene *c-fos*. Cellular localization of the immediate early gene *c-fos* following direct or indirect dopaminergic agents has helped to develop a better understanding of the dopaminergic modulation of basal ganglia output. For example, D1 dopamine receptor agonism in dopamine-depleted rats induces *c-fos* exclusively in neurons belonging to the striatonigral pathway, while D2 dopamine receptor antagonism in the intact animal induces *c-fos* in the striatopallidal pathway (Robertson *et al.*, 1992).

Studies investigating the amphetamine-induction of *c-fos* largely have focused on the basal ganglia and prefrontal cortex. These two brain areas receive direct dopaminergic innervation (Moghaddam and Bunney, 1989; Piazza *et al.*, 1991; Elsworth *et al.*, 1993) and have been shown to express a high level of dopamine receptor mRNA and bind dopaminergic ligands (Dearry *et al.*, 1990; Mansour *et al.*, 1990; Monsma *et al.*, 1990; Freneau *et al.*, 1991; Weiner *et al.*, 1991; Brouwer *et al.*, 1992; Levant *et al.*, 1992; Seeman *et al.*, 1992; Fox *et al.*, 1993; Gaspar *et al.*, 1995). However, the striatal neurons on which dopamine receptors are localized have an influence on thalamic activity, and ultimately the primary motor cortex, a region crucial to the production of integrated, planned movement (Hicks and D'Amato, 1970; Hicks and D'Amato, 1975; Price and Fowler, 1981).

The aim of this study was to compare the patterns of Fos protein in the striatum and primary motor cortex of the rat brain following amphetamine administration in the presence or absence of subtype-specific dopamine receptor antagonists. In efferent neurons of the primary motor cortex and the striatum, the amphetamine-mediated induction of *c-fos* was found to be dependent upon dopamine receptor

activation. However, D1 dopamine receptor antagonism is far less effective in blocking the induction of *c-fos* by amphetamine in the cortex than in the striatum. Furthermore, D2 dopamine receptor antagonism, while inducing *c-fos* in the striatum, did not produce any immunohistochemically-measurable Fos in the primary motor cortex, and produced a much greater inhibition of amphetamine-mediated *c-fos* induction in the primary motor cortex than did D1 receptor antagonism.

3.2. Materials and Methods

3.2.1. Pharmacological and Histological Preparation

Adult male Sprague Dawley rats (see Table 1 for the number of rats and sections in this study) were pretreated with either the D1-like dopamine receptor antagonist R(+)-SCH23390 hydrochloride (1.0 mg/kg, i.p.), the D2-like dopamine receptor antagonist S(-)-eticlopride hydrochloride (0.5 mg/kg, i.p.), combined antagonism (SCH23390 and eticlopride at 1.0 mg/kg and 0.5 mg/kg, i.p., respectively) or saline (i.p.) 150 minutes prior to perfusion. Two hours before perfusion, rats were administered S(+)-amphetamine sulfate (10 mg/kg, i.p.), or saline (i.p.). All drugs were purchased from Research Biochemicals International, Natick, MA. Transcardial perfusion with 4% paraformaldehyde in 10 mM phosphate buffered saline (PBS, pH 7.4) was performed at 4°C using approximately 400 ml/rat. Brains were removed and postfixed in 4% paraformaldehyde, cryoprotected in sucrose (15% and 30% in PBS) and frozen on dry ice. Tissue was cut into 20 µm-thick sections on a sliding microtome, and stored in storage solution (4.1 M glycerol, 5.4 M ethylene glycol, 0.02 M PBS) at -20°C.

Table 1

Drug Pretreatment	Drug Treatment	Rats	Sections
Saline	Saline	1	3
No Pretreatment	Saline	3	3-5
No Pretreatment	Amphetamine	4	4
SCH23390	Saline	3	5
SCH23390	Amphetamine	5	4-5
Eticlopride	Saline	3	2-4
Eticlopride	Amphetamine	5	4-5
SCH23390 + Eticlopride	Saline	2	2-3
SCH23390 + Eticlopride	Amphetamine	4	3-5

Table 1: Number of Rats and Tissue Sections Used in Study. See Section 3.2.1. for drug doses. "Rats" denotes the number of animals in each drug treatment group, corresponding number reflects number of tissue sections analyzed per animal.

3.2.2. Fos immunohistochemistry

Free-floating sections were washed in 10 mM PBS between incubations. The primary antibody solutions contained 0.1% Triton X-100 for cell permeabilization and bovine serum albumin (0.1 mg/ml) as a blocking agent. The rabbit polyclonal antibody to *c-fos* was used at a dilution of 1:500 (antibody cat # sc-52, Santa Cruz Biotechnology, Santa Cruz, CA). Fos antibody was detected using an anti-rabbit biotin/avidin labeling system (Vector Laboratories, Burlingame, CA). Following the biotin/avidin steps, each tissue section was reacted in a volume of 1 ml with 0.5 mg 3,3'-diaminobenzidine with 0.003% H₂O₂ in 1 mM PBS for visualization of the immunoreactivity. The addition of 1.12 μ M cobalt chloride hexahydrate and 676 nM nickelous ammonium sulfate hexahydrate provided significant signal intensification. The tissue was then mounted onto glass slides. Slides were Nissl counterstained for visualization of the cellular histology through tissue hydration, incubation in 0.5% cresyl violet for 5 minutes, dehydration in ethanol, and coverslipping utilizing the mountant DPX (Fluka, Buchs, Switzerland)

3.2.3. Data Analysis

Images from immunohistochemically-treated slides were captured using a Zeiss Axiophot photomicroscope equipped with a Zeiss MSP65 computer-controlled motorized stage (Zeiss, Oberkochen, Germany), a Zeiss ZVS-47E video camera (Zeiss, Thornwood, NY), a Sony Trinitron video monitor and a DEC 5000 workstation (Digital Equipment Corporation, Maynard, MA). All images were digitized using a 20X Zeiss Plan Neofluar objective with a resolution of 0.6 μ m/pixel in the x and y

axes. Photographic montages were created using Adobe Photoshop. Minimal contrast, brightness and color enhancements were applied to the images in order to obtain a product which most resembled the microscopic view, and to aid in counting.

The number of Fos-immunopositive neurons in 200X microscopic fields of the rostromedial striatum was determined using the method for quantitative digital analysis of immunohistochemical tissue described in Chapter 2. On the other hand, the number of Fos-immunopositive layer V pyramidal cells in the primary motor cortex was obtained by visual inspection. This was done in order to restrict the quantitation to cells in cortical layer V (using established morphometric criteria to identify the cortical lamina), and to eliminate non-pyramidal cells lacking an apical dendrite and general triangular shape. Two rats were removed from the analysis as they produced patterns of immunoreactivity vastly different from the other animals in their treatment group (one rat was pretreated and treated with saline and showed widespread Fos immunoreactivity, the other animal was pretreated with eticlopride and treated with saline and showed no measurable striatal Fos immunolabeling). Also note that saline-treated animals which were either not pretreated or pretreated with saline were considered a single drug treatment group. Significance between drug treatment groups was determined using a nested one way analysis of variance, followed by a Fisher's Protected Least Squared Difference post-hoc test with $p < 0.05$. This analysis compares the drug treatment groups while taking into account two important parameters: the variability between sections for each animal, as well as the variability between animals for each treatment group.

3.3. Results

The administration of amphetamine to rodents produced a variegated distribution of *c-fos* immunoreactivity in the striatum (Figure 7), consistent with previous reports (Graybiel *et al.*, 1990; Ruskin *et al.*, 1994). In the primary motor cortex, amphetamine induced the immediate early gene *c-fos* throughout the cortical layers. Analysis was restricted to the pyramidal cells of layer V due to their easily identifiable morphology and their known contribution to descending motor pathways (Figure 8). The administration of saline did not induce measurable *c-fos* in either the striatum (Figure 9) or the primary motor cortex (Figure 10)

SCH23390 (D1-like dopamine receptor antagonist) did not measurably increase the induction of *c-fos* in either the striatum (Figure 11) or the primary motor cortex (Figure 12). While pretreatment with SCH23390 completely prevented the striatal induction of *c-fos* following administration of amphetamine (Figure 13), there was only a small reduction in the amphetamine-mediated induction of *c-fos* in layer V of the primary motor cortex (Figure 14) which did not achieve statistical significance (see Table 3 at the end of this chapter).

Administration of eticlopride, a substituted benzamide and a D2-like dopamine receptor antagonist, increased *c-fos* immunoreactivity in the striatum (Figure 15). This finding is consistent with data from a study that combined immunohistochemical techniques with retrograde labeling of neuroanatomical pathways, demonstrating that D2-like dopamine receptor antagonists induce *c-fos* in the striatal neurons projecting to the globus pallidus (Robertson *et al.*, 1992). There was no difference in the amount of striatal Fos immunolabeling between

Figure 7



Figure 7: Immunohistochemical detection of the Fos protein in the striatum of rats treated with amphetamine. Arrows highlight Fos-immunopositive cells. Scale bar = 0.5 mm.

Figure 8

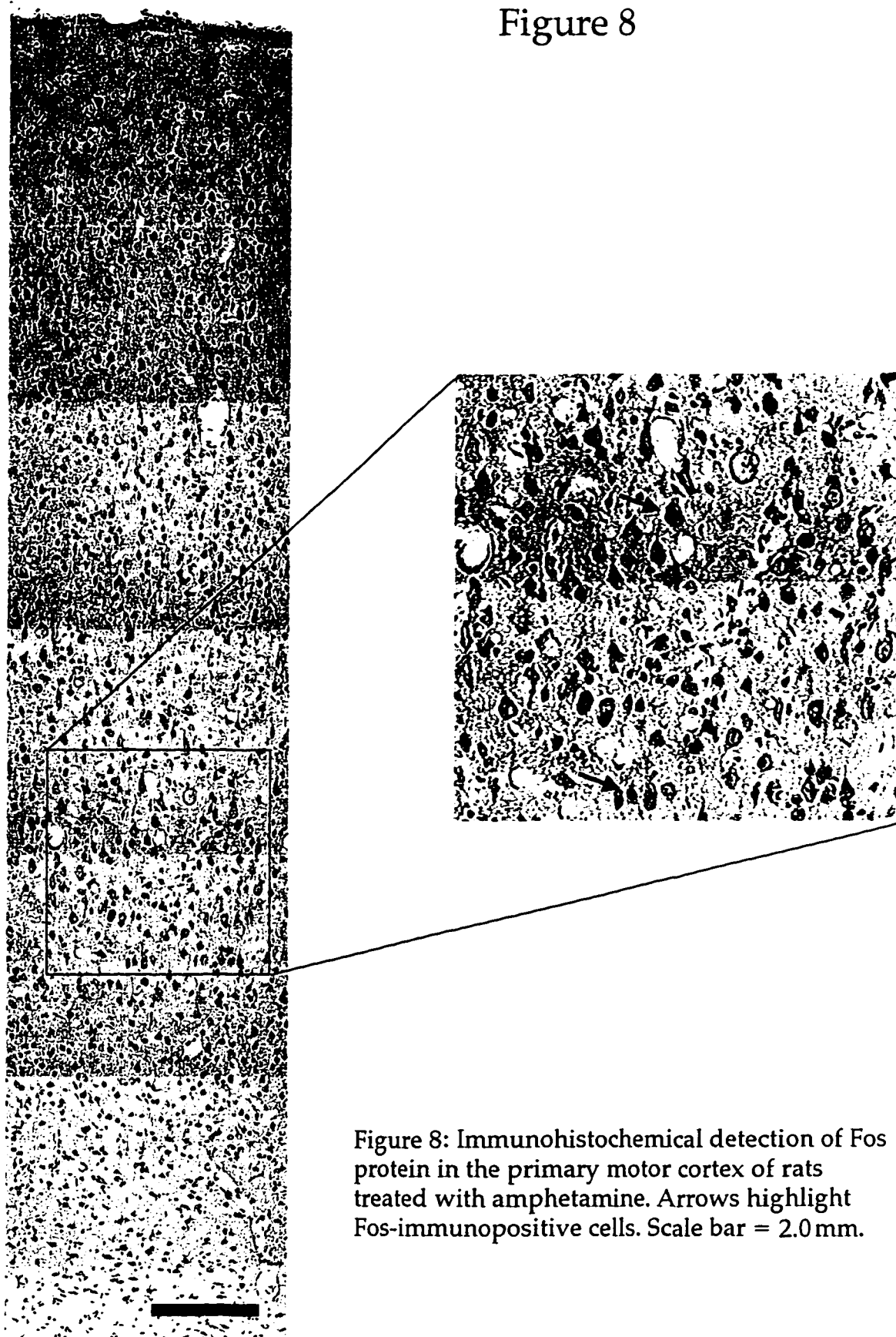


Figure 8: Immunohistochemical detection of Fos protein in the primary motor cortex of rats treated with amphetamine. Arrows highlight Fos-immunopositive cells. Scale bar = 2.0 mm.

Figure 9

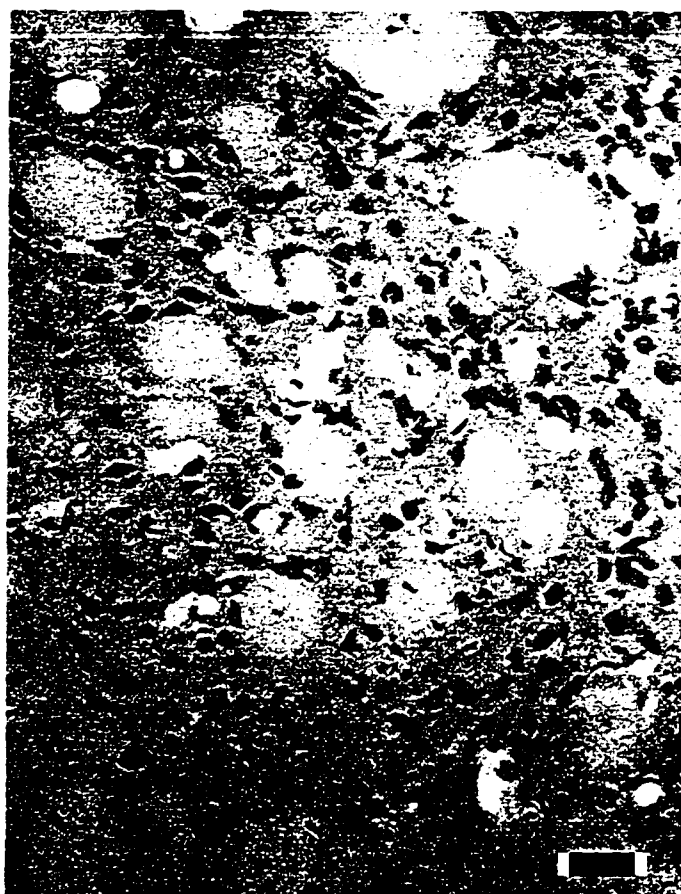


Figure 9: Immunohistochemical detection of the Fos protein in the striatum of rats treated with saline. Scale bar = 0.5 mm.

Figure 10

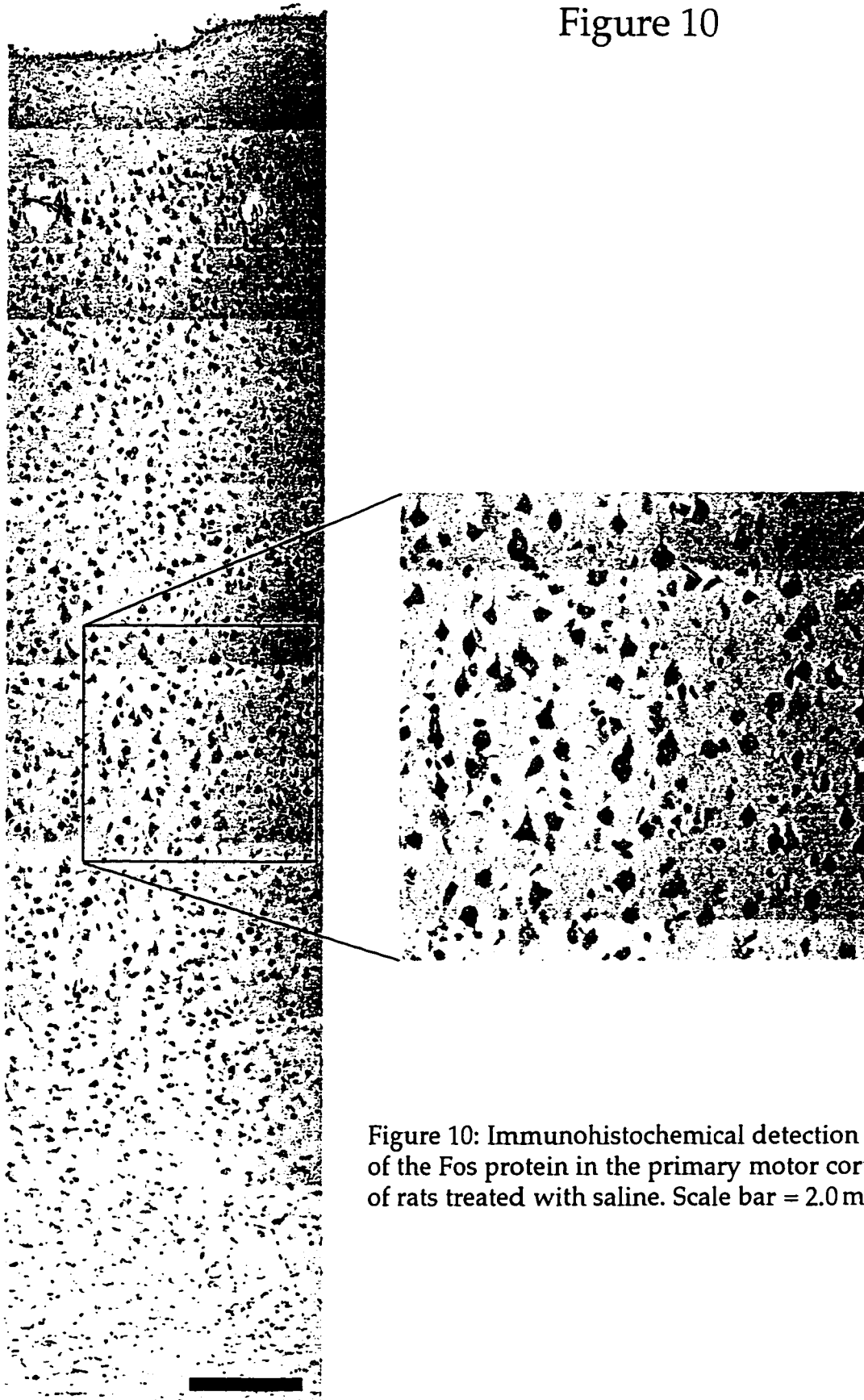


Figure 10: Immunohistochemical detection of the Fos protein in the primary motor cortex of rats treated with saline. Scale bar = 2.0 mm.

Figure 11



Figure 11: Immunohistochemical detection of the Fos protein in the striatum of rats pretreated with SCH23390 prior to saline administration. Scale bar = 0.5 mm.

Figure 12

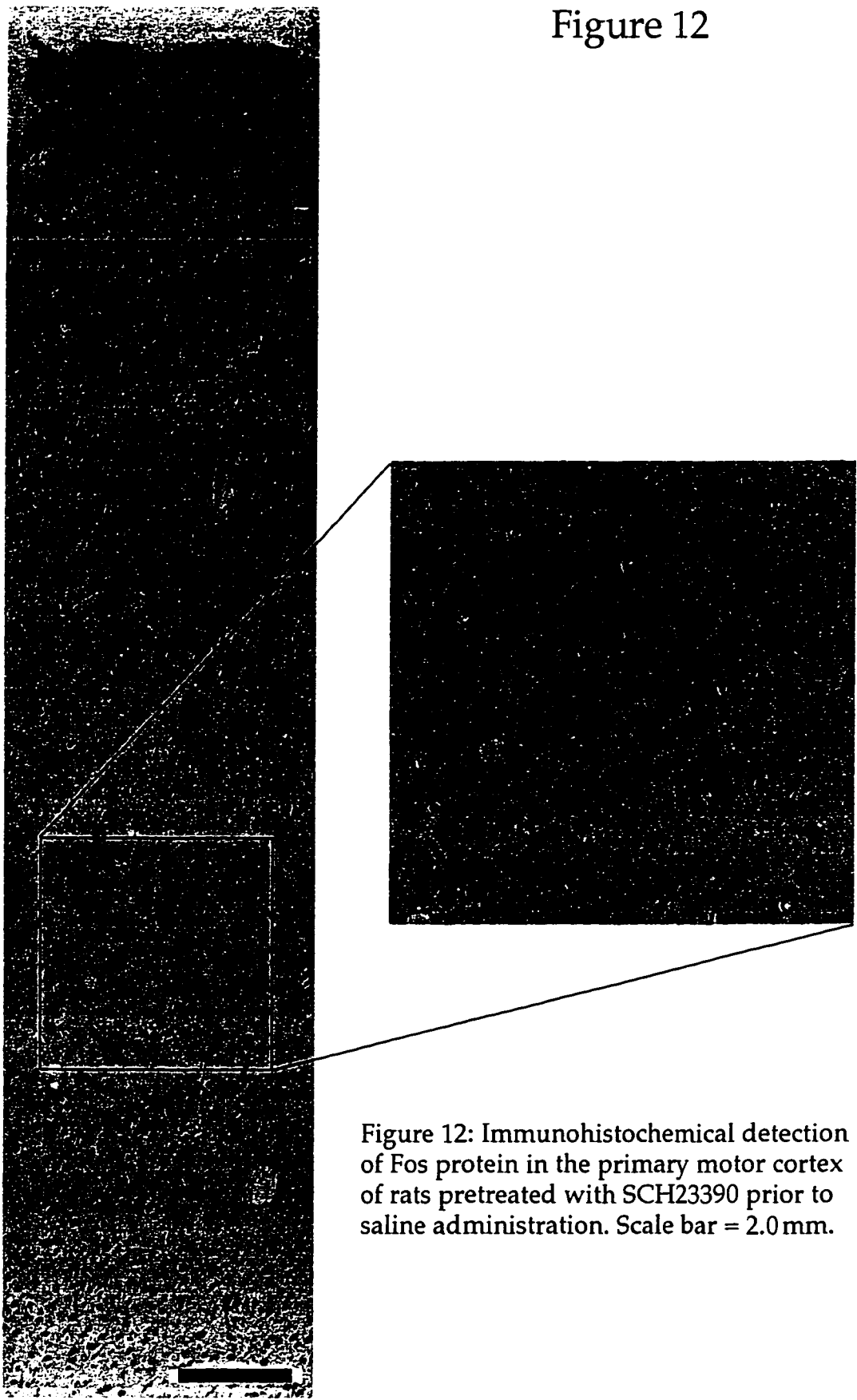


Figure 12: Immunohistochemical detection of Fos protein in the primary motor cortex of rats pretreated with SCH23390 prior to saline administration. Scale bar = 2.0 mm.

Figure 13

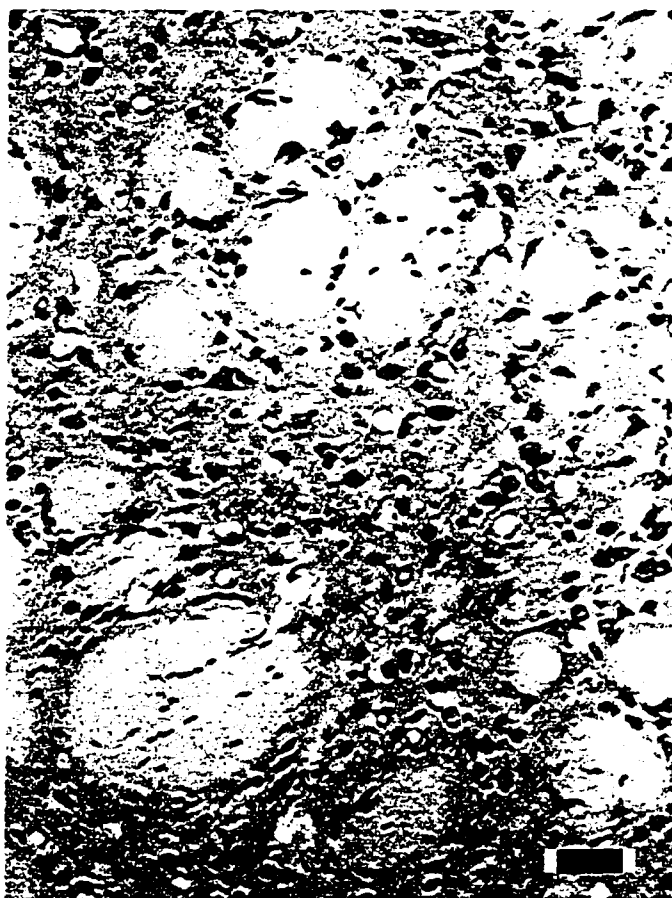


Figure 13: Immunohistochemical detection of Fos protein in the striatum of rats pretreated with SCH23390 prior to amphetamine administration. Scale bar = 0.5 mm.

Figure 14

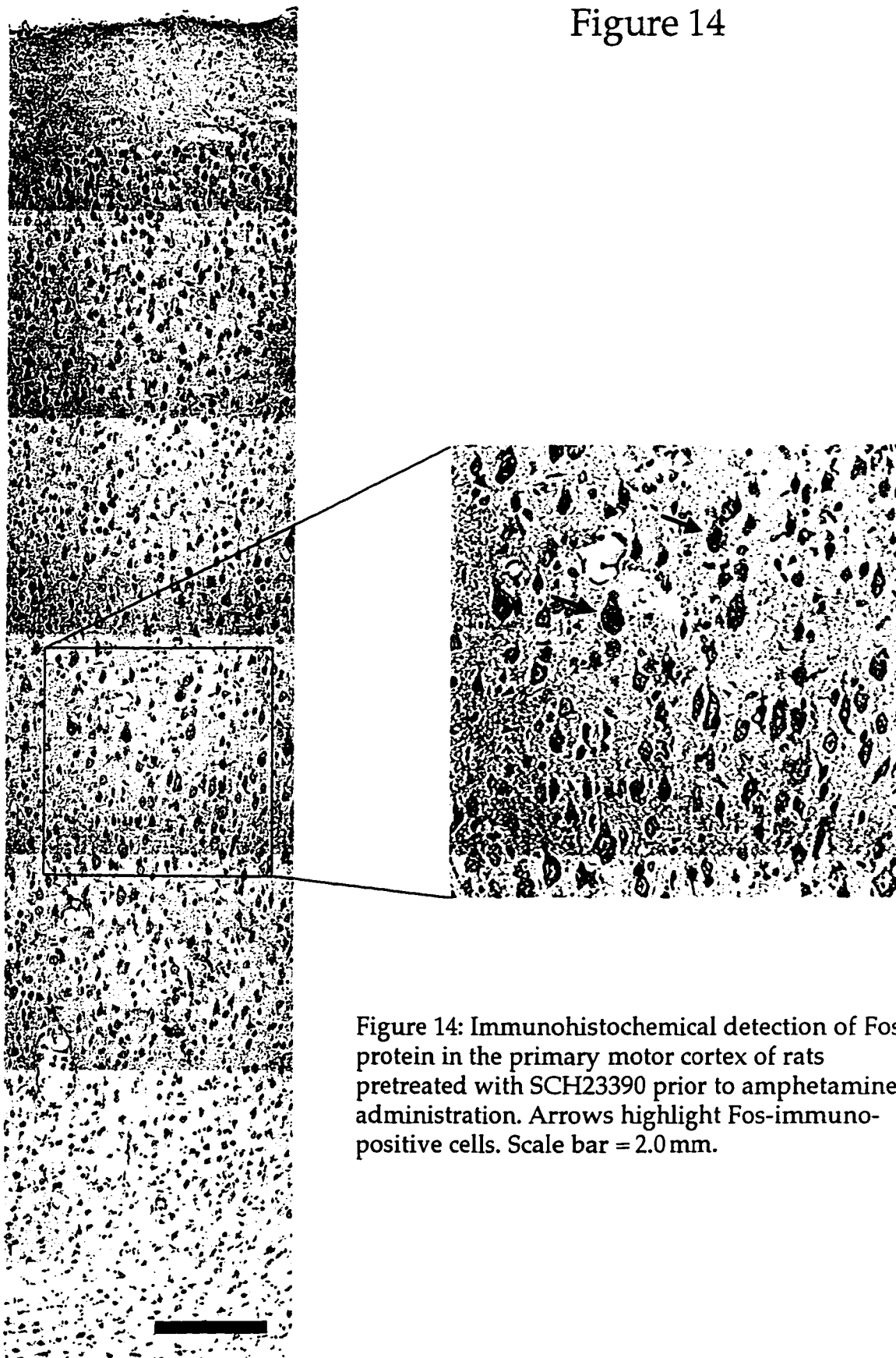


Figure 14: Immunohistochemical detection of Fos protein in the primary motor cortex of rats pretreated with SCH23390 prior to amphetamine administration. Arrows highlight Fos-immunopositive cells. Scale bar = 2.0 mm.

Figure 15



Figure 15: Immunohistochemical detection of Fos protein in the striatum of rats pretreated with eticlopride prior to saline administration. Arrows highlight Fos-immunopositive cells. Scale bar = 0.5 mm.

eticlopride-pretreated rats which were then administered amphetamine (Figure 16) versus eticlopride pretreated rats administered saline (Figure 15 and Table 2).

The effects of eticlopride on Fos immunohistochemistry in the primary motor cortex was opposite to the effects in the striatum. Eticlopride did not measurably increase *c-fos* immunolabeling in the primary motor cortex (Figure 17). However, eticlopride reduced the amphetamine-mediated induction of *c-fos* in layer V of the primary motor cortex (Figure 18) resulting in a statistically-significant decrease from either amphetamine alone (Figure 8) or when SCH23390 pretreatment preceded amphetamine administration (Figure 14 and Table 3).

Combination pretreatment with SCH23390 and eticlopride prior to saline administration did induce *c-fos* in the striatum (Figure 19), presumably due to D2-like dopamine receptor antagonism by eticlopride, although it was not found to be statistically significant from immunohistochemical analysis of tissue from saline-control rats (Table 2). However, the additional Fos immunolabeling which occurred when both antagonists preceded amphetamine administration (Figure 20) did achieve statistical significance in the striatum as compared with saline-control animals (Table 2). Neither drug treatment groups showed significant induction of *c-fos* in the primary motor cortex (Figures 21 and 22, Table 3).

Statistical significance between drug treatment groups is illustrated in Figures 23 and 24 with comparisons between all treatment groups presented in Tables 2 and 3.

Figure 16

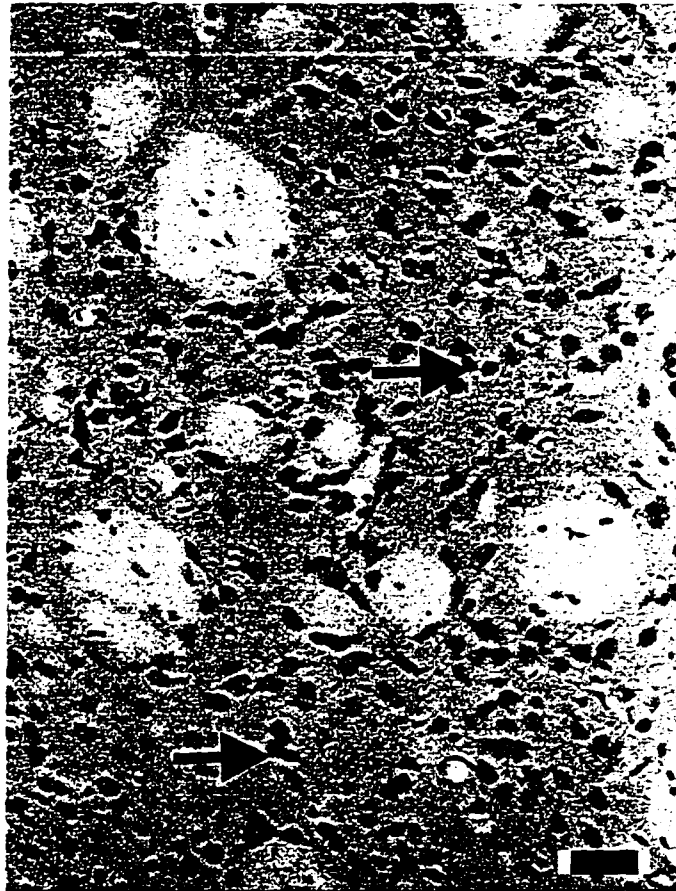


Figure 16: Immunohistochemical detection of Fos protein in the striatum of rats pretreated with eticlopride prior to amphetamine administration. Arrows highlight Fos-immunopositive cells. Scale bar = 0.5 mm.

Figure 17

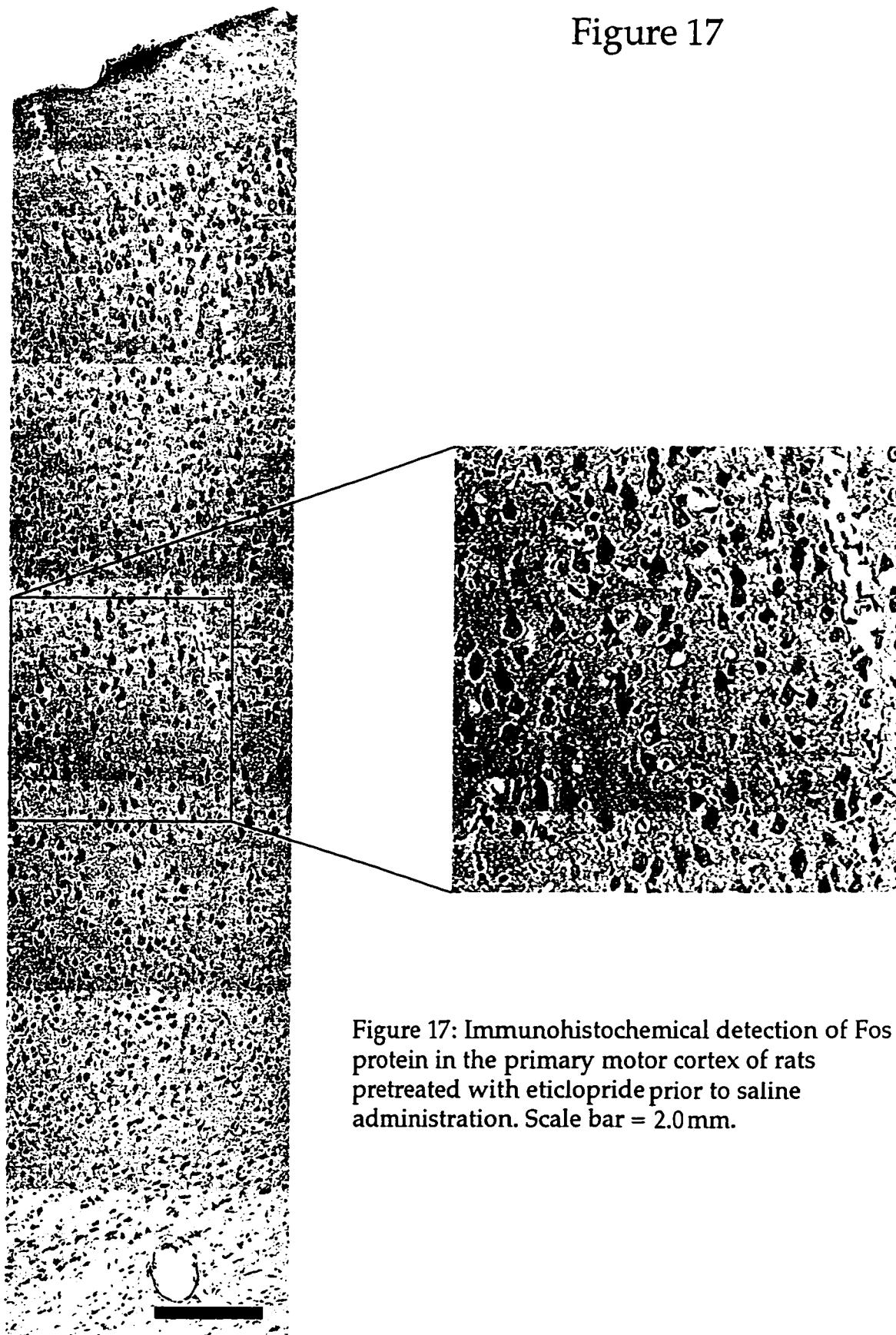


Figure 17: Immunohistochemical detection of Fos protein in the primary motor cortex of rats pretreated with eticlopride prior to saline administration. Scale bar = 2.0 mm.

Figure 18

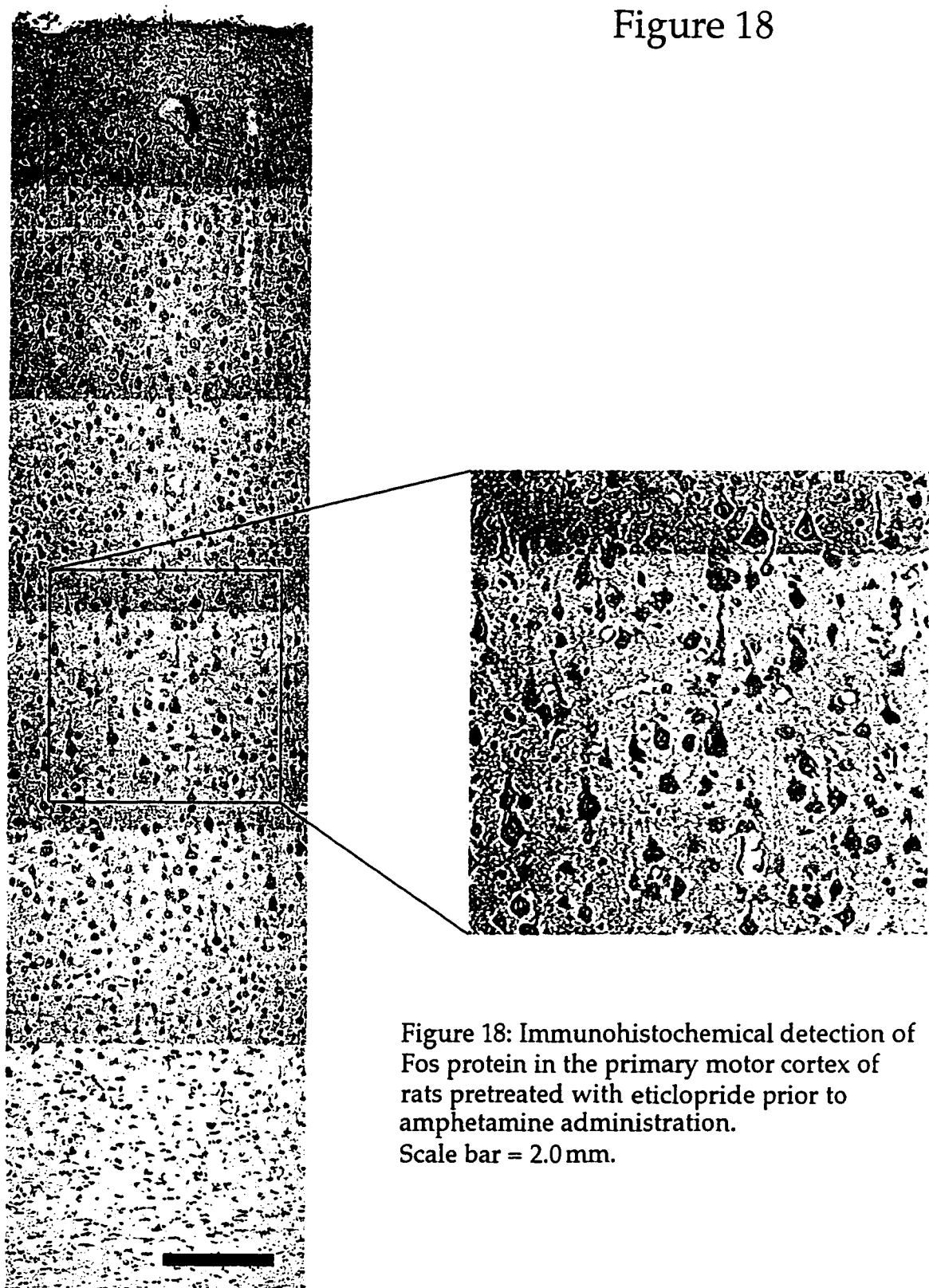


Figure 18: Immunohistochemical detection of Fos protein in the primary motor cortex of rats pretreated with eticlopride prior to amphetamine administration.
Scale bar = 2.0 mm.

Figure 19

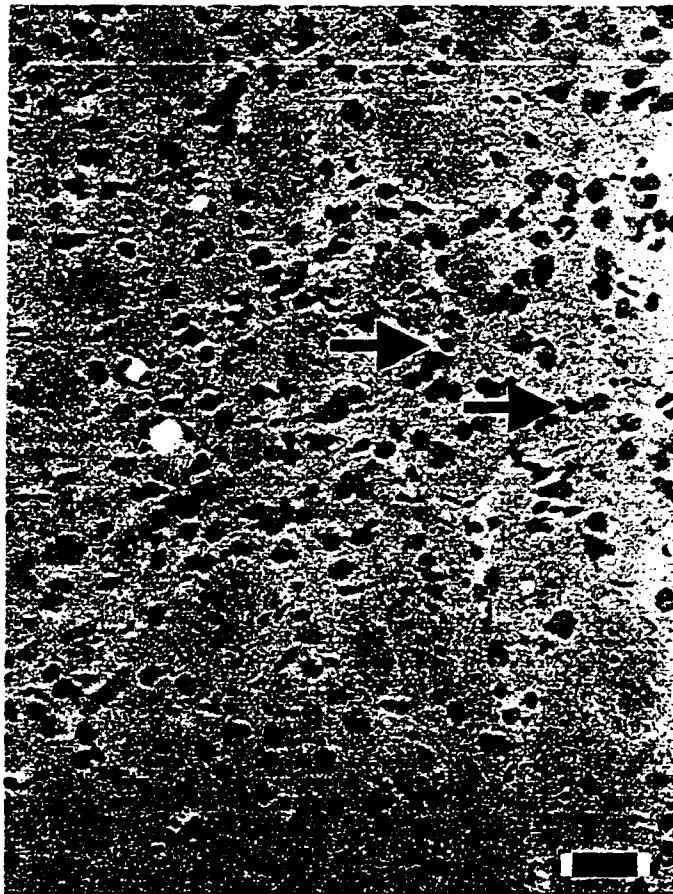


Figure 19: Immunohistochemical detection of the Fos protein in the striatum of rats pretreated with SCH23390 and eticlopride prior to administration of saline. Arrows highlight Fos-immunopositive cells. Scale bar = 0.5 mm.

Figure 20



Figure 20: Immunohistochemical detection of the Fos protein in the striatum of rats pretreated with SCH23390 and eticlopride prior to administration of amphetamine. Arrows highlight Fos-immunopositive cells. Scale bar = 0.5 mm.

Figure 21

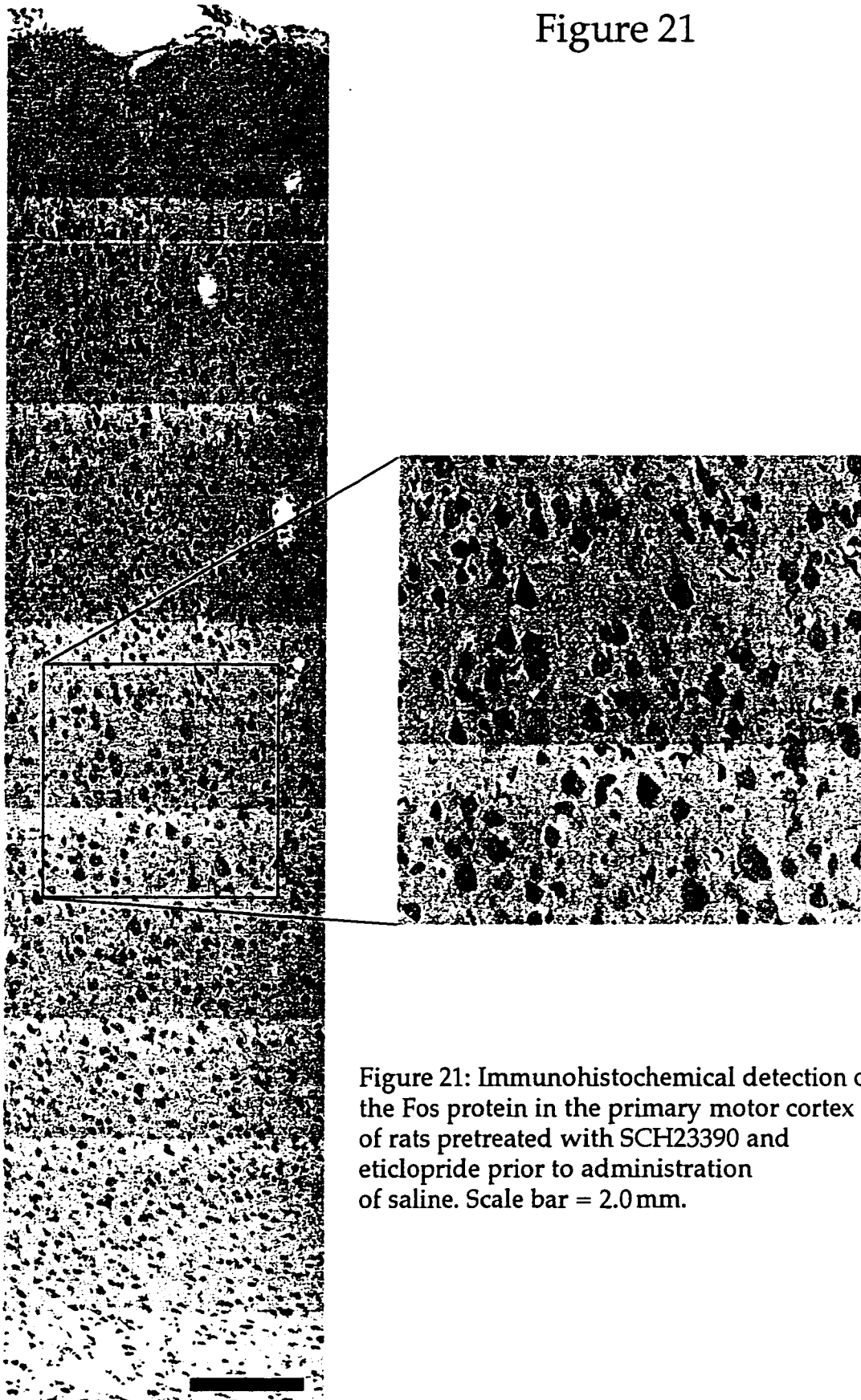


Figure 21: Immunohistochemical detection of the Fos protein in the primary motor cortex of rats pretreated with SCH23390 and eticlopride prior to administration of saline. Scale bar = 2.0 mm.

Figure 22

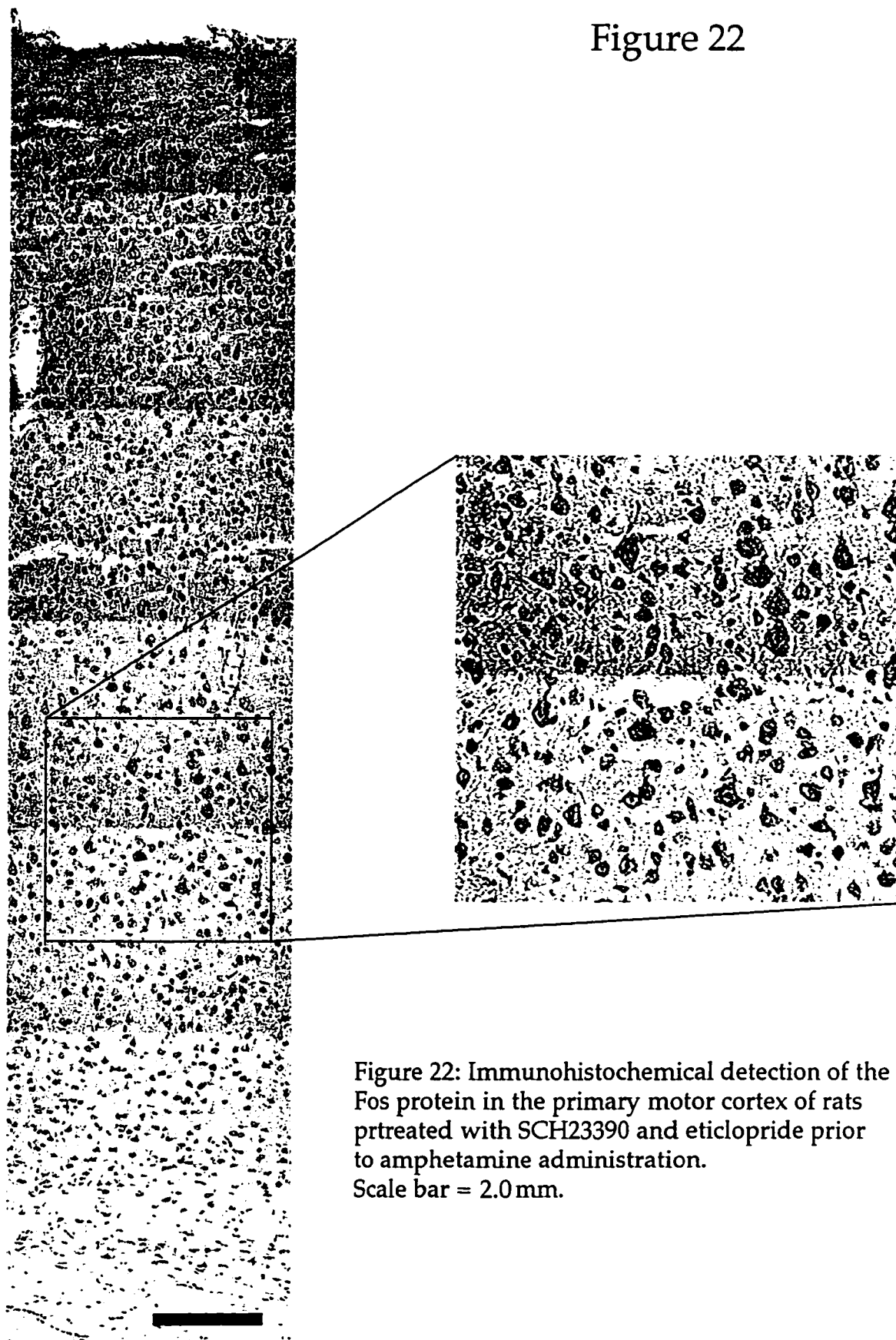


Figure 22: Immunohistochemical detection of the Fos protein in the primary motor cortex of rats pretreated with SCH23390 and eticlopride prior to amphetamine administration. Scale bar = 2.0 mm.

Figure 23

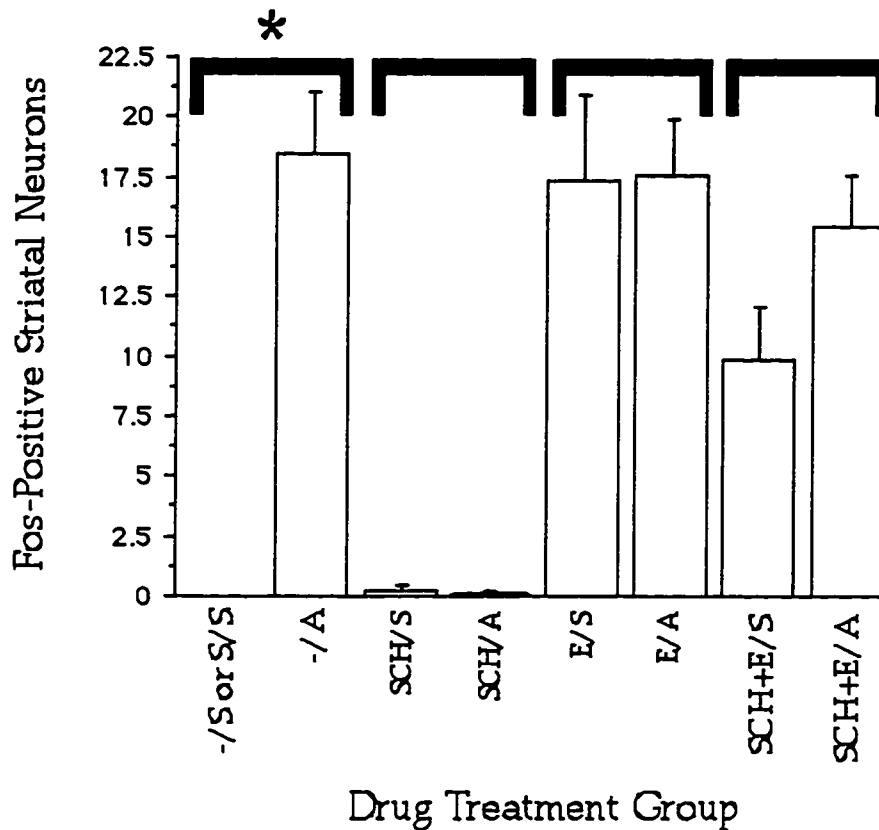


Figure 23: Fos in Striatum. Bar graph representing the number of Fos-immunopositive neurons from digitized images of 200X magnified sections of rostromedial striatum. All numbers shown are means $\pm$ S.E.M. for animals in that drug treatment group. Rats received either no pretreatment (-), saline pretreatment (S), D1-like dopamine receptor antagonist SCH23390 (1.0 mg/kg, i.p.) pretreatment (SCH), D2-like dopamine receptor antagonist eticlopride (0.5 mg/kg, i.p.) pretreatment (E) or pretreatment with both D1-like and D2-like dopamine receptor antagonists together 150 minutes prior to transcardial perfusion. 120 minutes prior to perfusion, rats received either amphetamine (A, 10 mg/kg, i.p.) or saline (i.p.). Fos immunopositive neurons were defined using the algorithm for threshold selection described in Chapter 2. Brackets compare rat subgroups with the same pretreatment and different drug treatments. Significance between drug treatment groups was determined using a nested one-way analysis of variance followed by a Fisher's Protected Least Squared Difference post-hoc test (* = $p < 0.05$). A statistical comparison of all of the drug treatment groups is presented in Table 2.

Table 2

	-/A	SCH/S	SCH/A	E/S	E/A	SCH+E/S	SCH+E/A
-/Sor S/S	+	-	-	+	+	-	+
-/A		+	+	-	-	-	-
SCH/S			-	+	+	-	+
SCH/A				+	+	-	+
E/S					-	-	-
E/A						-	-
SCH+E/S							-

Table 2: Comparison Between Drug Treatment Groups of the Induction of C-Fos in the Striatum. Fos-immunopositive neurons were counted as described in the Section 3.2 and shown graphically in Figure 23. Significance between drug treatment groups was determined using a nested one-way analysis of variance followed by a Fisher’s Protected Least Squared Difference post-hoc test (“+” = p<0.05, “-”= p>0.05).

Figure 24

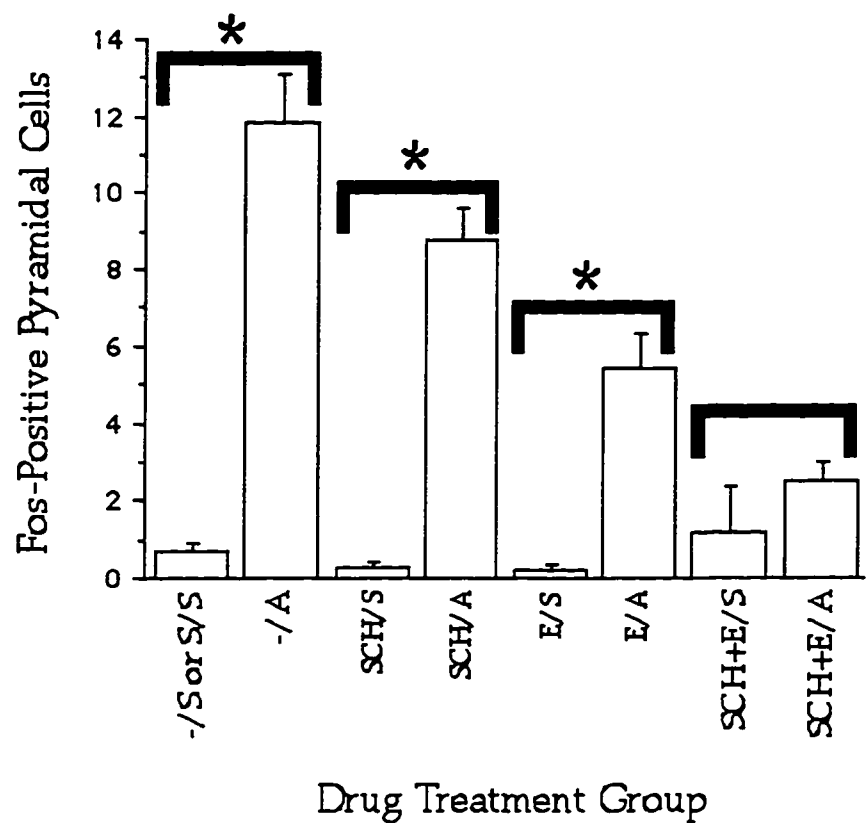


Figure 24: Fos in the Primary Motor Cortex. Bar graph representing the number of Fos-immunopositive layer V pyramidal cells from digitized images of 200X magnified sections through the primary motor cortex. All numbers shown are means $\pm$ S.E.M. for animals in that drug treatment group. The tissue sections used to generate this graph are the same as those used to generate Figure 23. Layer V of the primary motor cortex was determined using standard morphological criteria and the number of Fos-immunopositive neurons was assessed by visual inspection. Brackets compare rat subgroups with the same drug pretreatment and different drug treatments. Significance between drug treatment groups was determined using a nested one-way analysis of variance followed by a Fisher's Protected Least Squared Difference post-hoc test (* = $p < 0.05$). A statistical comparison of all of the drug treatment groups is presented in Table 3.

Table 3

	-/A	SCH/S	SCH/A	E/S	E/A	SCH+E/S	SCH+E/A
-/S or S/S	+	-	+	-	+	-	-
-/A		+	-	+	+	+	+
SCH/S			+	-	+	-	-
SCH/A				+	+	+	+
E/S					+	-	-
E/A						-	-
SCH+E/S							-

Table 3: Comparison Between Drug Treatment Groups of the Induction of C-Fos in the Primary Motor Cortex. Fos-immunopositive neurons were counted as described in the Section 3.2 and in Figure 24. Significance between drug treatment groups was determined using a nested one-way analysis of variance followed by a Fisher’s Protected Least Squared Difference post-hoc test (“+” = $p<0.05$, “-” = $p>0.05$).

3.4. Discussion

In this study, rats were pretreated with dopaminergic ligands prior to administration of amphetamine or saline in order to address the dopamine receptor-dependent mechanisms by which amphetamine leads to induction of the immediate early gene *c-fos*. Our findings demonstrate that induction of *c-fos* in layer V pyramidal cells of the primary motor cortex is dependent upon dopaminergic neurotransmission, as is striatal induction. However, this cortical induction displays a different dependence on D1-like and D2-like dopamine receptor subtype activation, which may reflect the molecular mechanisms through which dopamine influences subcortico-cortical circuits.

In order to address the role of dopamine receptor subtypes in the amphetamine induction of *c-fos*, rats were pretreated with either SCH23390, eticlopride, both drugs, or saline prior to administration of either amphetamine or saline. SCH23390 is a potent inhibitor of adenylate cyclase activity (Hyttel, 1983; Hyttel, 1984) and has been shown to label specifically D1-like dopamine receptors with 600-fold higher affinity for D1-like than D2-like receptors (Christensen *et al.*, 1984) with displacement of D2-selective drugs in the micromolar range (Hyttel *et al.*, 1983; Andersen and Gronvald, 1986; Andersen, 1988). SCH23390 has also been demonstrated to interact with 5HT₂ receptors with 23-fold lower affinity than D1 receptors (Christensen *et al.*, 1984). On the other hand, eticlopride is a substituted benzamide, a group of D2-like dopamine receptor antagonists whose binding is blocked with other D2-like dopamine receptor antagonists like haloperidol and *d,l*-sulpiride, but not with serotonin or adrenergic receptor blockers, and which shows a low IC₅₀ value for the displacement of the dopamine D2-like ligands [³H]spiperone

and [^3H]domperidone (Hall *et al.*, 1985; Kohler *et al.*, 1986). Although many dopaminergic ligands have some affinity for the 5HT₂ receptor, as discussed above for SCH23390, a comparison of various D₂-like dopamine receptor antagonists has shown that eticlopride has the highest D₂/5HT₂ selectivity ratio (Hall *et al.*, 1985; Kohler *et al.*, 1986).

Many studies involving administration of dopaminergic ligands, neurotoxic lesioning of dopaminergic neurons and immunohistochemistry have provided a framework for understanding how an amphetamine-mediated dopamine efflux leads to induction of *c-fos* in the striatum, as shown in Figure 7. For example, the indirect dopamine agonist cocaine and the direct but nonspecific dopamine agonist apomorphine both induce *c-fos* in the striatum preferentially in neurons projecting to the substantia nigra and expressing the D₁ dopamine receptor subtype (Young *et al.*, 1991; Berretta *et al.*, 1992; Cenci *et al.*, 1992). However, there is evidence to suggest that this induction, while dependent upon D₁ dopamine receptor activation, involves other dopaminergic and perhaps nondopaminergic mechanisms as well. The degree to which D₁ dopamine receptor agonism induces striatal *c-fos* in the non-sensitized striatum is still a matter of controversy. Absence of *c-fos* induction has been shown following administration of 4 mg/kg of the D₁-like dopamine receptor agonist SKF 38393 (Robertson *et al.*, 1989; Robertson *et al.*, 1992), sparse but widespread immunoreactivity following 10 mg/kg of the D₁-like dopamine receptor agonist SKF 81297 (Moratalla *et al.*, 1996) or 20 mg/kg SKF 38393 (La Hoste *et al.*, 1993), and a significant dose-dependent induction has been shown following incremental doses of the full D₁ dopamine receptor agonist A-77636 (Wirtshafter and Asin, 1994). In addition, antagonists against other

dopamine and nondopamine receptors inhibit the striatal induction of *c-fos* by dopaminergic ligands, such as eticlopride (Ruskin and Marshall, 1994) and NMDA receptor antagonist MK-801 (Torres and Rivier, 1993; Ziolkowska and Holtt, 1993).

There is evidence that the induction of *c-fos* in the striatum may occur concomitantly with or as a result of neuronal activation by dopamine. For example, D2 dopamine receptor agonism has a suppressive effect on striatal neurons, likely a result of increased potassium conductance (White and Wang, 1986; Hu *et al.*, 1990). Similarly, *c-fos* induction occurs in striatopallidal neurons, a subset of striatal efferent neurons which express the D2 dopamine receptor subtype, in the presence of a D2-like dopamine receptor antagonist such as haloperidol (Robertson *et al.*, 1992). This is supported by our results showing that the D2-like antagonist eticlopride induces *c-fos* in the rat striatum (Figure 15). On the other hand, D1 dopamine receptor agonism alone does not increase the spontaneous firing rate of striatal neurons, but rather increases glutamate-evoked neuronal activity in the striatum (Hu *et al.*, 1990). In other words, D1 dopamine receptor agonism promotes the increased neuronal activity in the striatum that is produced through another neurochemical event. Similarly, D1 dopamine receptor agonism alone may only minimally induce *c-fos* (Robertson *et al.*, 1989b; Robertson *et al.*, 1992), while necessary for the induction of *c-fos* produced by the indirect dopamine agonists amphetamine (Figure 13) and cocaine or the nonspecific direct dopamine agonist apomorphine, as previously described (Young *et al.*, 1991; Berretta *et al.*, 1992; Cenci *et al.*, 1992).

The induction of *c-fos* in the primary motor cortex may be a direct reflection of the effects of dopaminergic neurotransmission in the striatum on glutamatergic thalamocortical tone. As described previously, dopaminergic neurotransmission can affect both the firing rate as well as transcriptional activity of striatal neurons, and this data may be interpreted in light of known neuroanatomical connections in order to address how these changes may influence other components of the circuit. For example, it has been described in Chapter 1 that the striatopallidal neuron may contribute to thalamocortical excitation following D2-mediated suppression, as a result of a reduction in GABAergic neurotransmission in the globus pallidus (Nagy *et al.*, 1978; Jones and Mogenson, 1980; Scarnati *et al.*, 1983; Smith and Bolam, 1990). Therefore, D2 receptor antagonism, while inducing *c-fos* in the striatum, may reverse the decrease in GABAergic neurotransmission in the globus pallidus attributed to amphetamine-mediated dopaminergic agonism of striatopallidal D2 receptors. Indeed, D2 receptor antagonism reduces the induction of *c-fos* in the primary motor cortex by amphetamine in the present study (Figure 18). The inability of D2 receptor antagonism alone to induce *c-fos* in the primary motor cortex (Figure 17) as it does in the striatum (Figure 15) further suggests that increasing the activity of the striatopallidal neuron does not correspond with increased thalamocortical tone.

The hypothesis that amphetamine-mediated striatal dopamine efflux contributes to the induction of *c-fos* in the primary motor cortex suggests a role for the striatonigral neuron. This neuronal population has been shown to express D1 dopamine receptor mRNA (Le Moine *et al.*, 1991) and sends a GABAergic projection directly to the substantia nigra,

an output nucleus of the basal ganglia (Williams and Faull, 1985). For these two reasons the striatonigral neuron is likely to contribute to the dopamine-mediated suppression of neurons in the substantia nigra. However, our results suggest that it is unlikely that dopaminergic agonism of the striatonigral D1 receptor promotes *c-fos* induction in the primary motor cortex. D1 dopamine receptor antagonism with SCH23390 did block the striatal induction of *c-fos* by amphetamine (Figure 13) which has been demonstrated to occur in the striatonigral neuron (Berretta *et al.*, 1992). However, pretreatment with SCH23390 had only a minimal effect on the amphetamine-mediated induction of *c-fos* in the primary motor cortex (Figure 14), and furthermore, combined antagonism with the D2-like dopamine receptor antagonist did not produce a statistically-significant decrease in cortical *c-fos* induction (Figure 22) as compared with D2 dopamine receptor antagonism alone (Figure 18 and Table 3).

Striatal D2 dopamine receptor activity may indirectly influence the striatonigral neuron through modulation of cholinergic neurotransmission. The dopamine D2 receptor has been shown to be expressed in cholinergic interneurons (Le Moine *et al.*, 1990b; Aubry *et al.*, 1993). Acetylcholine release by these cells is regulated by dopamine, as D1 and D2 dopamine receptor agonism increases and decreases striatal acetylcholine levels, respectively (Agid *et al.*, 1975; Guyenet *et al.*, 1975; Drukarch *et al.*, 1990; Zocchi and Pert, 1993; Imperato *et al.*, 1994). There is evidence to suggest that increased striatal acetylcholine, such as found following administration of a D2-like dopamine receptor antagonist, decreases the activity of neurons which utilize the D1 subtype. For example, blockade of muscarinic receptors leads to a potentiation of D1-

mediated effects such as locomotion and induction of the immediate early gene *c-fos* in 6-OHDA-lesioned rats (Morelli *et al.*, 1993). Therefore, the possibility exists that dopaminergic neurotransmission in the striatum may influence the output of striatonigral neurons through indirect mechanisms such as modulation of synaptic acetylcholine.

The possibility also exists that a component of the induction of *c-fos* by amphetamine in the motor cortex is due to cortical dopamine receptor agonism. However, it is unlikely that this represents a major mechanism of action. Studies in which the noradrenergic projection to the cortex was lesioned (confirmed with dopamine- β -hydroxylase staining) have allowed investigators to use tyrosine hydroxylase (TH) immunohistochemistry to explore the dopaminergic innervation of the rat cortex. In addition to the well-documented projection from the ventral tegmental area/medial substantia nigra pars compacta to the medial prefrontal cortex, a low-intensity strip of TH-labeled neurons was found to terminate in the primary motor and somatosensory cortex (Berger *et al.*, 1985). Furthermore, *in situ* hybridization has demonstrated that both dopamine D1 and D2 dopamine receptor subtypes are expressed in the motor and parietal cortex (Gaspar *et al.*, 1995). However, since this dopaminergic innervation is sparse and the expression of dopamine receptor subtypes weak (and not found on the descending corticospinal neurons), it is unlikely that such a direct dopaminergic modulation of sensorimotor cortex plays a large role in dopamine receptor-dependent induction of *c-fos*.

The behavioral changes associated with administration of amphetamine to rodents are dependent upon D1-like and D2-like dopamine receptor-dependent signal transduction in the anatomical

circuitry subserving locomotion (Pijnenburg *et al.*, 1975a,b; Yurek *et al.*, 1993). Dissection of the molecular mechanisms through which neuroanatomical pathways respond to increased extracellular dopamine, in light of information concerning the role of each pathway in effecting behaviors, will provide insight into the role this neurotransmitter plays in movement.

4.0. Conclusions

The modulation of polysynaptic circuits by increased striatal dopamine is essential to the production of complex behaviors following the administration of amphetamine to rodents (Sharp *et al.*, 1987; Kuczenski *et al.*, 1991; Guix *et al.*, 1992). Evidence to support this role for dopamine includes the effects of neurotoxic lesioning of mesencephalic dopaminergic neurons and of pharmacological blockade of dopamine receptors prior to amphetamine administration (Kelly *et al.*, 1975; Arnt and Hyttel, 1984; Paul *et al.*, 1992; Yurek and Hipkens, 1993). However, the presence of multiple dopamine receptor subtypes on different neuronal populations in the striatum (Le Moine *et al.*, 1990a,b; Le Moine *et al.*, 1991) suggests several mechanisms through which dopaminergic stimuli may influence the motor pathways which are processed in the basal ganglia.

One signal transduction mechanism which is expressed in a dopamine receptor-dependent manner in striatal efferent neurons following the administration of amphetamine is the induction of the immediate early gene *c-fos* (Berretta *et al.*, 1992; Ruskin and Marshall, 1994). The present study focuses on the amphetamine induction of *c-fos* in efferent neurons of the striatum and the primary motor cortex, two components of the circuitry subserving movement. The significance of the findings of the present study has been interpreted in light of the segregation of dopamine receptor subtypes to efferent striatal neuronal populations, the electrophysiological effects of dopamine receptor agonism on the cells which express them, and the role dopamine receptor-expressing neurons play in the disinhibition of thalamic neurons and the activation of motor cortex.

Modulation of amphetamine-mediated *c-fos* induction in the primary motor cortex does not necessarily correlate with the behavioral responses to amphetamine. This caveat is important in interpreting experiments using *c-fos* induction to study the mechanisms involved in the effects of amphetamine. The nonspecific dopamine agonist apomorphine and the indirect dopamine agonist cocaine both induce *c-fos* in the striatonigral neuron in a D1-like dopamine receptor-dependent manner (Young *et al.*, 1991; Berretta *et al.*, 1992; Cenci *et al.*, 1992). The striatal induction of *c-fos* by the indirect dopamine agonist amphetamine is also D1-like dopamine receptor dependent (Figure 13). It is likely, therefore, that the *c-fos* induction by amphetamine in the striatum occurs in the striatonigral neuron. The amphetamine-mediated induction of *c-fos* in the primary motor cortex is largely independent of D1-like dopamine receptor stimulation (Figure 14). Thus dopaminergic input through the striatonigral pathway appears not to influence the activity of the pyramidal cells of motor cortex. Yet D1-like dopamine receptor antagonism has a dramatic effect on reducing amphetamine-mediated behavior (Yurek and Hipkens, 1993; Asin *et al.*, 1994), and D1-receptor deficient mice show no hyperactivity to the psychostimulant effects of cocaine, also known to be dopamine mediated (Xu *et al.*, 1994). Clearly, in order to develop an understanding of how an amphetamine-mediated dopamine efflux produces locomotor behaviors, it is important to address the molecular components necessary for information to be communicated to motor regions, such as shown by immediate early gene induction. However, the presence of Fos in neuronal populations important to motor behavior does not necessarily correlate with

locomotion, just as the lack of *c-fos* induction does not necessarily correlate with immobility.

The expression of *c-fos* itself may play a role in the motor effects of amphetamine. Experiments which suggest a role for the Fos protein in the production of the motor effects of psychostimulants have employed antisense oligodeoxynucleotide technology. Bilateral depletion of the Fos protein in the nucleus accumbens of cocaine-treated rats interferes with cocaine-induced hyperlocomotor activity (Heilig *et al.*, 1993). Furthermore, unilateral attenuation of the striatal expression of *c-fos* prior to administration of amphetamine results in a pattern of rotation similar to unilateral dopamine depletion (Hooper *et al.*, 1994). However, it is important to recognize that there is little evidence on the regulatory adaptations which result from chronic blockade of immediate early gene expression. Also, several studies suggest that *c-fos* induction is not the result of motor activity. Anesthetized, unilaterally dopamine-depleted rodents given the D1 agonist CY 208-243 show induction of *c-fos* despite the lack of the characteristic pattern of rotation which would occur in the awake animal (Robertson *et al.*, 1989b). Furthermore, there is little induction of *c-fos* in the striatum or primary motor cortex of saline-treated rats which exhibit the normal exploratory movements of rodents (Figures 9 and 10). Analysis of these studies suggests a potential role for the Fos protein in mediating dopamine-dependent behavior.

We have demonstrated that D2-like dopamine receptor activation is required for full induction of *c-fos* by amphetamine in the primary motor cortex. This finding is consonant with a variety of studies indicating the importance of D2-like dopamine receptor activation in motor activity. Clinically, the use of D2-like dopamine receptor

antagonists such as haloperidol as treatment for psychosis has been limited by the extrapyramidal side effects associated with the drugs (Baldessarini, 1990). Furthermore, chronic reduction in striatal D2 dopamine receptors through the use of antisense oligodeoxynucleotide administration has been demonstrated to reduce spontaneous locomotor activity and elicit catalepsy in rats (Zhang and Creese, 1993). As described in Chapter 3, the electrophysiological and molecular biological effects of D2-like dopamine receptor agonism on striatal output neurons may be through a variety of mechanisms, including direct suppression via dendritic D2 receptors on striatopallidal neurons or less direct suppression via regulation of acetylcholine. More detailed information involves addressing the role of neuron-selective dopamine receptor expression on amphetamine-mediated *c-fos* induction in the striatum and primary motor cortex, and behavior. The advent of new technology to eliminate dopamine receptor subtypes from subpopulations of neurons, as opposed to regional depletion through administration of antisense oligodeoxynucleotide or global elimination through the creation of receptor-deficient mutant mice, will provide more specific information concerning the role of dopamine receptor subtypes on selected pathways in the communication of dopaminergic information to different components of the motor circuit.

The present study suggests the necessity of dopamine receptor subtype activation for the communication of dopaminergic information through subcortico-cortical pathways. However, it is also clear that the striatal neurons which express dopamine receptors also coexpress neuropeptides. For example, a large number of D2 dopamine receptor-expressing striatopallidal neurons coexpress the neuropeptide enkephalin

(Le Moine *et al.*, 1990a). Alternatively, expression of the neuropeptide substance P has been shown to colocalize with the striatal neuron projecting to the substantia nigra (Jessell, 1978; Hanson *et al.*, 1981; Le Moine *et al.*, 1991). The expression of these neuropeptides is regulated by dopamine receptor activity (Li *et al.*, 1990; Engber *et al.*, 1992) and so not only may neuropeptide release play a role in amphetamine-mediated changes acutely, altered neuropeptide production may contribute to the chronic effects of amphetamine administration such as behavioral sensitization to the motor effects. Future experiments could address the role of selected neuropeptides in the molecular and behavioral effects of amphetamine, through the use of antisense technology or the creation of mutant mice lacking the expression of the neuropeptide, as previously described.

Behavioral changes resulting from administration of amphetamine to rodents are associated with D1- and D2 dopamine receptor-dependent signal transduction in the anatomical circuitry subserving locomotion. Dissection of the molecular mechanisms by which anatomical pathways respond to increased extracellular dopamine, in light of information concerning the role of each pathway in effecting behaviors, will help to reveal the way in which locomotor information is processed.

5.0. References

- Agid, Y., Guyenet, P., Glowinski, J., Beaujouan, J. C. and Javoy, F. (1975). Inhibitory influence of the nigrostriatal dopamine system on the striatal cholinergic neurons in the rat. *Brain Res.* **86**, 488-492.
- Agnati, L. F., Fuxe, K., Zini, I., Calza, L., Benfenati, F., Zoli, M., Hokfelt, T. and Goldstein, M., (1982). A new approach to quantitate the density and antigen contents of high densities of transmitter-identified terminals; immunocytochemical studies on different types of tyrosine hydroxylase immunoreactive nerve terminals in nucleus caudatus putamen of the rat. *Neurosci. Lett.* **32**: 253-258.
- Agnati, L. F. and Fuxe, K., (1984). Computer-assisted morphometry and microdensitometry of transmitter-identified neurons with special reference to the mesostriatal dopamine pathway: I. Methodological aspects. *Acta Physiol. Scand.* **532**: 5-36.
- Airila, Y. (1913). Über die Einwirkung verschiedener Erregungsmittel der Grosshirnrinde auf den Chloralhydratschlaf. *Arch. Int. Pharmacodyn. Thé.* **23**, 453-459.
- Alexander, G. E., DeLong, M. R. and Strick, P. L. (1986). Parallel organization of functionally segregated circuits linking basal ganglia and cortex. *Annu. Rev. Neurosci.* **9**, 357-381.
- Alexander, G. E. and Crutcher, M. D. (1990). Functional architecture of basal ganglia circuits: neural substrates of parallel processing. *Trends Neurosci.* **13**, 266-271.
- Andersen, P. H. and Gronvald, F. C. (1986). Specific binding of [3H]SCH23390 to dopamine D1 receptors in vivo. *Life Sci.* **38**, 1507-1514.
- Andersen, P. H. (1988). Comparison of the pharmacological characteristics of [3H]raclopride and [3H]SCH23390 binding to dopamine receptors in vivo in mouse brain. *Eur. J. Pharmacol.* **146**, 113-120.
- Antelman, S. M., Eichler, A. J., Black, C. A. and Kocan, D. (1980). Interchangeability of stress and amphetamine in sensitization. *Science* **207**, 329-331.

Anton, F., Herdegen, T., Peppel, P. and Leah, J. D. (1991). C-fos-like immunoreactivity in rat brainstem neurons following noxious chemical stimulation of the nasal mucosa. *Neuroscience* **44**, 409-420.

Archer, T., Fredriksson, A., Jonsson, G., Lewander, T., Mohammed, A. K., Ross, S. B. and Soderberg, U. (1986). Central noradrenaline depletion antagonizes aspects of d-amphetamine-induced hyperactivity in the rat. *Psychopharmacology* **88**, 141-146.

Ariano, M. A., Stromski, C. J., Smyk-Randall, E. M. and Sibley, D. R. (1992). D2 dopamine receptor localization on striatonigral neurons. *Neurosci. Lett.* **144**, 215-220.

Ariano, M.A. and Sibley, D.R. (1994). Dopamine receptor distribution in the rat CNS: elucidation using anti-peptide antisera directed against D_{1A} and D₃ subtypes. *Brain Res.* **649**, 95-110.

Arluison, M. M., Dietl, M. and Thibault, J. (1984). Ultrastructural morphology of dopaminergic nerve terminals and synapses in the striatum of the rat using tyrosine hydroxylase immunocytochemistry: A topographical study. *Brain Res. Bull.* **13**, 269-285.

Arnold, E. B., Molinoff, P. B. and Rutledge, C. O. (1977). The release of endogenous norepinephrine and dopamine from cerebral cortex by amphetamine. *J. Pharmacol. Exp. Ther.* **202**, 544-557.

Arnt, J. and Hyttel, J. (1984). Differential inhibition by dopamine D-1 and D-2 antagonists of circling behaviour induced by dopamine agonists in rats with unilateral 6-hydroxydopamine lesions. *Eur. J. Pharmacol.* **102**, 349-354.

Asin, K.E., Nikkel, A.L., Wirtshafter, D. (1994). Repeated D1 receptor agonist treatment blocks cocaine-induced locomotor activity and c-fos expression. *Brain Res.* **637**, 342-344.

Aubry, J.-M., Schulz, M.-F., Pagliusi, P. and Kiss, J. Z. (1993). Coexpression of dopamine D2 and substance P (neurokinin-1) receptor messenger RNAs by a subpopulation of cholinergic neurons in the rat striatum. *Neuroscience* **53**, 417-424.

Auer, R. N., (1994). Automated nerve fibre size and myelin sheath measurement using microcomputer-based digital image analysis: theory, method and results. *J. Neurosci. Methods* **51**: 229-238.

Augood, S. J., Westmore, K., McKenna, P. J. and Emson, P. C. (1993). Coexpression of dopamine transporter mRNA and tyrosine hydroxylase mRNA in ventral mesencephalic neurones. *Mol. Brain Res.* **20**, 328-334.

Baldessarini, R. J. (1990). Drugs and the Treatment of Psychiatric Disorders. The Pharmacological Basis of Therapeutics. New York, Pergamon Press. 383-435.

Battaglia, G., Norman, A. B., Hess, E. J. and Creese, I. (1985). D2 dopamine receptor-mediated inhibition of forskolin-stimulated adenylate cyclase activity in rat striatum. *Neurosci. Lett.* **59**, 177-182.

Berger, B., Verney, C., Alvarez, C., Vigny, A. and Helle, K. B. (1985). New dopaminergic terminal fields in the motor, visual (area 18b) and retrosplenial cortex in the young and adult rat. Immunocytochemical and catecholamine histochemical analyses. *Neuroscience* **15**, 983-998.

Berretta, S., Robertson, H. A. and Graybiel, A. M. (1992). Dopamine and glutamate agonists stimulate neuron-specific expression of Fos-like protein in the striatum. *J. Neurophysiol.* **68**, 767-77.

Bertler, A. (1961). Effect of reserpine on the storage of catechol amines in brain and other tissues. *Acta Physiol. Scand.* **51**, 75-83.

Blanc, G., Trovero, F., Vezina, P., Herve, D., Godeheu, A.-M., Glowinski, J. and Tassin, J.-P. (1994). Blockade of prefronto-cortical alpha1-adrenergic receptors prevents locomotor hyperactivity induced by subcortical d-amphetamine injection. *Eur. J. Neurosci.* **6**, 293-298.

Blanchard, V., Raisman-Vozari, R., Vyas, S., Michel, P. P., Javoy-Agid, F., Uhl, G. and Agid, Y. (1994). Differential expression of tyrosine hydroxylase and membrane dopamine transporter genes in subpopulations of dopaminergic neurons of the rat mesencephalon. *Mol. Brain Res.* **22**, 29-40.

Bolam, J. P. and Smith, Y. (1992). The striatum and the globus pallidus send convergent synaptic inputs onto single cells in the entopeduncular nucleus of the rat: A double anterograde labelling study combined with postembedding immunocytochemistry for GABA. *J. Comp. Neurol.* **321**, 456-476.

Bolender, R. P., Charleston, J., Mottet, K. and McCabe, J. T., (1991). Quantitative morphology of the nervous system: expanding horizons. *Anat. Rec.* **231**: 407-415.

Brouwer, N., Van Dijken, H., Ruiters, M. H. J., Van Willigen, J.-D. and Ter Horst, G. J. (1992). Localization of dopamine D2 receptor mRNA with non-radioactive in situ hybridization histochemistry. *Neurosci. Lett.* **142**, 223-227.

Brown, A. R., (1990). Combined immunocytochemical staining and image analysis for the study of lymphocyte specificity and function in situ. *J. Immunol. Methods* **130**: 111-121.

Bullitt, E. (1989). Induction of C-Fos-like protein within the lumbar spinal cord and thalamus of the rat following peripheral stimulation. *Brain Res.* **493**, 391-397.

Bullitt, E. (1990). Expression of C-fos like protein as a marker for neuronal activity following noxious stimulation in the rat. *J. Comp. Neurol.* **296**, 517-530.

Bunzow, J. R., Van Tol, H. H. M., Grandy, D. K., Albert, P., Salon, J., Christie, M., Machida, C. A., Neve, K. A. and Civelli, O. (1988). Cloning and expression of a rat D2 dopamine receptor cDNA. *Nature* **336**, 783-787.

Cador, M., Dullac, J. and Mormede, P. (1993). Modulation of the locomotor response to amphetamine by corticosterone. *Neuroscience* **56**, 981-988.

Callaway, C. W., Kuczenski, R. and Segal, D. S. (1989). Reserpine enhances amphetamine stereotypies without increasing amphetamine-induced changes in striatal dialysate dopamine. *Brain Res.* **505**, 83-90.

Campbell, B. A. and Fibiger, H. C. (1971). Potentiation of amphetamine-induced arousal by starvation. *Nature* **233**, 424-425.

Carlsson, A., Lindqvist, M., Magnusson, M. and Waldeck, B. (1958). On the presence of 3-hydroxytryptamine in brain. *Science* **127**, 471.

Carroll, M. E., France, C. P. and Meisch, R. A. (1979). Food deprivation increases oral and intravenous intake in rats. *Science* **205**, 319-321.

Cenci, M. A., Campbell, K., Wictorin, K. and Björklund, A. (1992). Striatal c-fos induction by cocaine or apomorphine occurs preferentially in output neurons projecting to the substantia nigra in the rat. *Eur. J. Neurosci.* **4**, 376-380.

Cerruti, C., Walther, D. M., Kuhar, M. J. and Uhl, G. R. (1993). Dopamine transporter mRNA expression is intense in rat midbrain neurons and modest outside midbrain. *Mol. Brain Res.* **18**, 181-186.

Chiu, R., Boyle, W., Meek, J., Smeal, T., Hunter, T. and Karin, M. (1988). The c-fos protein interacts with c-jun/AP1 to stimulate transcription of AP-1 responsive genes. *Cell* **54**, 541-552.

Christensen, A. V., Arnt, J., Hyttel, J., Larsen, J. J. and Svendsen, O. (1984). Pharmacological effects of a specific dopamine D-1 antagonist SCH23390 in comparison with neuroleptics. *Life Sci.* **34**, 1529-1540.

Cicirata, F., Angaut, P., Cioni, M., Serapide, M. F. and Papale, A. (1986). Functional organization of thalamic projections to the motor cortex. An anatomical and electrophysiological study in the rat. *Neuroscience* **19**, 81-99.

Colombi, M., Moro, L., Zoppi, N. and Barlati, S., (1993). Quantitative evaluation of mRNAs by in situ hybridization and image analysis: principles and applications. *DNA Cell Biol.* **12**: 629-636.

Cooper, D. M. F., Bier, C.M., Halford, M. K., Ahlijanian, K. and Zahniser, N. R. (1986). Dopamine, acting through D-2 receptors, inhibits rat striatal adenylate cyclase by a GTP-dependent process. *Mol. Pharmacol.* **29**, 113-119.

Creese, I. and Iversen, S. D. (1972). Amphetamine response after dopamine neurone destruction. *Nature New Biol.* **238**, 247-248.

Creese, I. and Iversen, S. D. (1973). Blockade of amphetamine-induced motor stimulation and stereotypy in the adult rat following neonatal treatment with 6-hydroxydopamine. *Brain Res.* **55**, 369-382.

Curran, T. and Franza, B. R. J. (1988). Fos and jun: the AP-1 connection. *Cell* **55**, 395-397.

Dahlström, A. and Fuxe, K. (1964). Evidence for the existence of monoamine-containing neurons in the central nervous system. *Acta Physiol. Scand.* **62**, 1-39.

De La Garza, R., Bergman, J. and Hartel, C. R. (1981). Food deprivation and cocaine self-administration. *Pharmacol. Biochem. Behav.* **15**, 141-144.

Dearry, A., Gingrich, J. A., Falardeau, P., Freneau, R. T. J., Bates, M. D. and Caron, M. G. (1990). Molecular cloning and expression of the gene for a human D1 dopamine receptor. *Nature* **347**, 72-76.

Deniau, J. M., Kita, H. and Kitai, S. T. (1992). Patterns of termination of cerebellar and basal ganglia efferents in the rat thalamus. Strictly segregated and partly overlapping projections. *Neurosci. Lett.* **144**, 202-206.

Di Chiara, G., Porceddu, M. L., Morelli, M., Mulas, M. L. and Gessa, G. L. (1979). Evidence for a GABAergic projection from the substantia nigra to the ventromedial thalamus and to the superior colliculus of the rat. *Brain Res.* **176**, 273-284.

Donoghue, J. P. and Wise, S. P. (1982). The motor cortex of the rat: cytoarchitecture and microstimulation mapping. *J. Comp. Neurol.* **212**, 76-88.

Donoghue, J. P. and Parham, C. (1983). Afferent connections of the lateral agranular field of the rat motor cortex. *J. Comp. Neurol.* **217**, 390-404.

Donoghue, J. P. and Herkenham, M. (1986). Neostriatal projections from individual cortical fields conform to histochemically distinct striatal compartments in the rat. *Brain Res.* **365**, 397-403.

Doyle, W., (1962). Operations useful for similarity-invariant pattern recognition. *J. Assoc. Comput. Mach.* **9**: 259-267.

Dragunow, M. and Robertson, H. A. (1987a). Generalized seizures induce c-fos protein(s) in mammalian neurons. *Neurosci. Lett.* **82**, 157-161.

Dragunow, M. and Robertson, H. A. (1987b). Kindling stimulation induces c-fos protein(s) in granule cells of the rat dentate gyrus. *Nature* **329**, 441-442.

Dragunow, M., Logan, B. and Lavery, R. (1991). 3,4-Methylenedioxymethamphetamine induces fos-like proteins in rat basal ganglia: reversal with MK-801. *Eur. J. Pharmacol.* **206**, 255-258.

Drukarch, B., Schepens, E. and Stoof, J. C. (1990). Muscarinic receptor activation attenuates D2 dopamine receptor mediated inhibition of acetylcholine release in rat striatum: indications for a common signal transduction pathway. *Neuroscience* **37**, 1-9.

Ebrahimi, A., Pochet, R. and Roger, M. (1992). Topographical organization of the projections from physiologically identified areas of the motor cortex to the striatum in the rat. *Neurosci. Res.* **14**, 39-60.

Edeleano, L. (1887). Über einige Derivate der Phenylmetacrylsäure und der Phenylisobuttersäure. *Ber. dt. chem. Ges.* **20**, 616a.

Ellinwood, E. H. J., Sudilovsky, A. and Nelson, L. M. (1973). Evolving behavior in the clinical and experimental amphetamine (model) psychosis. *Am. J. Psychiatry* **130**, 1088-1093.

Elsworth, J. D., Taylor, J. R., Berger, P. and Roth, R. H. (1993). Cocaine-sensitive and -insensitive dopamine uptake in prefrontal cortex, nucleus accumbens and striatum. *Neurochem. Int.* **23**, 61-9.

Enestrom, S. and Kniola, B., (1990). Quantitative ultrastructural immunocytochemistry using a computerized image analysis system. *Stain Technology* **65**: 263-278.

Engber, T. M., Boldry, R. C., Kuo, S. and Chase, T. N. (1992). Dopaminergic modulation of striatal neuropeptides: differential effects of D1 and D2 receptor stimulation on somatostatin, neuropeptide Y, neurotensin, dynorphin and enkephalin. *Brain Res.* **581**, 261-268.

Engberg, G. and Svensson, T. H. (1979). Amphetamine-induced inhibition of central noradrenergic neurons: a pharmacological analysis. *Life Sci.* **24**, 2245-2254.

Fischer, J. F. and Cho, A. K. (1979). Chemical release of dopamine from striatal homogenates: evidence for an exchange diffusion model. *J. Pharmacol. Exp. Ther.* **208**, 203-209.

Fox, C. A., Mansour, A., Thompson, R. C., Bunzow, J. R., Civelli, O. and Watson, S., Jr. (1993). The distribution of dopamine D2 receptor heteronuclear RNA (hnRNA) in the rat brain. *J. Chem. Neuroanat.* **6**, 363-373.

Freedman, J. E. and Weight, F. F. (1988). Single K⁺ channels activated by D-2 dopamine receptors in acutely dissociated neurons from rat corpus striatum. *Proc. Natl. Acad. Sci. USA* **85**, 3618-3622.

Freneau, R. T. J., Duncan, G. E., Fornaretto, M.-G., Dearry, A., Gingrich, J. A., Breese, G. R. and Caron, M. G. (1991). Localization of D1 dopamine receptor mRNA in brain supports a role in cognitive, affective, and neuroendocrine aspects of dopaminergic neurotransmission. *Proc. Natl. Acad. Sci. USA* **88**, 3772-3776.

Freund, T. F., Powell, J. F. and Smith, A. D. (1984). Tyrosine hydroxylase-immunoreactive boutons in synaptic contact with identified striatonigral neurons, with particular reference to dendritic spines. *Neuroscience* **13**, 1189-1215.

Fritz, P., Multhaupt, H., Hoenes, J., Lutz, D., Doerrer, R., Schwartzmann, P. and Tucek, H. V., (1992). Quantitative immunohistochemistry: Theoretical background and its application in biology and surgical pathology. *Prog. Histochem. Cytochem.* **24**: 1-53.

Fuxe, K., Agnati, L. F., Rosen, L., Bjelke, B., Cintra, A., Bortolotti, F., Tinner, B., Andersson, C., Hasselroth, U. and Steinbusch, H., (1991). Computer-assisted image analysis techniques allow a characterization of the compartments within the basal ganglia. Focus on functional compartments produced by d-amphetamine activation of the c-fos gene and its relationship to the glucocorticoid receptor. *J. Chem. Neuroanat.* **4**: 355-372.

Gardiner, T. W., Iverson, D. A. and Rebec, G. V. (1988). Heterogeneous responses of neostriatal neurons to amphetamine in freely moving rats. *Brain Res.* **463**, 268-274.

Gaspar, P., Bloch, B. and Le Moine, C. (1995). D1 and D2 receptor gene expression in the rat frontal cortex: cellular localization in different classes of efferent neurons. *Eur. J. Neurosci.* **7**, 1050-1063.

Gerfen, C. R. (1984). The neostriatal mosaic: compartmentalization of corticostriatal input and striatonigral output systems. *Nature* **311**, 461-464.

Gerfen, C. R. (1985). The neostriatal mosaic. I. Compartmental organization of projections from the striatum to the substantia nigra in the rat. *J. Comp. Neurol.* **236**, 454-476.

Gerfen, C. R., Baimbridge, K. G. and Miller, J. J. (1985). The neostriatal mosaic: Compartmental distribution of calcium-binding protein and parvalbumin in the basal ganglia of the rat and monkey. *Proc. Natl. Acad. Sci. USA* **82**, 8780-8784.

Gerfen, C. R. and Young, W. S. I. (1988). Distribution of striatonigral and striatopallidal neurons in both patch and matrix compartments: an in situ hybridization histochemistry and fluorescent retrograde tracing study. *Brain Res.* **460**, 161-167.

Gerfen, C. R., Engber, T. M., mahan, L. C., Susel, Z., Chase, T. N. and Monsma, F. J., Jr. (1990). D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science* **250**, 1429-1432.

Gerfen, C., McGinty, J. F. and Young, W. S. I. (1991). Dopamine differentially regulates dynorphin, substance P, and enkephalin expression in striatal neurons: in situ hybridization histochemical analysis. *J. Neurosci.* **11**, 1016-1031.

Giros, B., El Mestikawy, S., Bertrand, L. and Caron, M. G. (1991). Cloning and functional characterization of a cocaine-sensitive dopamine transporter. *FEBS Letters* **295**, 149-154.

Giros, B. and Caron, M. G. (1993). Molecular characterization of the dopamine transporter. *Trends Pharmacol. Sci.* **14**, 43-49.

Giros, B., Jaber, M., Jones, S. R., Wightman, R. M. and Caron, M. G. (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* **379**, 606-12.

Gold, M. S., Miller, N. S. and Jonas, J. M. (1992). Cocaine (and crack): neurobiology. Substance Abuse: A Comprehensive Textbook. Baltimore, Williams and Wilkins. 222-235.

Graham, A. and Aghajanian, G. K. (1971). Effects of amphetamine on single unit activity in a catecholamine nucleus, the locus coeruleus. *Nature* **234**, 100-102.

Grandy, D. K., Zhang, Y., Bouvier, C., Zhou, Q.-Y., Johnson, R. A., Allen, L., Buck, K., Bunzow, J. R., Salon, J. and Civelli, O. (1991). Multiple human D5 dopamine receptor genes: A functional receptor and two pseudogenes. *Proc. Natl. Acad. Sci. USA* **88**, 9175-9179.

Graybiel, A. M. and Ragsdale, C. W. J. (1978). Histochemically distinct compartments in the striatum of human, monkey and cat demonstrated by acetylcholinesterase staining. *Proc. Natl. Acad. Sci. USA* **75**, 5723-5726.

Graybiel, A. M., Ragsdale, C. W., Yoneoka, E. S. and Elde, R. P. (1981). An immunohistochemical study of enkephalins and other neuropeptides in the striatum of the cat with evidence that the opiate peptides are arranged to form mosaic patterns in register with the striosomal compartments visible by acetylcholinesterase staining. *Neuroscience* **6**, 377-397.

Graybiel, A. M. (1984). Correspondence between the dopamine islands and striosomes of the mammalian striatum. *Neuroscience* **13**, 1157-1187.

Graybiel, A. M., Moratalla, R. and Robertson, H. A. (1990). Amphetamine and cocaine induce drug specific activation of the c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum. *Proc. Natl. Acad. Sci. USA* **87**, 6912-6916.

Groenewegen, H. J., Berendse, H. W., Meredith, G. E., Haber, S. N., Voorn, P., Wolters, J. G. and Lohman, A. H. M. (1991). Functional anatomy of the ventral, limbic system-innervated striatum. The Mesolimbic Dopamine System: From Motivation to Action. John Wiley and Sons. 19-59.

Guix, T., Hurd, Y. L. and Ungerstedt, U. (1992). Amphetamine enhances extracellular concentrations of dopamine and acetylcholine in dorsolateral striatum and nucleus accumbens of freely moving rats. *Neurosci. Lett.* **138**, 137-140.

Guyenet, P. G., Agid, Y., Javoy, F., Beaujouan, J. C., Rossier, J. and Glowinski, J. (1975). Effects of dopaminergic receptor agonists and antagonists on the activity of the neo-striatal cholinergic system. *Brain Res.* **84**, 227-244.

Hall, R. D. and Lindholm, E. P. (1974). Organization of motor and somatosensory neocortex in the albino rat. *Brain Res.* **66**, 23-38.

Hall, H., Kohler, C. and Gawell, L. (1985). Some in vitro receptor binding properties of [3H]eticlopride, a novel substituted benzamide, selective for dopamine-D2 receptors in the rat brain. *Eur. J. Pharmacol.* **111**, 191-199.

Hanson, G. R., Alphs, L., Pradham, S. and Lovenberg, W. (1981). Response of striatonigral substance P systems to a dopamine receptor agonist and antagonist. *Neuropharmacology* **20**, 541-548.

Haracz, J. L., Tschanz, J. T., Greenberg, J. and Rebec, G. V. (1989). Amphetamine-induced excitations predominate in single neostriatal neurons showing motor-related activity. *Brain Res.* **489**, 365-368.

Heilig, M., Engel, J. A. and Soderpalm, B. (1993). C-fos antisense in the nucleus accumbens blocks the locomotor stimulant action of cocaine. *Eur. J. Pharmacol.* **236**, 339-340.

Herkenham, M. (1979). The afferent and efferent connections of the ventromedial thalamic nucleus in the rat. *J. Comp. Neurol.* **183**, 487-578.

Herkenham, M. and Pert, C. B. (1981). Mosaic distribution of opiate receptors, parafascicular projections and acetylcholinesterase in rat striatum. *Nature* **291**, 415-418.

Hicks, S. P. and D'Amato, C. J. (1970). Visual and motor function after hemispherectomy in newborn and mature rats. *Exp. Neurol.* **29**, 416-438.

Hicks, S. P. and D'Amato, C. J. (1975). Motor-sensory cortex-corticospinal system and developing locomotion and placing in rats. *Am. J. Anat.* **143**, 1-42.

Hoffman, B. B. and Lefkowitz, R. J. (1990). Catecholamines and sympathomimetic drugs. The Pharmacological Basis of Therapeutics. New York, Pergamon Press, Inc. 210-213.

Hökfelt, T. R., Martensson, R., Björklund, A., Kleinan, S. and Goldstein, M. (1984). Distributional maps of tyrosine-hydroxylase-immunoreactive neurons in the rat brain. Handbook of Chemical Neuroanatomy Vol. 2, Classical transmitters in the CNS Part I. Amsterdam, Elsevier. 277-379.

Hooper, M. L., Chiasson, B. J. and Robertson, H. A. (1994). Infusion into the brain of an antisense oligonucleotide to the immediate-early gene c-fos suppresses production of Fos and produces a behavioral effect. *Neuroscience* **63**, 917-924.

Hu, X.-T., Wachtel, S. R., Galloway, M. P. and White, F. J. (1990). Lesions of the nigrostriatal dopamine projection increase the inhibitory effects of D1 and D2 dopamine agonists on caudate-putamen neurons and relieve D2 receptors from the necessity of D1 receptor stimulation. *J. Neurosci.* **10**, 2318-2329.

Hunt, S. P., Pini, A. and Evan, G. (1987). Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. *Nature* **328**, 632-634.

Hurd, Y. L. and Ungerstedt, U. (1989a). Influence of a carrier transport process on in vivo release and metabolism of dopamine: dependence on extracellular Na⁺. *Life Sci.* **45**, 283-293.

Hurd, Y. L. and Ungerstedt, U. (1989b). Ca²⁺ dependence of the amphetamine, nomifensine, and Lu 19-005 effect on in vivo dopamine transmission. *Eur. J. Pharmacol.* **166**, 261-269.

Hyttel, J. (1983). SCH23390-the first selective dopamine D1 antagonist. *Eur. J. Pharmacol.* **91**, 153-154.

Hyttel, J. (1984). Functional evidence for selective dopamine D-1 receptor blockade by SCH 23390. *Neuropharmacology* **23**, 1395-1401.

Imperato, A., Obinu, M. C., Dazzi, L. and Gessa, G. L. (1994). Does dopamine exert a tonic inhibitory control on the release of striatal acetylcholine in vivo? *Eur. J. Pharmacol.* **251**, 271-279.

Ingham, C. A., Hood, S. H. and Arbuthnott, G. W. (1991). A light and electron microscopical study of enkephalin-immunoreactive structures in the rat neostriatum after removal of the nigrostriatal dopaminergic pathway. *Neuroscience* **42**, 715-730.

Jagoe, R., Steel, J. H., Vucicevic, V., Alexander, N., Van Noorden, S. V., Wootton, R. and Polak, J. M., (1991). Observer variation in quantification of immunocytochemistry by image analysis. *Histochemical Journal* **23**: 541-547.

Jessell, T. M. (1978). Substance P release from the rat substantia nigra. *Brain Res.* **151**, 469-478.

Joensson, L.-E., Gunne, L.-M. and Aenggaerd, E. (1969). Effects of alpha-methyl-tyrosine in amphetamine dependent subjects. *Pharmacol. Clin.* **2**, 27.

Jones, D. L. and Mogenson, G. J. (1980). Nucleus accumbens to globus pallidus GABA projection subserving ambulatory activity. *Am. J. Physiol.* **238**, R63-R69.

Jones, K. J. and Evinger, C. (1991). Differential neuronal expression of c-fos proto-oncogene following peripheral nerve injury or chemically-induced seizure. *J. Neurosci. Res.* **28**, 291-298.

Kebabian, J. W. (1978). Dopamine-sensitive adenylyl cyclase: a receptor mechanism for dopamine. Advances in Biochemical Psychopharmacology. New York, Raven Press. 131-154.

Kelly, P. H., Saviour, P. W. and Iversen, S. D. (1975). Amphetamine and apomorphine responses in rats following 6-OHDA lesions of the nucleus accumbens septi and corpus striatum. *Brain Res.* **94**, 507-522.

Kelly, P. H. and Iversen, S. D. (1976). Selective 6OHDA-induced destruction of mesolimbic dopamine neurons: abolition of psychostimulant-induced locomotor activity in rats. *Eur. J. Pharmacol.* **40**, 45-56.

Kelly, R. B. (1993). Storage and release of neurotransmitters. *Neuron* **10** (Suppl.), 43-53.

Kilpatrick, I. C., Starr, M. S., Fletcher, A., James, T. A. and MacLeod, N. K. (1980). Evidence for a GABAergic nigrothalamic pathway in the rat-I. Behavioral and biochemical studies. *Expl. Brain Res.* **40**, 45-54.

Kilty, J. E., Lorang, D. and Amara, S. G. (1991). Cloning and expression of a cocaine-sensitive rat dopamine transporter. *Science* **254**, 578-579.

King, G. R. and Ellinwood, E. H. J. (1992). Amphetamines and other stimulants. Substance Abuse: A Comprehensive Textbook. Baltimore, Williams and Wilkins. Second, ed. 247-270.

Kohler, C., Hall, H. and Gawell, L. (1986). Regional in vivo binding of the substituted benzamide [3H]eticlopride in the rat brain: evidence for selective labelling of dopamine receptors. *Eur. J. Pharmacol.* **120**, 217-226.

Kouzarides, T. and Ziff, E. (1988). The role of the leucine zipper in the fos-jun interaction. *Nature* **336**, 646-651.

Kowalski, C. and Giraud, P. (1993). Dopamine decreases striatal enkephalin turnover and proenkephalin messenger RNA abundance via D2 receptor activation in primary striatal cell cultures. *Neuroscience* **53**, 665-672.

Kubota, Y., Inagaki, S., Kito, S., Takagi, H. and Smith, A. D. (1986). Ultrastructural evidence of dopaminergic input to enkephalinergic neurons in rat striatum. *Brain Res.* **367**, 374-378.

Kuczenski, R., Segal, D. S. and Aizenstein, M. L. (1991). Amphetamine, cocaine and fencamfamine: relationship between locomotor and stereotypy response profiles and caudate and accumbens dopamine dynamics. *J. Neurosci.* **11**, 2703-2712.

Kuczenski, R. and Segal, D. S. (1992). Regional norepinephrine response to amphetamine using dialysis: comparison with caudate dopamine. *Synapse* **11**, 164-169.

Lacey, M. G., Mercuri, N. B. and North, R. A. (1987). Dopamine acts on D2 receptors to increase potassium conductance in neurons of the rat substantia nigra zona compacta. *J. Physiol.* **393**, 397-416.

LaHoste, G. J., Yu, J. and Marshall, J. F. (1993). Striatal fos expression is indicative of dopamine D1/D2 synergism and receptor supersensitivity. *Proc. Natl. Acad. Sci. USA* **90**, 7451-7455.

Landwehrmeyer, B., Mengod, G. and Palacios, J. M. (1993). Dopamine d3 receptor mRNA and binding sites in human brain. *Mol. Brain Res.* **18**, 187-192.

Large, C. H. and Stubbs, C. M. (1994). The dopamine D3 receptor: Chinese hamsters or Chinese Whispers? *Trends Pharmacol. Sci.* **15**, 46-47.

Le Moine, C., Normand, E., Guitteny, A. F., Fouque, B., Teoule, R. and Bloch, B. (1990a). Dopamine receptor gene expression by enkephalin neurons in rat forebrain. *Proc. Natl. Acad. Sci. USA* **87**, 230-234.

Le Moine, C., Tison, F., and Bloch, B. (1990b). D2 dopamine receptor gene expression by cholinergic neurons in the rat striatum. *Neurosci. Lett.* **117**, 248-252.

Le Moine, C., Normand, E. and Bloch, B. (1991). Phenotypical characterization of the rat striatal neurons expressing the D1 dopamine receptor gene. *Proc. Natl. Acad. Sci. USA* **88**, 4205-4209.

Le Moine, C. and Bloch, B. (1996). Expression of the D3 dopamine receptor in peptidergic neurons of the nucleus accumbens: comparison with the D1 and D2 dopamine receptors. *Neuroscience* **73**, 131-143.

Lester, J., Fink, S., Aronin, N. and DiFiglia, M. (1993). Colocalization of D1 and D2 dopamine receptor mRNAs in striatal neurons. *Brain Res.* **621**, 106-110.

Levant, B., Grigoriadis, D. E. and DeSouza, E. B. (1992). Characterization of [3H] quinpirole binding to D2-like dopamine receptors in rat brain. *J. Pharmacol. Exp. Ther.* **262**, 929-935.

Levesque, D., Diaz, J., Pilon, C., Martres, M.-P., Giros, B., Souil, E., Schott, D., Morgat, J.-L., Schwartz, J.-C. and Sokoloff, P. (1992). Identification, characterization and localization of the dopamine D3 receptor in rat brain using 7-[3H]hydroxy-N,N-di-n-propyl-2-aminotetralin. *Proc. Natl. Acad. Sci. USA* **89**, 8155-8159.

Li, S. J., Jiang, H. K., Stachowiak, M. S., Hudson, P. M., Owyang, V., Nanry, K., Tilson, H. A. and Hong, J. S. (1990). Influence of nigrostriatal dopaminergic tone on the biosynthesis of dynorphin and enkephalin in rat striatum. *Mol. Brain Res.* **8**, 219-225.

Liang, N. Y. and Rutledge, C. O. (1982). Comparison of the release of [3H]dopamine from isolated corpus striatum by amphetamine, fenfluramine and unlabelled dopamine. *Biochem. Pharmacol.* **31**, 983-992.

MacLeod, N. K., James, T. A., Kilpatrick, I. C. and Starr, M. S. (1980). Evidence for a GABAergic nigrothalamic pathway in the rat. *Exp. Brain Res.* **40**, 55-61.

Mansour, A., Meador-Woodruff, J. H., Bunzow, J. R., Civelli, O., Akil, H. and Watson, S. J. (1990). Localization of dopamine D2 receptor mRNA and D1 and D2 receptor binding in the rat brain and pituitary: an in situ hybridization-receptor autoradiographic analysis. *J. Neurosci.* **10**, 2587-2600.

McClelland, D. (1993). Macworld Photoshop 2.5 Bible. San Mateo, IDG Books Worldwide, Inc.

McGeorge, A. J. and Faull, R. L. M. (1987). The organization and collateralization of corticostriate neurones in the motor and sensory cortex of the rat brain. *Brain Res.* **423**, 318-324.

Memo, M., Carboni, E., Trabucchi, M., Carruba, M. O. and Spano, P. F. (1985). Dopamine inhibition of neurotensin-induced increase in Ca^{2+} -influx into rat pituitary cells. *Brain Res.* **347**, 253-257.

Memo, M., Carboni, E., Trabucchi, M., Carruba, M. and Spano, P. F. (1986). Dopaminergic inhibition of prolactin release and calcium influx induced by neurotensin in anterior pituitary is independent of cyclic AMP system. *J. Neurochem.* **47**, 1689-1695.

Merritt, J. E. and Brown, B. L. (1984). The possible involvement of both calcium and cyclic AMP in the dopaminergic inhibition of prolactin secretion. *Life Sci.* **35**, 707-711.

Mezin, P., Tenaud, C., Bosson, J. L. and Stoebner, P., (1994). Morphometric analysis of the peripheral nerve: advantages of the semi-automated interactive method. *J. Neurosci. Methods* **51**: 163-169.

Mize, R. R. (1989). The analysis of immunohistochemical data. Computer Techniques in Neuroanatomy. New York, Plenum Press. 333-372.

Mize, R. R., (1994). Quantitative image analysis for immunocytochemistry and in situ hybridization. *J. Neurosci. Methods* **54**: 219-237.

Moghaddam, B. and Bunney, B. S. (1989). Differential effect of cocaine on extracellular dopamine levels in rat medial prefrontal cortex and nucleus accumbens: comparison to amphetamine. *Synapse* **4**, 155-161.

Monsma, F. J. J., Mahan, L. C., McVittie, L. D., Gerfen, C. R. and Sibley, D. R. (1990). Molecular cloning and expression of a D1 dopamine receptor linked to adenylyl cyclase activation. *Proc. Natl. Acad. Sci. USA* **87**, 6723-6727.

Moratalla, R., Xu, M., Tonegawa, S. and Graybiel, A. M. (1996). Cellular responses to psychomotor stimulant and neuroleptic drugs are abnormal in mice lacking the D1 dopamine receptor. *Proc. Natl. Acad. Sci. USA* **93**, 14928-14933.

Morelli, M., Fenu, S., Cozzolino, A., Pinna, A., Carta, A. and Di Chiara, G. (1993). Blockade of muscarinic receptors potentiates D1 dependent turning behavior and c-fos expression in 6-hydroxydopamine-lesioned rats but does not influence D2 mediated responses. *Neuroscience* **53**, 673-678.

Morgan, J. I., Cohen, D. R., Hempstead, J. L. and Curran, T. (1987). Mapping patterns of c-fos expression in the central nervous system after seizure. *Science* **237**, 192-197.

Mrzljak, L., Bergson, C., Pappy, M., Huff, R., Levenson, R., and Goldman-Rakic, P. S. (1996) Localization of dopamine D4 receptors in GABAergic neurons of the primate brain. *Nature* **381**, 245-247.

Nagy, J. I., Carter, D. A. and Fibiger, H. C. (1978). Anterior striatal projection to the globus pallidus, entopeduncular nucleus and substantia nigra in the rat: the GABA connection. *Brain Res.* **158**, 15-29.

Nakanishi, H., Kita, H. and Kitai, S. T. (1987). Intracellular study of rat substantia nigra pars reticulata neurons in an in vitro preparation: Electrical properties and response characteristics to subthalamic stimulation. *Brain Res.* **437**, 45-55.

Neill, D. B., Grant, L. D. and Grossman, S. P. (1972). Selective potentiation of locomotor effects of amphetamine by midbrain raphé lesions. *Physiol. Behav.* **9**, 655-657.

O'Malley, K. L., Harmon, S., Tang, L. and Todd, R. D. (1992). The rat dopamine D4 receptor: sequence, gene structure, and demonstration of expression in the cardiovascular system. *The New Biologist* **4**, 137-146.

Olson, L., Seiger, A. and Fuxe, K. (1972). Heterogeneity of striatal and limbic dopamine innervation: Highly fluorescent islands in developing and adult rats. *Brain Res.* **44**, 283-288.

Onali, P., Olinas, M. C. and Gessa, L. (1985). Characterization of dopamine receptors mediating inhibition of adenylate cyclase activity in rat striatum. *Mol. Pharmacol.* **28**, 138-145.

Onodera, H., Kogure, K., Ono, Y., Igarashi, K., Kiyota, Y. and Nagaoka, A. (1989). Proto-oncogene c-fos is transiently induced in the rat cerebral cortex after forebrain ischemia. *Neurosci. Lett.* **98**, 101-104.

Ordway, R. W., Walsh, J. V. J. and Singer, J. J. (1989). Arachidonic acid and other fatty acids directly activate potassium channels in smooth muscle cells. *Science* **244**, 1176-1179.

Parent, A. and Hazrati, L.-N. (1995). Functional anatomy of the basal ganglia. I. The cortico-basal ganglia-thalamo-cortical loop. *Brain Res. Rev.* **20**, 91-127.

Paul, M. L., Graybiel, A. M., David, J.-C. and Robertson, H. A. (1992). D1-like and D2-like dopamine receptors synergistically activate rotation and c-fos expression in the dopamine-depleted striatum in a rat model of Parkinson's disease. *J. Neurosci.* **12**, 3729-3742.

Petrusz, P., (1983). Essential requirements for the validity of immunocytochemical staining procedures. *J. Histochem. Cytochem.* **31**: 177-179.

Piazza, P. V., Deminiere, J. M., Le Moal, M. and Simon, H. (1990). Stress- and pharmacologically-induced behavioral sensitization increases vulnerability to acquisition of amphetamine self-administration. *Brain Res.* **514**, 22-26.

Piazza, P. V., Rouge-Pont, F., Deminiere, J. M., Kharouby, M., Le Moal, M. and Simon, H. (1991). Dopaminergic activity is reduced in the prefrontal cortex and increased in the nucleus accumbens of rats predisposed to develop amphetamine self-administration. *Brain Res.* **567**, 2088-2092.

Pijnenburg, A. J. J., Honig, W. M. M. and Van Rossum, J. M. (1975a). Inhibition of d-amphetamine-induced locomotor activity by injection of haloperidol into the nucleus accumbens of the rat. *Psychopharmacologia* **41**, 87-96.

Pijnenburg, A. J. J., Honig, W. M. M. and Van Rossum, J. M. (1975b). Antagonism of apomorphine and amphetamine-induced stereotyped behaviour by injection of low doses of haloperidol into the caudate nucleus and nucleus accumbens. *Psychopharmacologia* **45**, 65-71.

Pizzi, M., D'Agostini, F., Da Prada, M., Spano, P. F. and Haefely, W. E. (1987). Dopamine D-2 receptor stimulation decreases the inositol triphosphate level of rat striatal slices. *Eur. J. Pharmacol.* **136**, 263-264.

Popovici, T., Represa, A., Crepel, V., Barbin, G., Beaudoin, M. and Ben-Ari, Y. (1990). Effects of kainic acid-induced seizures and ischemia on c-fos-like proteins in rat brain. *Brain Res.* **536**, 183-194.

Prewitt, J. M. S. and Mendelsohn, M. L., (1966). The analysis of cell images. *Ann. N.Y. Acad. Sci.* **128**: 1035-1053.

Price, A. W. and Fowler, S. C. (1981). Deficits in contralateral and ipsilateral forepaw motor control following unilateral motor cortical ablations in rats. *Brain Res.* **205**, 81-90.

Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J. and Sealfon, S. C. (1992). Sequence alignment of the G-protein coupled receptor superfamily. *DNA Cell Biol.* **11**, 1-20.

Raiteri, M., Levi, G. and Federico, R. (1974). D-amphetamine and the release of 3H-norepinephrine from synaptosomes. *Eur. J. Pharmacol.* **28**, 237-240.

Ramirez, O. A. and Wang, R. Y. (1986). Locus coeruleus norepinephrine-containing neurons: effects produced by acute and subchronic treatment with antipsychotic drugs and amphetamine. *Brain Res.* **362**, 163-173.

Ramm, P., (1994). Advanced image analysis systems in cell, molecular and neurobiology applications. *J. Neurosci. Methods* **54**: 131-149.

Robertson, G. S., Herrera, D. G., Dragunow, M. and Robertson, H. A. (1989a). L-Dopa activates c-fos in the striatum ipsilateral to a 6-hydroxydopamine lesion to the substantia nigra. *Eur. J. Pharmacol.* **159**, 99-100.

Robertson, H. A., Peterson, M. R., Murphy, K. and Robertson, G. S. (1989b). D1-dopamine receptor agonists selectively activate striatal c-fos independent of rotational behaviour. *Brain Res.* **503**, 346-349.

Robertson, G. S., Vincent, S. R. and Fibiger, H. C. (1992). D1 and D2 dopamine receptors differentially regulate c-fos expression in striatonigral and striatopallidal neurons. *Neuroscience* **49**, 285-296.

Robertson, G. S., Tetzlaff, W., Bedard, A., St.-Jean, M. and Wigle, N. (1995). c-fos mediates antipsychotic-induced neurotensin gene expression in the rodent striatum. *Neuroscience* **67**, 325-344.

Robinson, T. E. and Becker, J. B. (1986). Enduring changes in brain and behavior produced by chronic amphetamine administration: a review and evaluation of animal models of amphetamine psychosis. *Brain Res. Rev.* **11**, 157-198.

Robledo, P. and Feger, J. (1990). Excitatory influence of rat subthalamic nucleus to substantia nigra pars reticulata and pallidal complex: Electrophysiological data. *Brain Res.* **252**, 367-372.

Rosa-Kenig, A., Puotz, J. K. and Rebec, G. V. (1993). The involvement of D1 and D2 dopamine receptors in amphetamine-induced changes in striatal unit activity in behaving rats. *Brain Res.* **619**, 347-351.

Ruskin, D. N. and Marshall, J. F. (1994). Amphetamine- and cocaine-induced fos in the rat striatum depends on D2 dopamine receptor activation. *Synapse* **18**, 233-240.

Ryan, L. J., Young, S. J., Segal, D. S. and Groves, P. M. (1989). Antidromically identified striatonigral projection neurons in the chronically implanted behaving rat: relations of cell firing to amphetamine-induced behaviors. *Behav. Neurosci.* **103**, 3-14.

Sassone-Corsi, P., Ransone, L. J., Lamph, W. W. and Verma, I. M. (1988). Direct interaction between fos and jun nuclear oncoproteins: role of the 'leucine zipper' domain. *Nature* **336**, 692-695.

Scarnati, E., Campana, E. and Pacitti, C. (1983). The functional role of the nucleus accumbens in the control of the substantia nigra: electrophysiological investigations in intact and striatum-globus pallidus lesioned rats. *Brain Res.* **265**, 249-257.

Schiørring, E. (1977). Changes in individual and social behavior induced by amphetamine and related compounds in monkeys and man. Cocaine and Other Stimulants. New York, Plenum. 481-522.

Seeman, P., Guan, H.-c., Civelli, O., Van Tol, H. H. M., Sunahara, R. K. and Niznik, H. B. (1992). The cloned dopamine D2 receptor reveals different densities for dopamine receptor antagonist ligand. Implications for human brain positron emission tomography. *Eur. J. Pharmacol.* **227**, 139-146.

Seeman, P. and Van Tol, H. H. (1994). Dopamine receptor pharmacology. *Trends Pharmacol. Sci.* **15**, 264-70.

Segal, D. S. (1975). Behavioral characterization of d- and l-amphetamine: neurochemical implications. *Science* **190**, 475-477.

Segal, D. S. and Schuckit, M. A. (1983). Animal models of stimulant-induced psychosis. Stimulants: neurochemical behavioral and clinical perspectives. New York, Raven Press. 131-167.

Segal, D. S. and Kuczenski, R. (1987a). Behavioral and neurochemical characteristics of stimulant-induced augmentation. *Psychopharmacol. Bull.* **23**, 417-424.

Segal, D. S. and Kuczenski, R. (1987b). Individual differences in responsiveness to single and repeated amphetamine administration: behavioral characteristics and neurochemical correlates. *J. Pharmacol. Exp. Ther.* **242**, 917-926.

Segal, D. S. and Kuczenski, R. (1994). Behavioral Pharmacology of Amphetamine. Amphetamine and Its Analogs: Psychopharmacology, Toxicology and Abuse. New York, Academic Press. 115-150.

Senogles, S. E., Benovic, J. L., Amlaiky, N., Unson, C., Milligan, G., Vinitzky, R., Spiegel, A. M. and Caron, M. G. (1987). The D2-dopamine receptor of anterior pituitary is functionally associated with a pertussis toxin-sensitive guanine nucleotide binding protein. *J. Biol. Chem.* **262**, 4860-7.

Senogles, S. E., Spiegel, A. M., Padrell, E., Iyengar, R. and Caron, M. G. (1990). Specificity of receptor-G protein interactions; discrimination of Gi subtypes by the D2 dopamine receptor in a reconstituted system. *J. Biol. Chem.* **265**, 4507-4514.

Senogles, S. E. (1994). The D2 dopamine receptor isoforms signal through distinct Gi alpha proteins to inhibit adenylyl cyclase. A study with site-directed mutant Gi alpha proteins. *J. Biol. Chem.* **269**, 23120-7.

Sesack, S. R., Aoki, C. and Pickel, V. M. (1994). Ultrastructural localization of D2 receptor-like immunoreactivity in midbrain dopamine neurons and their striatal targets. *J. Neurosci.* **14**, 88-106.

Sharp, T., Zetterstrom, T., Ljungberg, T. and Ungerstedt, U. (1987). A direct comparison of amphetamine-induced behaviours and regional brain dopamine release in the rat using intracerebral dialysis. *Brain Res.* **401**, 322-330.

Sharp, F. R., Griffith, J., Gonzalez, M. F. and Sagar, S. M. (1989). Trigeminal nerve section induces Fos-like immunoreactivity (FLI) in brainstem and decreases FLI in sensory cortex. *Mol. Brain Res.* **6**, 217-220.

Sharp, F. R., Sagar, S. M., Hicks, K., Lowenstein, D. and Hisanaga, K. (1991). *c-fos* mRNA, fos and fos-related antigen induction by hypertonic saline and stress. *J. Neurosci.* **11**, 2321-2331.

Sheng, M. and Greenberg, M. E. (1990). The regulation and function of *c-fos* and other immediate early genes in the nervous system. *Neuron* **4**, 477-485.

Shimada, S., Kitayama, S., Lin, C.-L., Patel, A., Nanthakumar, E., Gregor, P., Kuhar, M. and Uhl, G. (1991). Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. *Science* **254**, 576-578.

Sirinathsinghji, D. J. S., Heavens, R. P. and Sikdar, S. K. (1988). In vivo studies on the dopamine re-uptake mechanism in the striatum of the rat: effects of benztropine, sodium and ouabain. *Brain Res.* **438**, 399-403.

Sloviter, R. S., Drust, E. G. and Connor, J. D. (1978). Evidence that serotonin mediates some behavioral effects of amphetamine. *J. Pharmacol. Exp. Ther.* **206**, 348-352.

Smith, Y. and Parent, A. (1988). Neurons of the subthalamic nucleus in primates display glutamate but not GABA immunoreactivity. *Brain Res.* **453**, 353-356.

Smith, A. D. and Bolam, J. P. (1990). The neural network of the basal ganglia as revealed by the study of synaptic connections of identified neurones. *Trends Neurosci.* **13**, 259-265.

Smith, Y., Bolam, J. P. and Krosigk, M. V. (1990). Topographical and synaptic organization of the GABA-containing pallidosubthalamic projection in the rat. *Eur. J. Neurosci.* **2**, 500-511.

Sonnenberg, J. L., Rauscher, R. J., Morgan, J. I. and Curran, T. (1989). Regulation of proenkephalin by Fos and Jun. *Science* **246**, 1622-1625.

Stern, R. (1889). Über die wirkung der hydronaphtylamine auf den tierischen organismus. *Virchows Arch. Path. Anat. Physiol.* **115**, 14-46.

Stoof, J. C. and Keabian, J. W. (1981). Opposing roles for D-1 and D-2 dopamine receptors in efflux of cyclic AMP from rat neostriatum. *Nature* **294**, 366-367.

Strange, P. G. (1994). Dopamine D4 receptors: curiouser and curiouser. *Trends Pharmacol. Sci.* **15**, 317-319.

Sulzer, D., Maidment, N. T. and Rayport, S. (1993). Amphetamine and other weak bases act to promote reverse transport of dopamine in ventral midbrain neurons. *J. Neurochem.* **60**, 527-535.

Sunahara, R. K., Guan, H.-C., O'Dowd, B. F., Seeman, P., Laurier, L. G., Ng, G., George, S. R., Torchia, J., Van Tol, H. H. M. and Niznik, H. B. (1991). Cloning of the gene for a human dopamine D5 receptor with higher affinity for dopamine than D1. *Nature* **350**, 614-619.

Tieman, D. G. (1989). Video enhancement techniques. Computer Techniques in Neuroanatomy. New York, Plenum Press. 285-331.

Torres, G. and Rivier, C. (1993). Cocaine-induced expression of striatal c-fos in the rat is inhibited by NMDA receptor antagonists. *Brain Res. Bulletin* **30**, 173-176.

Tschanz, J. T., Haracz, J. L., Griffith, K. E. and Rebec, G. V. (1991). Bilateral cortical ablations attenuate amphetamine-induced excitations of neostriatal motor-related neurons in freely moving rats. *Neurosci. Lett.* **134**, 127-130.

Ueki, A., Uno, M., Anderson, M. and Yoshida, M. (1977). Monosynaptic inhibition of thalamic neurons produced by stimulation of the substantia nigra. *Experientia* **33**, 1480-1481.

Uemura, Y., Kowall, N. W. and Moskowitz, M. A. (1991). Focal ischemia in rats causes time-dependent expression of c-fos protein immunoreactivity in widespread regions of ipsilateral cortex. *Brain Res.* **552**, 99-105.

Van Tol, H. H. M., Bunzow, J. R., Guan, H.-C., Sunahara, R. K., Seeman, P., Niznik, H. B. and Civelli, O. (1991). Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. *Nature* **350**, 610-614.

Veening, J. G., Cornelissen, F. M. and Lieven, P. A. J. M. (1980). The topical organization of the afferents to the caudatoputamen of the rat: A horseradish peroxidase study. *Neuroscience* **5**, 1253-1268.

Vennari, J., (1994). Image processing and the thresholding operation. *SciTech. Journal* **4**: 22-23.

von Krosigk, M., Smith, Y., Bolam, J. P. and Smith, A. D. (1992). Synaptic organization of GABAergic inputs from the striatum and the globus pallidus onto neurons in the substantia nigra and retrorubral field which project to the medullary reticular formation. *Neuroscience* **50**, 531-49.

Walter, R. J. J. and Berns, M. W. (1986). Digital image processing and analysis. *Video Microscopy*. New York, Plenum Press. 327-392.

Weiner, D. M., Levey, A. I., Sunahara, R. K., Niznik, H. B., O'Dowd, B. F., Seeman, P. and Brann, M. R. (1991). D1 and D2 dopamine receptor mRNA in rat brain. *Proc. Natl. Acad. Sci. USA* **88**, 1859-1863.

Weszka, J. S., (1978). A survey of threshold selection techniques. *Computer Graphics and Image Processing* **7**: 259-265.

White, F. J. and Wang, R. Y. (1986). Electrophysiological evidence for the existence of D-1 and D-2 dopamine receptors in the rat nucleus accumbens. *J. Neurosci.* **6**, 274-280.

Willemse, F., Nap, M., de Kok, L. B. and Eggink, H. F., (1992). Image analysis in immunohistochemistry: Factors with a possible influence on performance of VIDAS Version 2.0, a commercially available true color image analysis system. *Anal. Quant. Cytol. Histol.* **15**: 136-143.

Williams, M. N. and Faull, R. L. M. (1985). The striatonigral projection and nigrotectal neurons in the rat. A correlated light and electron microscopic study demonstrating a monosynaptic striatal input to identified nigrotectal neurons using a combined degeneration and horseradish peroxidase procedure. *Neuroscience* **14**, 991-1010.

Williams, S., Evan, G. I. and Hunt, S. P. (1990). Changing patterns of c-fos induction in spinal neurons following thermal cutaneous stimulation in the rat. *Neuroscience* **36**, 73-81.

Wilson, C. J. (1987). Morphology and synaptic connections of crossed corticostriatal neurons in the rat. *J. Comp. Neurol.* **263**, 567-580.

Wirtshafter, D. and Asin, K. E. (1994). Interactive effects of stimulation of D1 and D2 dopamine receptors on Fos-like immunoreactivity in the normosensitive rat striatum. *Brain Res. Bull.* **35**, 85-91.

Xu, M., Hu, X.-T., Cooper, D. C., Moratalla, R., Graybiel, A. M., White, F. J. and Tonegawa, S. (1994). Elimination of cocaine-induced hyperactivity and dopamine-mediated neurophysiological effects in dopamine D1 receptor mutant mice. *Cell* **79**, 945-955.

Young, S. T., Porrino, L. J. and Iadarola, M. J. (1991). Cocaine induces striatal c-fos-immunoreactive proteins via dopaminergic D1 receptors. *Proc. Natl. Acad. Sci. USA* **88**, 1291-1295.

Yurek, D. M. and Hipkens, S. B. (1993). Intranigral injections of SCH 23390 inhibit amphetamine-induced rotational behavior. *Brain Res.* **623**, 56-64.

Zaczek, R., Culp, S. and De Souza, E. B. (1990). Intrasyntosomal sequestration of [3H]-amphetamine and [3H]-methylene dioxymphetamine: Characterization suggests the presence of a factor responsible for maintaining sequestration. *J. Neurochem.* **54**, 195-204.

Zaczek, R., Culp, S., Goldberg, H., McCaan, D. J. and De Souza, E. B. (1991a). Interactions of [3H]amphetamine with rat brain synaptosomes. I. Saturable sequestration. *J. Pharmacol. Exp. Ther.* **257**, 820-829.

Zaczek, R., Culp, S. and De Souza, E. B. (1991b). Interactions of [3H]amphetamine with rat brain synaptosomes. II. Active transport. *J. Pharmacol. Exp. Ther.* **257**, 830-835.

Ziolkowska, B. and Holtt, V. (1993). The NMDA receptor antagonist MK-801 markedly reduces the induction of c-fos gene by haloperidol in the mouse striatum. *Neurosci. Lett.* **156**, 39-42.

Zocchi, A. and Pert, A. (1993). Increases in striatal acetylcholine by SKF-38393 are mediated through D1 dopamine receptors in striatum and not the frontal cortex. *Brain Res.* **627**, 186-192.

Zoltay, G. and Cooper, J. R. (1994). Presynaptic modulation by dopamine and GABA opens a potassium channel in rat cortical, striatal and hippocampal synaptosomes via eicosanoids. *Neurochem. Int.* **25**, 345-8.