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Activation of Immediate Early Genes By Drugs of Abuse

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Contents

Introduction

Reinhard Grzanna and Roger M. Brown

A major goal in drug abuse research is to determine the neurobiological mechanisms by which drugs of abuse produce tolerance, dependence, and addiction. These behavioral manifestations of drug abuse have been attributed to longlasting neuroadaptations in central nervous system (CNS) neurons. The nature and extent of these neuroadaptations remain to be characterized, but investigators agree that they are the result of drug-induced alterations in neuronal gene expression.

The recent discovery that cocaine and amphetamine rapidly and transiently induce immediate early genes (IEGs) provided the most direct indication that drugs of abuse can profoundly influence gene expression. Following their description a few years ago, IEGs and their protein products became recognized as important links by which extracellular signals can produce alterations in gene transcription. Turning on IEG expression by drugs of abuse may be the initial step by which drugs alter the expression of late genes to produce longlasting changes in neuronal functions. Thus, studies of the effects of drugs of abuse on IEGs may hold the key to providing the answer to how drugs of abuse produce long-term changes in neurons. Studies of this class of genes also should provide a powerful approach to explore correlations between drug-induced changes in behavior and the neuronal systems in which drugs permanently alter gene expression.

Studies of the effects of drugs of abuse on IEGs are greatly facilitated by the availability of methods to visualize the activity states of these genes in tissue sections by immunohistochemistry and by in situ hybridization histochemistry. This has opened a new and promising approach to define drug-induced, permanent changes in the CNS and to identify the neuronal circuitries in which they occur.

The chapters in this volume were presented at a conference on June 3-4, 1991, in Rockville, MD, organized by the National Institute on Drug Abuse. The main goal of this conference was to assess the potential impact of studies of IEGs in the field of drug abuse research. The contributors to this volume reviewed

current developments in the field and discussed to what extent studies of the effects of drugs of abuse on IEGs are likely to provide new insights into the molecular underpinning of drug-seeking behavior.

Among the topics discussed were the specificity of the activation of IEGs by drugs of abuse, the nature of the long-term alterations following the induction, and the possible relationship between the observed changes in IEG activity and behavior. Many of the questions discussed remain unresolved. Yet, there was excitement about the remarkable specificity in the patterns of IEG activation induced by different drugs in the CNS. The participants expressed confidence about the prospects of linking drug-induced IEG induction to specific alterations in peptide gene expression in identified populations of CNS neurons.

This volume reflects the considerable optimism among drug abuse researchers that studies of IEGs offer a new path to identify the long-term effects of drug exposure in the CNS. The chapters reveal a sense of confidence that studies of the action of drugs of abuse on IEGs will greatly facilitate efforts to identify the neuronal systems critically involved in the development of drug addiction and the nature of the changes produced within these systems.

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Regulation of Immediate Early Gene Expression

Brent H. Cochran

INTRODUCTION

Recently, much excitement has been generated by the finding that various immediate early genes (IEGs) are expressed in neurons in response to a variety of neurotropic stimuli. Part of the reason these findings have generated so much interest is that earlier work has suggested that long-term changes in nervous system behavior require changes in gene expression (Goelet et al. 1986). Thus, since changes in IEG expression are coupled to neuronal activity and neurotransmitter release, it may now be possible, using in situ techniques that provide resolution at the single-cell level, to correlate the physiological state of a neuron with behavioral and physiological outputs. This chapter reviews what is currently known about IEGs and speculates on what roles these genes might be playing in the operation of the nervous system.

IEGs (sometimes called early-response genes or primary-response genes) were first identified not in the nervous system but through the study of cell growth regulation. During the 1970s it became apparent that polypeptide growth factors were key modulators of cell growth and differentiation of multicellular organisms. Early studies had suggested that the mitogenic effects of serum growth factors were mediated in part by the ability of these factors to regulate gene expression (Smith and Stiles 1981). To identify such genes, several investigators set out to clone them by differential or subtractive cDNA cloning. The first of such genes cloned were those induced by the platelet-derived growth factor (PDGF) in 3T3 fibroblasts (Cochran et al. 1983). The genes cloned in this study displayed regulatory properties that are characteristic of most early-response genes now known. These genes are induced rapidly within 1 hour of growth factor treatment and are regulated at the level of transcription (Cochran et al. 1988). They are induced by polypeptide growth factors that bind to cell surface receptors in the absence of new protein synthesis. Therefore, the induction of these genes is a primary response to events at the cell surface and not secondary to other waves of gene expression or changes in growth state of the cell. The ability of early-response genes to be

induced in the absence of new protein synthesis distinguishes this set of genes from other genes, which may be called late-response genes (those that are expressed only if protein synthesis is allowed). Late-response genes are likely to be targets of transcription factors induced in the early part of the response. Subsequently, it was found that some of the IEGs are the cellular homologs of various retroviral oncogenes, including those for c-*myc,* c-*fos,* and c-*jun* (Cochran et al. 1984; Greenberg and Ziff 1984; Kelly et al, 1983; Lamph et al. 1988; Muller et al. 1984; Ryder and Nathans 1988). Studies using antisense RNAs or oligonucleotides or antibodies have shown that the expression of c-*fos* and proto-oncogenes is necessary for the entry of quiescent fibroblasts into the cell cycle (Heikkila et al. 1987; Holt et al. 1988, 1988; Nishikura and Murray 1987; Wickstrom et al. 1988). Microinjection or overexpression of c-myc by itself can potentiate the response of fibroblasts to other growth factors (Armelin et al. 1984; Kaczmarek et al. 1985). However, the finding that some IEGs can also be induced in cells such as neurons that have terminally differentiated indicates that the IEGs may have roles to play in processes other than cell growth regulation (Bravo et al. 1985; Curran and Morgan 1985; Greenberg et al. 1985; Kruijer et al. 1985).

A wide variety of early-response genes have been identified from studies of serum growth factor-stimulated fibroblasts (Almendral et al. 1988; Cochran et al. 1983; Lau and Nathans 1985, 1987; Lim et al. 1987). The total number of such genes is unclear but is probably in the range of 50 to 100. It can be seen from table 1, which lists some of these genes, that there are a wide variety of gene products encoded by early-response genes. The two best characterized classes of such genes are transcription factor genes and secreted gene products. However, these are not the only type of gene products represented. Others such as ß-actin and *rho*-B encode cytoskeletal and signal transduction molecules, respectively (Greenberg and Ziff 1984; Jahner and Hunter 1991 a). The best characterized of these genes are transcription factors, which can be subdivided into several distinct categories. C-Fos and c-Jun are DNA-binding proteins that form heterodimers via their leucine zipper domains (Bohmann et al. 1987; Chiu et al. 1988; Landschulz et al. 1988; O'Shea et al. 1989; Rauscher et al. 1988a, 1988b; Vinson et al. 1989). Each of these genes has several related homologs (i.e., *jun*-B, *jun*-D, *fra*-1, and *fos*-B), which all can form heterodimers with opposite members of the family (Hai and Curran 1991; Halazonetis et al. 1988; Nakabeppu et al. 1988). In addition, *c-jun* can form a homodimer. Thus, the number of dimeric complexes formed in the cells by this family of gene products is larger than the number of genes that encode them. Almost all these homodimeric and heterodimeric complexes can bind to the activator protein-1 (AP-1) consensus site (TGACTCA), although the affinity of a given complex for a given site can vary (Hai and Curran 1991; Kovary and Bravo 1991; Ryseck and Bravo 1991). Different *fos/jun* complexes have

TABLE 1. *A list of representative early-response genes**

Gene	Possible Function	References
C-*myc*	Transcriptional modulator? Helix loop helix	Kelly et al. 1983; Murre et al. 1989
JE	Cytokine	Cochran et al. 1983; Rollins et al. 1988
KC, MGSA, gro	Cytokine	Anisowicz et al. 1987; Cochran et al. 1983; Richmond et al. 1988
C-*fos*	Transcription factor Leucine zipper	Cochran et al. 1984; Greenberg and Ziff 1984; Kruijer et al. 1984; Muller et al. 1984
Fra-1	Transcription factor Leucine zipper	Cohen and Curren 1988
Fos-*B*	Transcription factor Leucine zipper	Zerial et al. 1989
Zif/268, egr-1 , TIS8, KROX24, NGFIA	Transcription factor Zinc finger	Christy et al. 1988; Lemaire et al. 1988; Milbrandt 1987; Sukhatme et al. 1988
KROX20, *egr*-2	Transcription factor Zinc finger	Chavrier et al. 1989
NGFIB, TIS1, *nur77*	Steroid receptor Superfamily	Hazel et al. 1988; Milbrandt 1988
C-*jun*	Transcription factor Leucine zipper	Lamph et al. 1988; Ryder and Nathans 1988
Jun-B	Transcription factor Leucine zipper	Ryder et al. 1988
ß-actin	Cytoskeletal component	Greenberg and Ziff 1984
SRF	Transcription factor	Norman et al. 1988
2'-5' oligo-adenylate synthetase	Enzyme	Garcia-Blanco et al. 1989
Rho-B	Ras-like G protein	Jahner and Hunter 1991 a

*For a more comprehensive list, see Herschman (1991).

different effects on transcription, with some, such as *c-jun,* being more positively acting and others, such as *jun*-B, tending to be more negatively acting (Bohmann et al. 1987; Chiu et al. 1988, 1989; Nakabeppu and Nathans 1991; Schonthal et al. 1989; Schutte et al. 1989). In addition, many of these complexes can be modified posttranslationally, especially by phosphorylation, and these phosphorylations can affect the function of the complex (Barber and Verma 1987; Boyle et al. 1991; Lamph et al. 1990; Ofir et al. 1990; Binetruy et al. 1991). Thus, through combinatorial action, the *fos/jun* gene family can potentially exert a broad range of effects on the transcriptional program of the cell.

However, the *fos/jun* family are not the only transcription factors that are early-response genes, Other early-response genes that appear to be transcription factors are *zif/268,* which encodes a zinc finger protein (Christy et al. 1988); *nur77,* which is related to the steroid receptor family of transcription factors (Hazel et al. 1988; Milbrandt 1988); and the c-myc gene, which has both a helix-loop-helix domain and a leucine zipper (Murre et al. 1989). All these genes are likely to be involved in the regulation of late-response genes, which then presumably alter physiology and function of the cell. Secreted proteins that are IEGs are KC/gro, which appears to be a melanocyte growth factor (Anisowicz et al. 1987; Richmond et al. 1988), and JE, which is a member of a large family of monocyte chemotractant factors (Oquendo et al. 1989; Rollins et al. 1989). The functions of these secreted factors are not specific to fibroblasts because expression of JE and KC has been detected in monocytes (Orlofsky and Stanley 1987).

In addition to studies on fibroblasts, investigators have cloned genes from other cell types that are induced early in response to various growth and differentiation stimuli. Typically, it has been found that many of the genes induced in T cells by mitogens, in liver cells by partial hepatectomy, or by nerve growth factor (NGF) in PC12 cells are the same ones that are induced by PDGF in 3T3 cells (Greenberg et al. 1985; Kujubu et al. 1987; Thompson et al. 1986; Zipfel et al. 1989). Genes that are commonly induced in many different cell types by many different agents typically include c-*fos,* c-*jun,* and the c-*myc* proto-oncogenes (Bartel et al. 1989; Greenberg et al. 1985; Kelly et al. 1983; Mechta et al. 1989; Thompson et al. 1986). In addition, in each cell type there appears to be a class of early-response genes that have a pattern of expression restricted to a particular cell type. For instance, in activated T cells, there is a rapid induction of interleukin-2 (IL-2) and its receptor, both of which are gene products that are specifically expressed in T cells (Greene et al. 1989; Taniguchi 1988). Similarly, in PC12 cells there are unique cytoskeletal proteins that are induced early in the response to NGF (Leonard et al. 1988).

REGULATION OF EARLY-RESPONSE GENES

Although the typical early-response gene is induced rapidly and transiently, the magnitude and the time course of induction can vary. For instance, c-*fos* transcription can be detected within 5 minutes of stimulation, but expression is typically shut off at the transcriptional level by 1 hour. The messenger RNA for c-*fos* is unstable, and the persistance of the message is less than 2 hours (Greenberg and Ziff 1984; Muller et al. 1984). This could be compared with the JE gene in fibroblast, which is transcriptionally induced rapidly by 30 minutes, but which is still being transcribed up to 6 hours postinduction (Cochran et al. 1988). Absolute levels of induction of these genes can vary greatly; for example, the c-*myc* gene is induced only to a level of 30 to 50 copies per cell, whereas the c-*fos* gene is present transiently at 20 times that level (Cochran et al. 1988; Kelly et al. 1983). For individuals of the same gene family such as the *fos*-related genes, the time courses can also vary. The time course of the *fra* genes appears to be slower and longer than those of *fos;* this may have a physiological significance for the type of AP-1 complexes present in the cell at a given time, since it has been shown that the subunits of AP-1 complex can interchange rapidly in solution (Kovary and Bravo 1991; Ryseck and Bravo 1991; Sonnenberg et al. 1989a). The mechanisms responsible for the transient expression of IEGs also may vary. One mechanism by which transient expression is maintained is autorepression of a promoter by its own gene product. For instance, c-*fos* and in some cases c-*myc* can autoregulate their own synthesis (Penn et al. 1990; Sassone-Corsi et al. 1988a). Another mechanism by which expression may be shut off may be simply the down-regulation of the second signal stimuli, either at the level of the receptor or at the level of second messenger.

Most IEGs are capable of responding to a variety of extracellular stimuli. In fibroblasts these agents include not only growth factors such as PDGF, fibroblast growth factor, and epidermal growth factor (EGF), but also agents such as phorbol esters and bombesin that can induce IEG expression through non-tyrosine-kinase-type receptors (Lim et al. 1987; Rabin et al. 1986). Calcium ionophores and agents that affect cyclic AMP (cAMP) levels within the cell can induce expression of some IEGs (Almendral et al. 1988; Morgan and Curran 1986; Ran et al. 1986; Sheng et al. 1988). In PC12 cells, it has been shown that IEGs can be induced by neurotransmitters, by membrane depolarization, and by various manipulations of extracellular ions and ion channels (Curran and Morgan 1985; Greenberg et al. 1985; Kruijer et al. 1985; Morgan and Curran 1986). Moreover, a variety of recent studies have shown that activated oncogenes such as *ras* and *src* can induce many of these same early-response genes (Jahner and Hunter 1991b; Stacey et al. 1987). This observation is not surprising considering that many of the activated oncogenes are components of signal transduction pathways.

Not all IEGs are coordinately regulated. Different stimuli can elicit different patterns of expression of the IEGs in the same cell type. C-*fos* is induced readily by phorbol esters in fibroblasts, but the JE and KC genes are not (Hall and Stiles 1987). Conversely, in 3T3 fibroblasts, interleukin-1 can induce JE and KC but have little effect on c-*fos* (Hall et al. 1989). Moreover, a given stimulus can have dramatically different effects on different cell types. In PC12 cells, c-*fos* is induced by calcium ionophores as well as by EGF (Greenberg et al. 1985; Kruijer et al. 1985). However, in Balb/c-3T3 cells, calcium ionophores and EGF are very weak inducers of *fos* (Hall and Stiles 1987). In addition, it is clear that different stimuli can induce a similar set of genes in a given cell and yet have different effects. This can be dramatically visualized in studies of PC12 cells, for which EGF is a mitogen and NGF can induce differentiation. Both of these factors can induce c-*fos* and c-*myc* in these cells, but only NGF produces neurite outgrowth (Greenberg et al. 1985). The difference between these responses must result from differing second signals generated by these two factors, even though some of the second signals are likely to overlap. Differences in signals could include the activation of different kinases and phosphotylation of different substrates as well as activation of genes uniquely by one factor or the other. To understand the complex regulation of IEGs, it will be necessary to understand both the second signals generated by any given stimulus and the regulatory elements that control the expression of each of these genes.

Second messenger systems to which early-response genes can respond are numerous and varied. The fact that phorbol esters such as phorbol myristoy acetate can induce c-*fos* and c-*jun* indicates that activation of protein kinase C is one of the pathways by which IEGs are induced (Lamph et al. 1988; Rabin et al. 1986). The observation that a calcium ionophore such as A23187 can induce c-*fos* in some cells indicates that calcium can be a second messenger for the induction of some early-response genes, In PC12 cells there is evidence to suggest that the opening of a voltage-gated calcium channel can induce c-*fos* (Morgan and Curran 1986). IEGs such as c-*fos* and *jun*-B can respond to agents such as forskolin and isobutyl methyl xanthine, which increase intracellular cAMP concentrations (Berkowitz et al. 1989; Fisch et al. 1989a; Sassone-Corsi et al. 1988b). In addition, it is clear that second signal pathways activated by other kinases can induce early-response genes. This is indicated by the fact that cotransfection or microinjection of genes such as *c-raf* and activation of a temperature-sensitive *c-src* gene can induce a broad spectrum of early-response genes (Jahner and Hunter 1991b; Jamal and Ziff 1990; Kaibuchi et al. 1989). Moreover, activated *ras,* a G protein, can induce at least c-*fos* (Fukumoto et al. 1990; Gauthier et al. 1990; Stacey et al. 1987). Thus, the ras-activated signaling pathway appears to be another mechanism by which some early-response genes can be activated.

Physiological activation of early-response genes is likely mediated by several of these second signal pathways operating simultaneously in a ceil. For instance, the PDGF receptor is known to activate a variety of these pathways and directly phosphorylate on tyrosine several proteins involved in signaling pathways (Williams 1989). PDGF stimulates phosphatidyl inositide turnover, thereby activating protein kinase C. The PDGF receptor can also bind to GAP, a modulator of the *ras* pathway, and in addition can activate c-*raf* (Molloy et al. 1989; Morrison et al. 1989). Thus, a network of second signals leads to the activation of the appropriate set of early-response genes in any given cell type in response to a given stimulus. Most of those pathways have not been worked out in great detail and are likely to be quite complicated. This is the case even in the unicellular yeast *Saccharomyces cerevisiae.* Mating pheromones are extracellular peptides that regulate gene expression in yeast. A variety of second signaling molecules similar to those found in mammalian cells have been implicated in the yeast signal transduction process; these include G proteins and a variety of protein kinases, including one related to the CDC28 kinase (for a review, see Herskowitz 1989). However, even with the power of yeast genetics, these pathways are not yet fully understood.

TRANSCRIPTIONAL REGULATORY ELEMENTS OF IEGs

Ultimately, a complete understanding of the induction of IEGs will have to come from the study of transcriptional regulatory elements of these genes and the factors that interact with them. Currently, the c-*fos* proto-oncogene is the best characterized of the early-response genes at the level of transcriptional regulation. Analysis of the upstream regulatory elements of the c-*fos* gene reveals a complex array of regulatory elements that can respond to one or more second messenger signals. A diagram of the c-*fos* promoter is shown in figure 1. Upstream of the transcriptional start site is a TATA consensus box to which the transcription factor TFIID should bind (Buratowski et al. 1988; van Straaten et al. 1983). This element is characteristic of most RNA polymerase II promoters. Upstream of the TATA box at -65 is a Ca/CRE box, which serves at least three functions in the promoter. This element contributes to basal levels of expression (Fisch et al. 1987; Gilman et al. 1986; Runkel et al. 1991), can respond to increases in cAMP and in PC12 cells can mediate responses to intracellular calcium (Berkowitz et al. 1989; Fisch et al. 1989a; Sassone-Corsi et al. 1988b; Sheng et al. 1988). A variety of transcription factors could bind to this site, but the best evidence would suggest that the CREB factor, which was initially isolated as a cAMP response element-binding protein, can likely mediate both the response to cAMP and calcium (Sassone-Corsi et al. 1988b; Sheng et al. 1990). Recent work has shown that this factor gets phosphotylated by the CaM II kinase in PC12 cells that have undergone depolarization (Dash et al. 1991; Sheng et al. 1991).

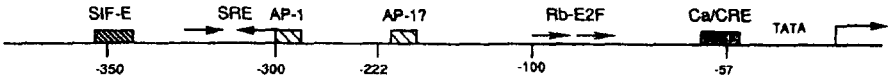

FIGURE 1. *The structure of the c-fos promoter. See text for explanation of each element.*

Upstream of this element at approximately -100 are two direct repeat elements that also have multiple functions. There is evidence that these elements are involved in maintenance of basal expression of *c-fos* (Fisch et al. 1987; Gilman et al. 1986; Runkel et al. 1991). In addition, sequences that contain these elements have been shown to be responsive to a cotransfected retinoblastoma gene. Overexpression of the retinoblastoma gene can repress transcription of *fos* through this site (Robbins et al. 1990). Recently, it has been suggested that the transcription factor E2F, which was originally characterized as a factor that bound upstream of adenovirus early genes that are transactivated by the adenovirus transforming gene E1A, can bind to the retinoblastoma responsive element (Chellappan et al. 1991). This is of interest since it has been shown that E2F can form a complex with the retinoblastoma gene product. Furthermore, E2F has a serum-stimulated DNA-binding activity and is involved in the regulation of other serum-inducible genes in fibroblasts, including c-*myc* and dihydrofolate reductase (Blake and Azizkhan 1989; Mudryj et al. 1990). However, although these two intriguing factors interact with this site, it has not yet been shown that this element plays a crucial role in the response of c-*fos* to extracellular stimuli such as growth factors, phorbol esters, and cAMP. Moreover, it is quite clear that *c-fos* is readily inducible in cells that contain a functional retinoblastoma gene product and that only overexpression of retinoblastoma causes a repression of *fos* in a transient assay. Further upstream, at -200 and -290, are two potential AP-1 sites. The one at -290 has been shown to be functional and can bind proteins other than the Fos/Jun family complex (Fisch et al. 1989b; Velcich and Ziff 1990).

Centered around -310 of the human *c-fos* promoter is a region of dyad symmetry that has been termed the "serum response element" (SRE). It has been shown that this element is sufficient by itself to mediate induction of a heterologous gene by serum and phorbol esters (Gilman et al. 1986; Greenberg et al. 1987; Treisman 1985). The serum response factor (SRF) is a 67-kd phosphoprotein that binds to the core of the dyad symmetry element (Gilman et al. 1986; Norman et al. 1988; Treisman 1987). This core region has been called a CArG box and is also found upstream of several other early-response genes as well as in muscle-specific enhancers (Miwa and Kedes

1987; Phan-Dinh-Tuy et al. 1988; Rivera et al. 1990; Walsh 1989). In A431 cells the SRF has a mildly inducible DNA-binding activity (Prywes and Roeder 1986), but in general this factor appears to be constitutively bound to the CArG box (Herrera et al. 1989; Treisman 1986). Although the SRF is a phosphoprotein, to date no phosphorylation changes have been shown that correlate with the induction of the *c-fos* gene. It is likely that the SRF is the anchoring member of a complex of factors that bind to the SRE. One such factor that has been characterized is $p62^{TCF}$ (for ternary complex factor), which appears to bind to the SRE only in conjunction with the SRF and makes contacts with the 5' side of the SRE (Schroter et al. 1990; Shaw et al. 1989a). Mutations in the 5' flank of the dyad symmetry region (outside the CArG box) reduce the ability of the SRE to confer responses to phorbol esters, but these same mutant elements still retain the ability to respond to serum (Graham and Gilman 1991; Shaw et al. 1989a). In addition, the SRE can mediate autorepression of *c-fos* transcription (Konig et al. 1989; Sassone-Corsi et al. 1988a; Shaw et al. 1989b). Cotransfection of a constitutively produced *c-fos* gene with a reporter gene that is driven by the SRE greatly reduces the expression of a linked reporter gene. Thus, the rapid burst of transcription of c-fos can be explained by the fact that the activation of the *c-fos* promoter stimulates production of the c-Fos protein, which is then able to repress its own transcription, It is not yet known whether this repression is a direct or indirect effect. The C terminus of the c-Fos protein is required for this repression and is often mutated in the viral form of the gene (Ofir et al. 1990). Understanding the transcription factor complexes that bind near and around the SRE is essential for understanding the regulation of *c-fos* and several other early-response genes (de Belle et al. 1991).

Upstream of the SRE at -346 is a sequence called the sis/PDGF-inducible factor (SIF) element. A transcription factor with an inducible DNA-binding activity specifically binds to this element after treatment of 3T3 cells with PDGF (Hayes et al. 1987). Genomic footprinting indicates that in A431 cells treated with EGF there is also an inducible DNA-binding activity that interacts with this element (Herrera et al. 1989). The SIF element confers induction onto the *c-fos* promoter in the absence of the SRE but responds specifically to PDGF and not to serum or phorbol esters (Wagner et al. 1990).

Most of these regulatory elements are capable of showing some activity outside of the context of the *fos* promoter. However, the context within which they lie is important as well. For instance, the SIF element requires sequences between -222 and -100 of the *c-fos* gene to respond to PDGF. Furthermore, moving the SRE closer to the promoter weakens its response to PDGF and phorbol esters but not its response to serum (Wagner et al. 1990). Moreover, there are cell type-specific differences in the ability of these elements to respond to a given

stimulus. It has been shown that there are differences between the response of the SRE to TPA in 3T3 cells and Hela cells (Siegfried and Ziff 1989).

In addition to these upstream transcription factors, there is evidence from at least two of the IEGs that there is regulation of transcriptional termination. A transcription termination site has been found at the end of the first exon of both *c-fos* and *c-myc* (Bentley and Groudine 1986; Lamb et al. 1990; Nepveu et al. 1967; Nepveu and Marcu 1986). The mechanisms by which transcriptional termination is regulated in mammalian cells is not clear, but recent studies of the human immunodeficiency virus Tat protein indicate that there are promoter-specific mechanisms for regulating transcriptional termination of mammalian genes (Marciniak et al. 1990). However, it is not known how these termination sites are regulated by external cellular signals,

SUMMARY

From this brief overview of the regulation of the c-*fos* promoter, it can be seen that the regulation of early-response genes is a complex affair. Therefore, it is not easy to predict from the upstream sequence of a given early-response gene exactly which elements are responsible for responding to what signals in a given cell type. However, from studies of other early-response genes, it is clear that several of the elements found upstream of c-*fos* appear frequently and are important in the regulation of other early-response genes. For instance the *zif/268* gene has four separate CArG boxes that are similar to *fos.* These CArG boxes can function in the serum and TPA response, but interestingly, they are not imbedded in a region of dyad symmetry as in *c-fos* (Christy and Nathans 1989). Upstream of the *c-jun* oncogene is an AP-1 site that can modulate the expression and induction of this gene and is responsive to TPA (Angel et al. 1988). Moreover, other studies suggest that cAMP-mediated signals can repress induction of c-jun through this element (de Groot et al. 1991; Mechta et al. 1989). This would explain why in certain circumstances, such as depolarization of PC12 cells or in the striatum in response to cocaine, there is an uncoupling of the induction of *c-jun* and *jun-B.* Depolarization induces *c-fos* and *jun*-B but not *c-jun;* however, growth factors such as NGF can induce all three genes in the same cell (Bartel et al. 1989). Upstream of the *jun*-B gene there does not appear to be an SRE, but there is a new element that can be responsive to both cAMP and phorbol esters (de Groot et al. 1991). Genes such as *c-myc,* JE, and KC have no consensus SREs upstream, and the regulatory elements responsible for the induction of these genes have not been clearly identified (Rollins et al. 1988). However, there is some evidence from the *c-myc* gene that the E2F binding sites are important for its regulation by serum (Mudryj et al. 1990; Sacca and Cochran 1990). In addition, there are two SIF sites upstream of the c-*myc* proto-oncogene (B.H. Cochran and T.E. Hayes,

unpublished results). Upstream of the *nur-77* gene there are no SREs, but there are four potential calcium/CRE-like elements (Watson and Milbrandt 1989). Thus, although most of these early-response genes can be isolated from a differential screening of cDNA library in response to a single inducer such as PDGF, the regulation of each is unique. In addition, there is much more to understand about how some of the early-response genes such as that of IL-2 or the IL-2 receptor are expressed as both tissue-specific and IEGs. It is likely that the tissue-specific expression of early-response genes is due to a combination of chromatin accessibility of the gene and interaction with universal regulatory elements as well as tissue-specific regulatory factors (Greene et al. 1989; Phan-Dinh-Tuy et al. 1988).

The finding that many early-response genes are induced in the nervous system in response to neurotransmitters and other physiological stimuli may indicate that these genes have an important role to play in the function of the nervous system. (For review, see Sheng and Greenberg [1990] and other chapters in this monograph.) The functions of the early-response genes are undoubtedly as varied as the gene products. However, many of these genes do appear to be transcriptional regulatory factors and therefore are likely to modulate the expression of other genes. Work on identifying the targets of these transcription factors is still in the early stages. Interestingly, there is already evidence that two important neurotransmitter genes, the proenkephalin and the prodynorphin genes, may be regulated by an AP-1 complex (Naranjo et al. 1991; Sonnenberg et al. 1989b). Thus, one of the roles of early-response genes in the nervous system may be to regulate the levels of neurotransmitters in a particular neuron.

One role that has been suggested for these genes is that they mediate between short-term signaling and long-term changes in the nervous system (Morgan and Curran 1989). This is especially intriguing, since many studies have implicated cAMP in the formation of long-term memory through transcriptional changes in the nervous system (Goelet et al. 1986), tiowever, as the induction of IEGs is transient, it is not yet clear how these genes would mediate such long-term changes. They could be regulating the expression of structural genes that could contribute to the formation of synapses and other structural changes within neurons. Alternatively, early-response genes could serve as a reset mechanism that functions to replenish and restore supplies of neurotransmitters that have been utilized. Another possible role for early-response genes would be to modulate in the intermediate term the temporal patterns of spike trains of given neurons. Since these genes are expressed on a time scale of hours, they could contribute to short-term adaptive changes in the activity of a given neuron. Obtaining the answers to questions like these should be the focus of future research.

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Everything Activates C-fos—How Can It Matter?

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INTRODUCTION

This chapter addresses often-raised questions concerning the specificity and biological significance of drug-induced immediate early gene (IEG) expression, especially c-fos expression, in the nervous system. The definition of cellular IEGs and basic information about their induction are described elsewhere in this volume and are well reviewed in Morgan and Curran (1991) and Sheng and Greenberg (1990).

lmmunohistochemical detection of c-Fos protein has become a widely used tool to map activation of cells in the nervous system in response to many drugs and other stimuli. The widespread use of this approach has been facilitated by the availability of antisera recognizing c-Fos and, more particularly, by the properties of the *c-fos* gene, mRNA, and protein. IEGs, including *c-fos,* are activated rapidly by multiple intracellular second messenger systems and, therefore, by multiple primary neural stimuli. Under basal (unstimulated) conditions, many IEGs, including *c-fos,* have nearly undetectable levels of mRNA and protein; this lack of background expression contributes to the sensitivity of the technique, making inductions readily detectable by visual inspection. Given its simplicity, sensitivity, cellular resolution, and utility with respect to diverse stimuli, c-Fos immunohistochemistry has been applied to many paradigms, including several relevant to the field of substance abuse such as administration of opiates (Chang et al. 1988; Chang and Harlan 1990), cocaine (Graybiel et al. 1990; Young et al. 1991; Hope et al., in press), and amphetamine (Graybiel et al. 1990; Nguyen et al., in press). In addition, induction of c-*fos* mRNA has been studied in opiate withdrawal (Hayward et al. 1990).

Given the many stimuli that activate c-fos expression, questions have often been raised about the biological specificity and significance of immunohistochemically detected c-*Fos.* Such concerns have been worsened

on occasion by the implication made by some investigators that induction of c-fos mRNA or protein indicates significant phenotypic alterations in the *fos*-expressing neurons. Much of this latter concern could be alleviated if researchers were conceptually clear as to whether they were using *c-fos* as a marker of activation of second messenger pathways, whether they were investigating its biological functions within neurons, or both.

Like all intracellular signaling molecules, the *c-fos* gene product may have a significant biological effect depending on the state of many other molecules in the cell at the time of its induction. Moreover, under circumstances of normal stimulation, the functions of IEGs such as *c-fos* are likely to be involved more often in homeostasis than in change (e.g., coupling new synthesis of peptides to secretion) (Morgan and Curran 1991). However, even mechanisms that are primarily homeostatic under certain circumstances may contribute to significant functional plasticity in the nervous system under others. For example, one important hypothesis in the drug abuse field is that the state of dependence that occurs with repetitive administration of opiates or psychostimulants reflects adaptive changes within target neurons that result in altered cellular and synaptic function. An important candidate mechanism for the adaptive changes underlying the cellular basis of dependence is that repetitive drug administration drives intracellular signaling systems, including expression of IEGs, in such a fashion that the overall pattern of gene expression within the target neuron is altered in ways that significantly alter cellular function.

C-FOS EXPRESSION MAY BE ACTIVATED BY MULTIPLE PATHWAYS

C-fos has been shown to be induced by a variety of stimuli relevant to signal transduction within the nervous system, including growth factors (Fisch et al. 1987; Rivera and Greenberg 1990), and multiple intracellular messenger pathways such as those activated by depolarization (Sheng et al. 1988, 1990) and calcium entry (Fisch et al. 1987; Sheng et al. 1988), the cyclic AMP (cAMP)/protein kinase A pathway (Sassone-Corsi et al. 1988; Fisch et al. 1989), the protein kinase C pathway (Fisch et al. 1987; Gilman 1988), and others. C-*fos* expression appears to be blocked by agents that facilitate the hyperpolarization of neurons (Morgan et al. 1987). Since the stimulation of many neurotransmitter receptor types within the nervous system leads to activation of calcium-dependent protein kinases, protein kinase A, or one or more isoforms of protein kinase C, it is not surprising that many neural stimuli or drugs that affect excitatory or G protein-linked synaptic transmission also activate *c-fos.* Moreover, the activation of more than one of these pathways may produce synergistic activation of gene expression (Sheng et al. 1990).

If carefully interpreted, this convergence of multiple signaling pathways on activation of *c-fos* may, in fact, prove to be advantageous in investigations of neural circuitry in that the particular neurotransmitter receptor types mediating the signal need not be known ahead of time. However, in analyzing the neural circuitry and receptor types involved with IEG activation, both synergy and cross-talk among second messenger pathways within cells must be taken into account.

IF EVERYTHING ACTIVATES C-FOS, HOW CAN IT BE SIGNIFICANT?

IEGs and their protein products can be conceptualized as components of a molecular cascade that transduces signals from cell surface receptors to the nucleus. Thus, IEGs such as c-*fos* have been termed "third messengers" in signal transduction cascades if neurotransmitters are designated as intercellular "first messengers" and small molecules such as cAMP as intracellular "second messengers" (Morgan and Curran 1991); alternatively, IEGs have been termed "fourth messengers" if second messenger-dependent protein kinases are designated as the intervening "third messengers" (Hyman and Nestler, in press). In either case, characterizing the biological actions of IEGs within particular neurons has many analogies with understanding the functions of other molecules within signal transduction cascades, such as the second messenger, cAMP, a ubiquitous molecule, the biological significance of which is rarely questioned. Thus, for example, in considering the biological specificity and function of cAMP within a cell, it is necessary to determine the threshold for activating cAMP-dependent protein kinase, the available substrates for this kinase within the cell, the basal phosphorylation status of these substrates, the activity of specific phosphodiesterases and phosphoprotein phosphatases, cross-talk with other second messenger systems, and subcellular compartmentalization of cAMP and protein kinase A. Thus, although many neurotransmitters activate cAMP, based on these parameters its cellular effects are specific as well as biologically critical to cellular function.

Similarly, investigations of the biological specificity and function of IEGs must address such issues as what particular cells the IEG is induced in, which potential target genes are transcriptionally active in those cells, what partners exist within those cells for dimer formation with the protein product of the IEG, what other proteins in the cell will compete for binding to DNA, and what the effect is of the dimers formed on target gene expression. Experiments that would address these issues include colocalization of IEGs coinduced with putative target genes within identified neurons (Naranjo et al. 1991), demonstration that the temporal appearance of the activator protein-1 (AP-1) proteins of interest precede induction of the putative target gene in response to

the relevant stimulus (Sonnenberg et al. 1989a; Naranjo et al. 1991), demonstration that the proteins of interest bind specifically to the target gene with high affinity in vitro, and demonstration that the IEG activates (or represses) the target gene following cotransfection (Sonnenberg et al. 1989a; Naranjo et al. 1991). Because of difficulties in culturing and transfecting many primary cell types within the nervous system, evidence that a particular IEG plays a role in the cell type of interest has been difficult to obtain to date. Additional experiments that could strengthen the interpretation of a causal interaction between IEG and target might include inhibition of expression of specific IEGs using antisense oligonucleotides or inhibition of transcription factor function by microinjection of specific antibodies into cells prior to stimulation.

THE PROENKEPHALIN GENE IS A POTENTIAL TARGET FOR IEG-MEDIATED ACTIVATION

The proenkephalin gene is widely investigated as a model of stimulus-induced gene expression in brain and as a potential target gene for regulation by IEGs. Hyman and colleagues (1988), Comb and colleagues (1988), and Sonnenberg and colleagues (1989a) have demonstrated that the AP-1 transcription factor (of which *c-fos* is a component; see Morgan and Curran 1991) binds the proenkephalin promoter and that AP-1 proteins can positively and negatively regulate proenkephalin gene expression (Sonnenberg et al. 1989a; Kobierski et al. 1991).

The proenkephalin enhancer has been resolved into three closely spaced DNA regulatory elements (figure 1) by detailed mutational analysis and DNAse I footprinting with affinity purified protein (Hyman et al. 1988, 1989; Comb et al. 1988). Within the enhancer, the element most distal from the TATA box is called ENKCRE-1. It contains the sequence TGGCGTA and binds NF-I-like proteins (Chu et al. 1991). The element that is closest to the TATA box contains the sequence CCGCCGGC and binds nuclear factor AP-2 (Comb

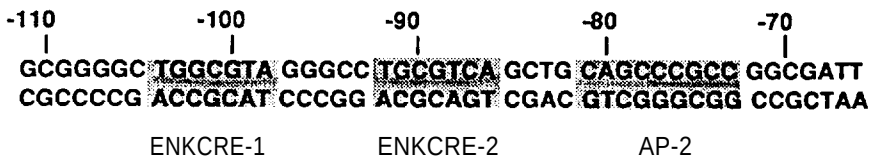

FIGURE 1. *The proenkephalin second messenger-inducible enhancer. The sequence of the enhancer is shown; the sequences of the boxed regulatory elements were determined by mutational analysis.*

et al. 1988; Hyman et al. 1989). The middle element, ENKCRE-2, contains the sequence TGCGTCA. Multimers of this sequence can confer cAMP and Ca++ induction on a minimal proenkephalin promoter in a fashion similar to the observed regulation of the endogenous proenkephalin gene in many cell types (Nguyen et al. 1990; Kobierski et al. 1991). The ENKCRE-2 sequence is similar to the consensus binding site for CREB (TGACGTCA) and for AP-1 proteins (TGAC/GTCA). Although ENKCRE-2 contains the CGTCA motif that is required for cAMP regulation in many genes (Hyman et al. 1988), it binds AP-1 proteins with very high affinity (Hyman et al. 1988; Comb et al. 1988; Kobierski et al. 1991).

Stimulus paradigms in brain, in which both IEGs and the proenkephalin gene are known to be regulated, include seizures (White and Gall 1987; White et al. 1987; Gall 1988), antipsychotic drug administration (Sabol et al. 1983; Tang et al. 1983; Sivam and Hong 1986; Romano et al. 1987), and precipitated opiate withdrawal (Lightman and Young 1987). Seizures induced by electrical or chemical means (e.g., pentylenetetrazol) produce dramatic effects on expression of proenkephalin in the dentate gyrus of the hippocampus and in entorhinal cortex. Seizures increase levels of enkephalin immunoreactivity and decrease levels of dynorphin immunoreactivity in mouse hippocampus (Gall 1988). Electrical stimulation has also been shown to increase proenkephalin mRNA levels and to decrease prodynorphin mRNA levels in rat hippocampus (Morris et al. 1988). Because electrical stimulation and seizures also induce *c-fos* and other IEGs (Morgan et al. 1987; White and Gall 1987; Dragunow and Robertson 1987) with a time course that precedes proenkephalin mRNA accumulation, the proenkephalin gene has been investigated as a model of IEG-target gene interaction in seizure paradigms. Based on the temporal relationship of induction, on the observation that AP-1 binding activity is induced by seizures, and on activation of the proenkephalin promoter in undifferentiated F9 cells by cotransfected c-*fos* and c-*jun,* Sonnenberg and colleagues (1989a) concluded that proenkephalin is likely to be a target gene for c-Fos and c-Jun induced in hippocampal neurons by seizures.

Although heterodimers of c-Fos and c-Jun may regulate proenkephalin in F9 cells, the many proteins that can regulate gene expression via binding to AP-1 sites (including Fos, Jun, and ATF family members) suggests that for AP-1 -regulated genes such as proenkephalin, the relevant transcriptional regulators may differ from cell type to cell type and, even within a given cell type, may vary under different circumstances. It is also important to recall that not all proteins that bind to AP-1 sites are IEGs. Some, such as *jun-D,* are constitutively expressed, with little if any change in level of expression following stimulation. This is relevant in the present context because it has recently been shown that *jun*-D can positively regulate proenkephalin gene

expression in response to cAMP and Ca++ in a fashion that mimics physiologic regulation in many cell types (Kobierski et al. 1991).

THE PROENKEPHALIN GENE CAN BE REGULATED AS AN IEG IN ITS OWN RIGHT

The cAMP- and Ca++-inducible ENKCRE-2 site within the proenkephalin gene is quite similar to the cAMP- and Ca++-inducible calcium-response element that is involved with the rapid induction of *c-fos* in response to depolarization (Sheng et al. 1988). Indeed, in transformed cell types, such as C6 glioma cells and PC12 cells, the induction of proenkephalin gene expression by second messengers is quite similar to the induction of *c-fos:* It is rapid, transient, and independent of new protein synthesis. In fact, in such cell types the regulation of proenkephalin gene expression in response to second messengers is essentially indistinguishable from that of *c-fos* and other IEGs.

Activation of the proenkephalin promoter has been studied in C6 glioma cell lines, which also express the endogenous rat proenkephalin gene (Yoshikawa and Sabol 1986), by stably transfecting the plasmid pENKAT-12, which contains human proenkephalin gene regulatory sequences (Comb et al. 1986). In pENKAT-12, human proenkephalin gene sequences from nucleotides -193 to +70 (with respect to the mRNA cap site), containing the proenkephalin second messenger-inducible enhancer, are fused to the bacterial chloramphenicol acetyltransferase (CAT) transcription unit. The best characterized of the C6 glioma stably transfected lines is the C6-D2 line (Nguyen et al. 1990). When C6-D2 cells are treated with forskolin (10 µM) and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IMX) (0.5 mM), CAT mRNA correctly initiated from the proenkephalin promoter is detectable by S1 analysis within 15 minutes (figure 2); mRNA levels peak by 90 to 120 minutes and then rapidly decline. By 6 hours after addition of forskolin, levels of correctly initiated mRNA have returned to basal levels, Moreover, the protein synthesis inhibitors cycloheximide (100 µM) or anisomycin (10 µM) added to the cells just prior to addition of forskolin and IMX have no effect on onset of mRNA appearance, consistent with activation being caused by a rapid posttranslational modification of preexisting proteins (figure 3). This pattern of activation is consistent with second messenger-dependent posttranslational modification (phosphorylation) of a preexisting transcription factor such as *jun*-D (Kobierski et al. 1991).

IEG-TARGET INTERACTIONS IN CHRONIC STIMULATION PARADIGMS MUST TAKE DESENSITIZATION INTO ACCOUNT

Proenkephalin mRNA and peptides have also been shown to be induced by antipsychotic drugs in rat striatum following 2 to 3 weeks of treatment (Sabol

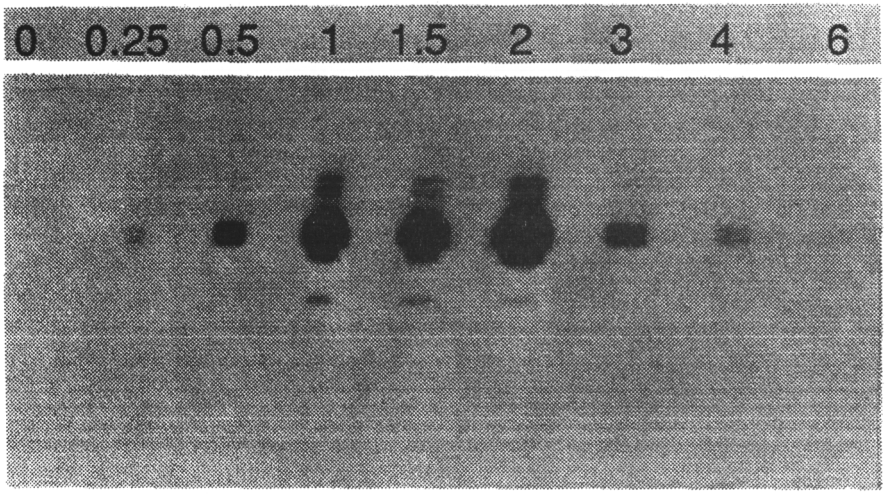

FIGURE 2. *S1 analysis showing the time course of accumulation of correctly initated mRNA from pENkAT-12 in stably transfected C6 glioma ceils (C6-D2). The protected band is 70 bp as expected for this probe. The numbers represent time in hours after replacing the growth media with low serum media (0.1 percent fetal bovine serum) containing 10 μM forskolin and 0.5 mM IMX. Rapid transcriptional changes are more easily seen with pENKAT- 12 than with the endogenous proenkephalin gene because the CAT mRNA is relatively unstable; therefore, basal levels of mRNA are low, leaving changes unmasked.*

et al. 1983; Tang et al. 1983; Romano et al. 1987; Le Moine et al. 1950). Because haloperidol has also been shown to induce expression of *c-fos, zif/* 268, and other IEGs in striatum (Dragunow et al. 1990; Miller 1990; Robertson and Fibiger 1992; Nguyen et al., in press), a role for IEGs in the regulation of proenkephalin gene expression is under investigation. However, in such chronic stimulation paradigms an additional difficulty must be taken into account: IEG expression is transient, and over a time course of minutes to hours, the composition of AP-1 complexes is altered (Sonnenberg et al. 1989b). Indeed, not only does *c-fos* expression rapidly return to basal levels, it also becomes refractory to further activation (desensitization). The same is true of cAMP-inducible proenkephalin gene expression in C6 glioma cells. As shown in figure 2, forskolin and IMX-stimulated levels of pENKAT-12 mRNA decline to undetectable levels by 6 hours after the onset of stimulation. To determine whether expression had been desensitized, either the stable cAMP analog cpt-

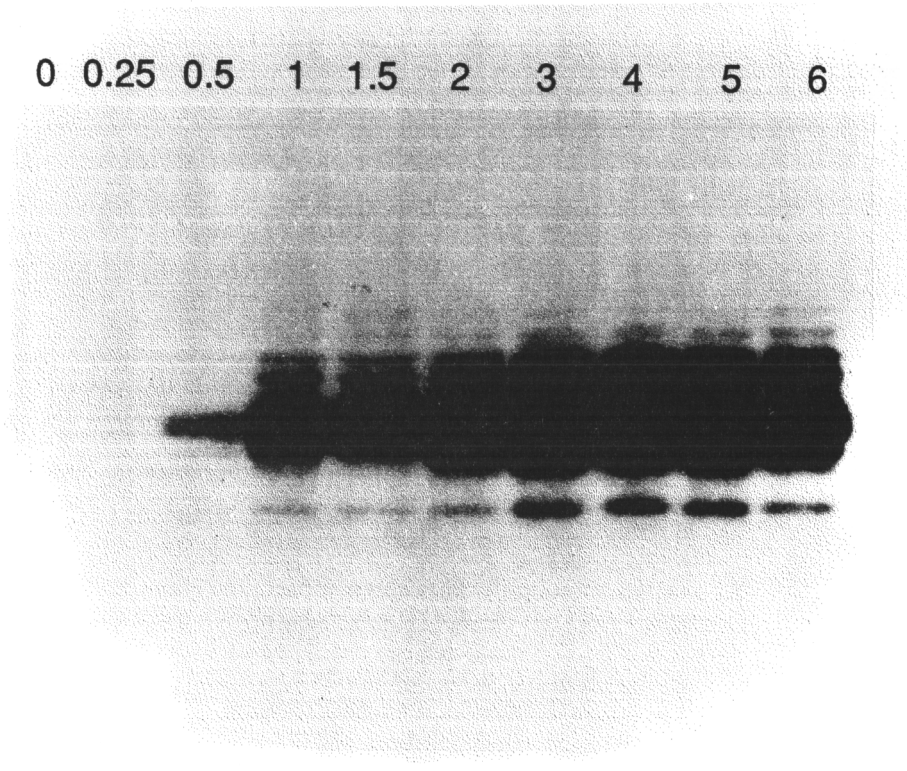

FIGURE 3. *Independence from new protein synthesis. S1 analysis showing the time course of accumulation of correctly initiated mRNA from pENKAT-in C6-D2 cells in response to forskolin (10 µM) and IMX (0.5 mM). Anisomycin (10 µM) was added just before the forskolin and IMX. Anisomycin causes superinduction of proenkephalin mRNA levels, consistent with inhibition of synthesis of a cAMP-inducible transcriptional repressor or inhibition of synthesis of a labile mRNAse.*

cAMP (200 µM) or the phorbol ester 12-0-tetradecanoyl-phorbol-14 acetate (TPA) (50 nM) was added 90 minutes after the addition of forskolin and IMX, by which time transcriptional activation appears to have peaked (figure 4). Cpt-cAMP, which is a potent activator of proenkephalin gene expression (figure 4 inset), had no effect on proenkephalin expression when cells were pretreated with forskolin and IMX for 90 minutes (i.e., the addition of cpt-cAMP did not produce a new peak of mRNA synthesis or in any way prolong the time course of mRNA accumulation). On the other hand, TPA, which activates protein kinase C rather than the cAMP-dependent protein kinase, did produce a new

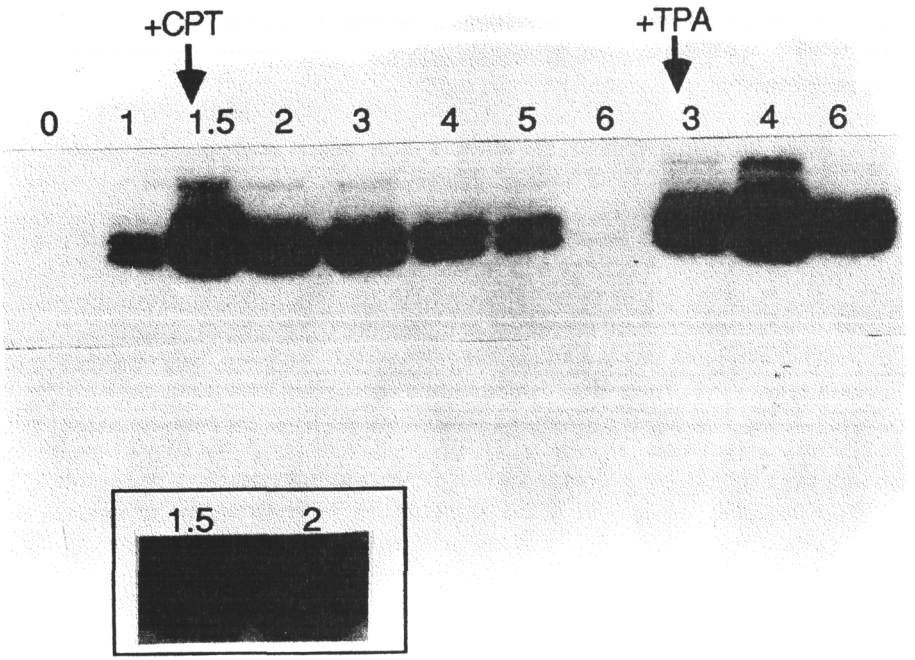

FIGURE 4. *Desensitization of proenkephalin gene expression. S1 analysis in which two parallel sets of plates of C6-D2 cells were treated and assayed. In both sets of plates, forskolin (10 µM) and IMX (0.5 mM) were added at time 0. In the set of plates represented by the first 8 lanes (times 0 to 6 hours from the left), cpt-cAMP (200 µM) was added 1.5 hours after forskolin and IMX. The addition of cpt-cAMP has no effect on the normal time course of mRNA accumulation (compare with figure 2); thus, by the 6-hour time point (4.5 hours after addition of cpt-cAMP), no correctly initiated mRNA is observed. In the set of plates represented by the last 3 lanes, TPA (50 nM) was added instead of cpt-cAMP 1.5 hours after forskolin and IMX. The time points shown are for 3, 4, and 6 hours total (1.5, 2.5, and 4.5 hours after addition of TPA, respectively). Compared with the corresponding cpt-cAMP-treated plates, there is a new burst of transcription with maximal RNA accumulation 2.5 hours after the addition of TPA and appreciable mRNA remaining after 6 hours total (4.5 hours after the addition of TPA). The inset demonstrates the effectiveness of cpt-cAMP (200 µM) on induction of pENKAT-12 in C6-D2 cells not pretreated with forskolin or IMX after 1.5 and 2 hours.*

burst of mRNA synthesis. These data argue that the signal transduction pathway for cAMP-inducible proenkephalin gene expression has become desensitized.

With these problems in mind, the authors have begun studying IEG-target interactions in chronic drug treatment paradigms, which are critical to the field of substance abuse research. Although studies of IEG-target interactions in response to cocaine are in progress, we have begun with the study of haloperidol because it is known to regulate the proenkephalin gene, whereas other target genes are likely to be relevant to cocaine administration. In rats given daily intraperitoneal injections of haloperidol, 2 mg/kg for 2 weeks, the proenkephalin gene is induced twofold to threefold, as previously reported (Sabol et al. 1983; Tang et al. 1983). However, at this time point *c-fos* mRNA is undetectable, and *jun*-D mRNA has diminished 20 to 40 percent compared with saline-treated animals (Nguyen et al., unpublished data). The phosphorylation status of constitutively expressed AP-1 proteins in this paradigm are currently being investigated; however, at the present time the mechanism by which proenkephalin gene expression is activated in brain by a chronic drug stimulus remains unknown.

SUMMARY

It is probably safe to say that the regulation of *c-fos* expression by drug treatment and other stimulus paradigms has biological specificity and mechanistic significance. However, as this brief essay makes clear, the immunohistochemical detection of *c-fos* is only the tip of the biological iceberg. IEGs deserve to be studied as critical components of the intracellular signaling machinery that may transduce intercellular signals, including those produced by drugs of abuse, into longer term changes in cellular function. However, the study of IEG expression must be related to biologically significant target genes and must be complemented by the study of phosphorylation of constitutively expressed transcription factors. Moreover, as illustrated by the proenkephalin gene, the regulation of genes that play important roles in differentiated cell function may differ depending on which cell types and stimulus conditions are investigated. Finally, attention must be paid to the complexities of gene regulation under conditions of repetitive and chronic stimulation if phenomena such as the neural substrates of drug dependence are to be understood.

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Immediate Early Genes: Their Involvement in Physiological and Pathological Responses in the Nervous System

Michael D. Hayward, Tom Corran, and James I. Morgan

INTRODUCTION

The Cellular Immediate Early Gene Response

The application of an extracellular stimulus to a cell has two types of general consequences: a rapid, usually short-lived, response that is protein synthesis independent and a more persistent action that frequently requires gene transcription. The latter finding led to a search for the signaling molecules that couple a stimulus to the transcriptional machinery of the cell. A quantum leap in understanding this problem came with the discovery that several proto-oncogenes, including c-*fos,* c-*jun,* and c-*myc,* are rapidly (within minutes) induced by a diverse array of extracellular stimuli. Since these genes encode nuclear proteins, it was supposed that they might be involved in stimulus-dependent regulation of transcription (Curran and Morgan 1987; Lau and Nathans 1987). Subsequent studies have confirmed and expanded this basic notion

Cellular Immediate Early Genes and Learning

For the neurobiologist, alterations in gene expression have long been supposed to underlie several key aspects of neurophysiology and, indeed, neuropathology. For example, there are many studies dating back to the early 1960s indicating that protein synthesis is essential for memory acquisition in both vertebrates and invertebrates (for reviews, see Barondes 1965, 1970, 1975; Agranoff 1972; Goelet et al. 1986). Indeed, based on the prevailing literature of the time, Barondes (1975) suggested that memory acquisition was composed of two processes. The first process began immediately upon training (i.e., it had no measurable lag), lasted for hours, and involved posttranslational

modification of existing substrates. However, he went on to state that, “Process II begins within minutes after training and augments for hours or longer; Process II is mediated by the synthesis of proteins.” Although the properties of Process II fit those of cellular immediate early (CIE) genes, it required another decade until this notion could be extended by the specific suggestion that genes such as *c-fos* might mediate this process (Goelet et al. 1986). Several studies have been aimed at testing this hypothesis in mammalian models of learning, notably long-term potentiation (LTP) (Douglas et al. 1988; Cole et al. 1989; Wisden et al. 1990). Although it was thought initially that *c-fos* expression did correlate with the induction of LTP, subsequently conditions could be defined whereby LTP could be established in the absence of an induction of *c-fos (e.g.,* Cole et al. 1989). However, another CIE gene, *egr*-1 (also known as NGFI-A, zif/268, and krox-24), was consistently induced in the hippocampus by the stimulus that elicits LTP (Cole et al. 1989; Wisden et al. 1990). Since many CIE genes have been identified, it may be no simple matter to determine which, if any, are critical in a process such as LTP. Indeed, until strategies become available in vertebrates that permit specific interference with CIE gene responses, the absolute involvement of particular genes of this class in neurophysiological responses must remain an open issue.

CIE Genes and Kindling

Although it has drawn the most attention, learning is by no means the only example in the nervous system of a process that requires a window of protein synthesis concomitant with a stimulus for the establishment of a long-lasting effect. For example, kindling is a widely used model of human complex partial epilepsy that appears to require protein synthesis for its establishment. The kindled state is induced by repeated administration of subconvulsant electrical stimuli to various regions of the central nervous system (CNS). Treatment with protein synthesis inhibitors during periods of stimulation either blocks or attenuates the development of kindling (Cain et al. 1980; Jonec and Wasterlain 1979). Unlike the genesis of LTP, the induction of CIE genes has a close temporal and spatial correlation with the kindling stimulus and the establishment of the kindled state (Dragunow and Robertson 1987, 1988; Shin et al. 1990). Indeed, both kindling and related seizure paradigms have suggested potential target genes and biological consequences for CIE gene induction (reviewed in Morgan and Curran 1991a, 1991b).

Kindling is known to bring about alterations in the levels of several neuromodulatory peptides, neurotransmitter receptors, and neurotrophic factors. These alterations and their relationship to CIE gene products have provided several clues to CIE gene function. First, the overall transcriptional

changes in hippocampus elicited by seizures are consistent with the activation of mechanisms that would tend to counteract the transduction of excitatory signals (reviewed in McNamara et al. 1987) (i.e., inhibitory neurotransmission is augmented and excitatory neurotransmission is depressed). For example, there appears to be an up-regulation of γ-aminobutyric acid (GABA) receptors and a down-regulation of muscarinic receptors. There is not yet any direct evidence to suggest that CIE genes mediate the transcription of these particular genes, although appropriate increases in specific CIE genes have been observed in the neuronal populations in which these changes occur. However, in a second situation there are biochemical data to support the involvement of CIE gene products in the regulation of neuropeptide gene expression in hippocampus. It is known that proenkephalin transcription is increased in the granule cells of the dentate gyrus following seizures (for reviews, see McNamara et al. 1987; Morgan and Curran 1991a, 1991b). The timing of this induction occurs just after Fos and Jun increase in these same cells (Sonnenberg et al. 1989a, 1989b). Furthermore, the proenkephalin promoter can be cooperatively transactivated in transient transfection assays by Fos and Jun (Sonnenberg et al. 1989b). These data have been interpreted in terms of a homeostatic response, whereby recruitment of the CIE genes replenishes enkephalin stores that would be depleted by the high levels of firing encountered during seizures (i.e., stimulus-transcription coupling is an integral component of stimulus-secretion coupling).

CIE Genes and Neurotrophic Factors

A third rapid consequence of seizure is the induction, or increased transcription, of neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). This again appears to occur in the same cells in which there is an elevation of CIE gene expression. In addition to the relatively rapid responses cited above, seizures and excitatory stimuli also elicit axonal sprouting in the CNS (Sutula et al. 1988). This has led to the idea that there is a general trophic response to stimulation in brain that has, as one of its early components, the induction of CIE genes and growth factors (reviewed in Morgan and Curran 1991b). In this model, neurotrophic factors such as BDNF may be either CIE genes (as may be the case for NGF) or targets for CIE gene products (Gall and lsackson 1989; Hengerer et al. 1990). It is supposed that these genes underlie the physical growth response in the nervous system following seizure (Sutula et al. 1988; Gall and lsackson 1989; Morgan and Curran 1991b). In a wider sense, such an activity-driven growth response in the nervous system might be viewed as a general process that provides for long-term neuronal plasticity in both normal and neuropathological situations.

FOS, JUN, AND AP-1 IN THE NERVOUS SYSTEM

The CIE genes *c-fos* and *c-jun* encode nuclear phosphoproteins, Fos and Jun, respectively, that are components of the transcription factor activator protein-i (AP-1) (reviewed in Curran and Franza 1988). Fos and Jun dimerize via a leucine zipper-motif, an a-helical domain within both proteins that contains a heptad repeat of leucine residues (Landschultz et al. 1988). The dimeric complexes bind cooperatively to the AP-1 DNA consensus sequence (TGACTCA) via domains rich in basic amino acids. Fos and Jun both contain such a domain; therefore, each protein in the complex provides a half site for DNA binding. The AP-1 sequence is known to be essential for both basal and stimulated expression of some genes, and AP-1 binding at this element can modulate transcriptional activity (reviewed in Curran and Franza 1988; Morgan and Curran 1991a).

Several genes have been described that are related closely to either *c-fos* or c-jun (e.g., *fra*-1, *fra*-2, *fos*-B, *jun*-B, *jun*-D). The protein products can also participate in AP-1 dimeric complexes (reviewed in Kerppola and Curran 1991). In general, all Jun family members can dimerize with all Fos family members, In addition, all Jun (but not Fos) family members can form heterodimers and homodimers with themselves, although the apparent affinity of these complexes for the AP-1 site is lower than that of Fos family-Jun family heterodimers. The situation has become more complicated with the discovery that the leucine-zipper and basic DNA-binding motifs are present in several other families of DNA-binding proteins. This so-called basic-zipper family includes such proteins as the cyclic AMP response element binding proteins (CREBs) and the activating transcription factors (ATFs). These proteins interact with DNA sequences other than the AP-1 consensus, such as the cyclic AMP response element (CRE) (TGACGTCA) (Hai and Curran 1991; Kerppola and Curran 1991). Furthermore, CREBs and ATFs form dimers with themselves as well as with selective members of the Fos and Jun families (Hai and Curran 1991). Thus, in some cases members of the Fos and Jun families may be components of an AP-1 complex, whereas in other circumstances they may be part of a complex interacting with a CRE. Obviously, this situation provides for a considerable degree of mixing and matching and may be one way that diversity is generated within the CIE response.

A second feature of the CIE gene response is that it has a temporal component. In the rodent nervous system, pentylenetetrazole (PTZ) seizures elicit a rapid and transient increase in the mRNAs encoding c-*fos* and c-*jun* (Morgan et al. 1987; Sonnenberg et al. 1989a, 1989b); *fos* and *jun* mRNAs reach maximum values within 30 to 60 minutes following seizure and subsequently decline to basal values by 2 to 3 hours. As anticipated, Fos accumulates in the brain for

1 to 2 hours and then disappears by about 4 hours postseizure. Although AP-1-like DNA-binding activity rises during the first hour after seizure, as would be expected, the levels remain elevated for at least 8 hours, despite the absence of Fos. This additional AP-1 activity is contributed by a series of inducible Fos-related proteins that appear with a delay following seizure and disappear with a slower kinetic (Sonnenberg et al. 1989a). This means that AP-1 complexes have a dynamic composition over time and suggests that the CIE gene response is part of a temporally regulated program that may span many hours following even a brief stimulus. The precise functional significance of the different AP-1 complexes is not known at present; however, it may provide a way of targeting the expression of different sets of genes over time or sequentially inducing and repressing a set of genes (reviewed in Morgan and Curran 1989).

FOS MAPPING STUDIES OF DRUGS OF ABUSE

These considerations have resulted in the widespread use of Fos immunohistochemistry as a mapping technique to identify the pathways and cells involved in particular responses in vivo (reviewed in Morgan and Curran 1991 a). It has even been proposed that Fos immunohistochemistry provides a form of activity map analogous to that obtained with 2-deoxyglucose (Sagar et al. 1988; Morgan and Curran 1991a). Of particular relevance has been the application of Fos mapping to the study of drugs of abuse. Here, the general concept has been that drug dependence, a long-term phenomenon, is elicited by repetitive administration of a stimulus (the drug) and in some senses is analogous to learning (i.e., do drugs of abuse induce a CIE response and could CIE gene products be involved in the establishment of the dependent state?).

Morphine Regulation of C-*fos*

Acute administration of morphine induces Fos-like immunoreactivity (FLI) in the caudate putamen of rat (Chang et al. 1988), a region rich in μ-type opiate receptors. In addition, FLI can be detected in the ventromedial nucleus of the hypothalamus (Chang and Harlan 1990); in contrast to the caudate, the hypothalamus contains predominantly κ-type opiate receptors and few μ-type receptors (Mansour et al. 1987). Thus, both classes of opiate receptors may be capable of transducing signals that can induce *c-fos.* Another morphine-sensitive brain structure, the locus coeruleus (LC), does not respond to acute administration of morphine with an induction of *c-fos.* This is not surprising since morphine depresses the firing rate of neurons in LC (Aghajanian 1978). However, during chronic morphine treatment, firing rates in LC recover to normal levels; whereafter, abrupt cessation of morphine or administration of naloxone precipitates a withdrawal, and firing rates increase severalfold above

those seen in control and chronically treated animals (Aghajanian 1978; Christie et al. 1987). The withdrawal results in an induction of *fos* and *jun* in LC and several other regions of the CNS (Hayward et al. 1990). The increased firing of neurons within LC during withdrawal is compatible with the notion that c-fos is an activity-driven gene. However, 2-deoxyglucose uptake does not change in other brain regions where *fos* is also induced by opiate withdrawal, showing that there is not an absolute coincidence between activity, as measured by 2-deoxyglucose, and *fos* induction (Wooten et al. 1982; Kimes and London 1989; reviewed in Morgan and Curran 1991a). What role CIE genes play during withdrawal is not known at present. However, several possibilities are suggested. For example, the tyrosine hydroxylase (TH) gene can be regulated in some systems by Fos and Jun (Gizang-Ginsberg and Ziff 1990). Furthermore, the regulation of TH levels in a few highly specific regions of the CNS has been implicated in the physical effects of morphine (and cocaine) (Guitart et al. 1990; Beitner-Johnson and Nestler 1991). Indeed, morphine induces changes in TH in several brain regions, including LC (Hayward et al. 1990). Thus, AP-1 complexes may provide a mechanistic link that couples morphine to alterations in TH levels and thereby catecholamine biosynthesis

Cocaine and Amphetamine Regulatlon of C-*fos*

The psychomotor stimulants, methamphetamine and cocaine, can act indirectly as dopamine agonists to elevate prodynorphin-derived peptides in the striatum (Hanson et al. 1987; Sivam 1989). Since it had been suggested that Fos and Jun act in the regulation of expression of several neuropeptide genes, including preprodynorphin and preproenkephalin (White and Gall 1987; Draisci and ladarola 1989; Sonnenberg et al. 1989b), several studies have undertaken the analysis of immediate early (IE) gene expression following treatment with amphetamines and cocaine (e.g., Graybiel et al. 1990; Young et al. 1991). Acute cocaine induces FLI and *c-fos* mRNA in striatum, olfactory tubercle, the islands of Calleja, and nucleus accumbens. Since this induction was blocked by SCH 23390, it is inferred that cocaine is acting via D1-dopamine receptors (Young et al. 1991). Likewise, amphetamine, as well as two D1-dopamine receptor agonists, SKF 38393 and CY 208-243, induce *fos* in the striatum via the D1-dopamine receptor (Robertson et al. 1989; Graybiel et al. 1990). D2-dopamine receptor agonists such as LY 171555 do not induce *fos* in striatum (Robertson et al. 1989), although this agent, like D1-dopamine agonists, can elicit rotational behavior in rats bearing unilateral lesions in the substantia nigra. That is, activation of D1-dopamine, but not D2-dopamine, receptors results in *fos* induction in striatum. Furthermore, rotational behavior can be separated from an induction of *c-fos.* Surprisingly, haloperidol, a neuroleptic with dopamine antagonist properties, also induces *c-fos* in striatum (Miller 1990). Since the action of haloperidol is blocked by the D2-dopamine receptor agonist

LY 171555, it is inferred that inhibition of D2-dopamine receptors either in striatum or in neurons that project to it elicit *fos* induction, perhaps by a disinhibition of striatal neurons,

Although both amphetamine and cocaine induced *fos* in striatum, they did so with different anatomical distributions (Graybiel et al. 1990). Furthermore, there were pharmacological distinctions in the mechanisms linking the two agents to *fos* expression, suggesting that they acted via the same transmitter system but in different cell populations. For example, in the caudatoputamen, acute and chronic reserpine pretreatment suppressed subsequent c-fos induction by cocaine but not amphetamine, suggesting that the two drugs utilize different stores of monoamines (Graybiel et al. 1990).

Fos-lacZ **Transgenic Mice**

To advance the use of *fos* mapping, a line of transgenic mice were bred that carry a *fos-lacZ* transgene (Smeyne and colleagues 1992). There were several reasons for doing this. First, the technique would be less time consuming and costly than immunohistochemistry and in situ hybridization. Second, it would avoid the ambiguity of cross-reaction of antisera with Fos-related proteins. Third, it would serve to define the regulatory elements required for c-*fos* expression in vivo. Fourth, it would provide the first step in devising an inducible gene system for use in mice. Fifth, by mutational analysis of the *fos* promoter in the context of a fusion gene, it may be possible to derive insights into the second messengers and transcription factors critical in specific neurons following a particular challenge.

A fusion gene was constructed that comprised bacterial *lacZ* (ß-galactosidase) fused in-frame into exon 4 of c-*fos* that was tested by stable transfection into B104 neuroblastoma cells (Schilling et al. 1991). The gene contained all the known 5'-upstream regulatory sequences of c-fos as well as all introns and exons (which also contain intragenic regulatory sequences) and several kilobases of 3' flanking genomic DNA. Thus, the fusion gene also includes the polyadenylation consensus sequence from *c-fos* and the Shaw-Kamen element believed to confer the rapid degradation of *c-fos* mRNA (Schilling et al. 1991). Furthermore, since the fusion is in the fourth exon of *c-fos,* the fusion protein retains the leucine-zipper motif and other sequences that direct the protein to the nucleus. It was expected that inducible nuclear ß-galactosidase activity would be observed from the construct. This was deemed important since it would greatly improve the sensitivity and resolution of the technique when applied in animo. In a stably transfected B104 neuroblastoma cell line, inducible nuclear (ß-galactosidase activity could be demonstrated following treatment with serum, phorbol ester, and dibutyryl cyclic AMP, as well as

α- and β-adrenergic agonists (Schilling et al. 1991). Thus, activation of both the protein kinase A and protein kinase C pathways are efficiently coupled to expression of the fusion gene. The induction of c-fos and the fusion gene was both rapid and transient, but the fusion protein had a slightly greater half-life than endogenous Fos (Schilling et al. 1991). Therefore, it was concluded that this fusion gene, when tested in culture, behaved like endogenous *fos* and was appropriate for use in transgenic mice.

Several lines of transgenic mice bearing the *fos-lacZ* transgene have been derived (Smeyne et al. 1992). Three areas of research have been performed to date and these are summarized here.

Constitutive Sites of *Fos-LacZ* Expression

In unstimulated mice, several tissues express the transgene constitutively. Such tissues include the skin, root sheath cells of the hair follicle, ossifying chondrocytes, and cells that give rise to the nail. This suggested that constitutive expression of the transgene (and *c-fos)* is associated with cells that are in terminal differentiation and are destined to die. Subsequently, this notion has been extended by the observation of constitutive transgene expression in other cells that exhibit programed cell death in vivo. What the relationship is between expression of *c-fos* and cell death is unknown at the present.

Expression of *Fos-LacZ* In the Developing Nervous System

During postnatal development, constitutive expression of *fos-LacZ* is readily detected in the nuclei of neurons in several regions of the CNS. Notable examples include, but are not limited to, mitral cells of the developing olfactory bulb, CA1 pyramidal neurons of the hippocampus, neurons in the cingulate cortex, and neurons within the parafascicular nucleus of the thalamus. Many of these sites are undergoing active synaptogenesis at the times that this expression is observed. However, it is unknown whether Fos is involved in, or responsive to, the synaptogenesis or plasticity of these neurons during this period.

Induced Expression of *Fos-LacZ* in the Nervous System

There are several sites of expression of the transgene in unstimulated mice during the daytime, such as the raphe and reticular formation (Smeyne et al. 1992). It has been suggested that these sites of expression might be related to the state of arousal/sleep (Smeyne et al. 1992). However, following several forms of neural stimulation there ensues a rapid and transient induction of the

transgene that can be detected as nuclear ß-galactosidase activity. For example, both PTZ and kainic acid seizures evoke a widespread, transient induction of the transgene in overlapping, but not identical, populations of neurons. These differences in distribution were not so apparent when studied by Fos immunohistochemistry since the antibodies react with related proteins that are induced to high levels over protracted periods following seizure (Morgan et al. 1987; Sonnenberg et al. 1989a, 1989c). The transgene product, like Fos, has a relatively short half-life, and so a precise map of Fos expression is obtained. This has also pointed to the surprising fact that, in some neurons, Fos-like proteins must be induced with little or no Fos expression. That differences in patterns of Fos induction exist between related stimuli should come as no surprise; in fact, it would be surprising if such differences did not exist. It has only been the lack of specificity of the reagents that has heretofore obscured these differences.

Recently, the analysis of gene induction in the *fos-lacZ* transgenic mouse has been extended to examine other forms of stimulation, Light exposure during presumptive night will induce the transgene in the suprachiasmatic nucleus of the hypothalamus, as it does cognate c-*fos.* Thus, the transgene is capable of responding to a physiological stimulus. The *fos-lacZ* transgene is also responsive to drugs of abuse. In preliminary experiments both cocaine and amphetamines have induced the gene in caudatoputamen; however, the latter agent is the more efficient of the two. In addition, amphetamine, but not cocaine, elicits a considerable induction of the gene in other regions of the CNS that are presently being investigated. Therefore, future studies will be aimed at establishing whether the transgenic mice can be useful in the assessment of dependence and withdrawal in chronic models of drug abuse.

PERSPECTIVE ON THE APPLICATION OF IE GENES IN THE STUDY OF DRUGS OF ABUSE

A combination of Fos immunohistochemistry, in situ hybridization, and now histochemistry for ß-galactosidase in transgenic mice provides a novel and precise method for assessing certain aspects of the physiology of drugs of abuse. These include identifying sites within the CNS that show an induction of the IE response as a result of acute drug administration as well as sites that exhibit a response upon withdrawal. Since Fos mapping does not necessarily equate with activity mapping as determined by 2-deoxyglucose, this may be important in directing attention to regions of the nervous system that were previously unsuspected of playing any role in drug responses and dependence. The use of conventional neuropharmacologicai reagents in these systems can resolve some aspects of the synaptology of the IE response to drugs. Thus, it may provide a means to understand some of the signaling molecules and

pathways involved in drug responses. In addition, it points to potential target genes that might underlie dependence, such as neuropeptide genes (e.g., proenkephalin and prodynorphin), neurotransmitter enzymes (e.g., TH), neurotrophic factors and their receptors (e.g., NGF, BDNF, and *trk*B, as well as genes encoding proteins involved in modifying the extracellular matrix (e.g., collagenase and stromelysin). At the structural level these genes may contribute to a general synaptic growth response that modifies the behavior of a neural circuit over the long term (i.e., the CIE genes might elicit a chain of transcriptional events that culminate in the sprouting of axons with concomitant changes in synaptic density, size, and efficiency).

Several criticisms have been leveled at the significance of CIE genes. First, many researchers find it difficult to see any specificity in the system since it is apparently induced quite ubiquitously in most cell types by a diverse array of agents (reviewed in Morgan and Curran 1991a). Thus, it might be argued that this is merely a cellular stress mechanism, analogous to the induction of heat shock proteins, designed to protect neurons when they are at risk for neurotoxic damage. This argument is becoming less valid. First, these genes are induced by normal physiological stimuli, not just the pharmacological and traumatic stimuli used in early experiments. Second, many new CIE genes have been discovered, some of which show restricted sites of expression and are preferentially induced by certain agents in a particular cell type. Third, the complex, differential, posttranslational modification of CIE gene products, combined with their staggered induction patterns and multiple dimerization properties, provides a startling array of diversity. That is, it is likely that diversity is generated at one level by a combination of a relatively few restrictively induced CIE gene products interacting with many more ubiquitously induced members. In addition, specificity is determined by various protein-protein interactions between induced and constitutive proteins as well as their subsequent posttranslational modification. The presence or absence of particular kinases, resident transcription factors, as well as specific signaling pathways will dictate this latter aspect of the response.

Second, although the scenario outlined above nicely accounts for several aspects of neurophysiology and neuropathology, it is unproven except for correlations. Herein lies a fundamental difficulty in pursuing the function of CIE genes in higher vertebrates, namely, the ability to specifically eliminate them or their action in vivo. Since many of the CIE genes encode proteins that are expressed naturally during development, it is likely that if they were to be eliminated by homologous recombination the animals would die in utero. Therefore, more subtle ways must be designed to impair their function or expression. At one level this could be viewed as a novel branch of pharmacology (i.e., designing agents that specifically impair the CIE

response while leaving short-term responses to a stimulus intact). Such agents might target one of the later elements in the signal transduction cascade between the stimulus and the CIE gene promoter. For instance, it has been shown that certain types of benzodiazepines can greatly augment the IE response, whereas calcium channel blockers and calmodulin antagonists suppress it (Curran and Morgan 1985; Morgan and Curran 1986; reviewed in Morgan and Curran 1991a, 1991b). Other agents might target the CIE gene product by, for example, impeding their binding to DNA or a partner protein or modifying posttranslational events (Curran and Morgan 1986). The use of transdominant suppressor molecules in transgenic mice might provide such an avenue. Until such issues are clarified, the precise role that the CIE genes fulfill and the utility of regulating their expression and function in vivo will remain an open question.

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Immediate Early Gene Activation and Long-Term-Changes in Neural Function: A Possible Role in Addiction?

Harold A. Robertson

INTRODUCTION

Psychostimulants such as cocaine and amphetamine have a range of pharmacological effects on the central nervous system (CNS). The most important of these effects, those related to addictive behavior, appear to depend on dopaminergic mechanisms (Wise 1984). Thus, a receptor for cocaine has been described (Ritz et al. 1987) and sequenced (Shimada et al. 1991; Kilty et al. 1991) that appears to be a site on the dopamine transporter on dopamine nerve terminals. Furthermore, there is evidence from animal studies to suggest that dopamine function in the nucleus accumbens plays an important part in intracranial self-stimulation and self-administration of cocaine (Roberts and Koob 1982). Finally, there is evidence that the dopaminergic mechanisms in the nucleus accumbens may be a final common pathway for many, if not all, substances that produce addictive behavior (Di Chiara and Imperato 1988). Therefore, it seems clear that dopaminergic mechanisms play a central role in the addictive properties of cocaine and other psychostimulant drugs. These compounds have their effects on dopamine either by increasing its release into the synapse (amphetamine) or by blocking the reuptake of dopamine from the synapse (cocaine), the net effect in either case being an increase in dopamine levels in the synapse. It should be noted that this explanation for the actions of cocaine and amphetamine is almost certainly incomplete. Other agents (e.g., tricyclic antidepressants, tyramine) share these actions and do not produce addiction. The obvious question is, What does the dopamine accumulation following cocaine and/or amphetamine do that produces addiction? It seems reasonable to assume that dopamine produces some sort of change in neural systems that leads to a long-term change in function. It is known that the changes that occur are robust and persistent. In animals, the sensitization and reverse tolerance produced by

psychostimulant drugs last many weeks and perhaps months. In humans, the effects of psychostimulant use are still evident after years of abstinence (Koob and Bloom 1988).

The mechanism underlying long-term changes in the brain is unknown. The phenomenon of long-term potentiation is interesting but, in general, only lasts for a few weeks at the most and decays over that period. By contrast, the phenomenon of kindling in animals is generally regarded as representing a permanent change in neuronal function. However, the biological basis of kindling is largely unknown, although there are suggestions that it could involve activation (or inhibition) of gene expression in parts of the limbic system, including the hippocampus. Kindling is a process whereby repeated subthreshold stimulation (electrical or chemical) will lead to a gradual increase in response. Thus, repeated subconvulsant doses of a chemical convulsant agent will also induce kindling (for reviews on kindling, see Goddard 1983 and Cain 1989). In the case of kindled seizures, this process culminates in a major motor seizure. Most importantly, once an animal is kindled, it apparently remains in the kindled state for the remainder of its life (about 33 months is the longest known duration). Moreover, the kindled state, unlike long-term potentiation, appears to depreciate little with time (for a review, see Cain 1989).

Kindling can also be induced chemically by agents such as cocaine. The kindling-like effects of cocaine have been known longer than researchers have been aware of kindling (Downs and Eddy 1932). Post and colleagues (1975) drew attention to the idea that the local anesthetic actions of cocaine might produce a process of "pharmacological kindling" during repeated high-dose drug administration. In many ways, electrophysiological kindling was reminiscent of the behavioral sensitization that is characteristic of cocaine. Cocaine-induced behavioral sensitization is longlasting and shows cross-sensitization to other stimuli and shows, at least in part, a dependence on the interval between injections just as kindling does (Post and Contel 1981; Post and Weiss 1988). The search for a mechanism to explain the persistent changes that are characteristic of electrophysiological kindling led to a consideration of the possible role of gene activation in this process. Since electrophysiological kindling is a permanent change in neuronal function, it was reasoned that the genetic material might be involved. Experiments demonstrated that the immediate early gene (IEG) c-*fos* was rapidly and transiently expressed in the dentate gyrus and other parts of the hippocampus after a kindling stimulus (Dragunow and Robertson 1987a) or following induction of seizure activity by chemical convulsants (Morgan et al. 1987; Dragunow and Robertson 1987b). Subsequent work has shown that induction of IEGs is a consequence of neuronal activation in many parts of the CNS

(see reviews by Robertson and Dragunow 1990; Morgan and Curran 1991; Robertson et al. 1991 a).

IEG INDUCTION AND THE BRAIN

Activation of IEGs is not a unique property of neurons; indeed, IEGs were first characterized in nonneuronal cells during attempts to identify genes that might respond to growth factors (Cochran et al. 1983). This resulted in the description of a class of genes whose transcription was rapidly (in minutes) but transiently (usually for a few hours at most) activated following growth factor application. Using differential screening of cDNA libraries from growth-factor stimulated cells, many IEGs have been characterized, and the number of these, now at about 100, continues to grow. In the absence of stimulation, the expression of IEGs is generally low. Following stimulation, transcriptional activation is rapid and transient, and the mRNAs transcribed from these genes often have short half-lives. The subsequent shutoff of transcription requires new protein synthesis; IEGs are therefore superinduced in the presence of protein synthesis inhibitors (Sheng and Greenberg 1990). The crucial step linking these growth-activated IEGs to neuronal events was accomplished when several groups at about the same time demonstrated that not only growth factors but also neurotransmitters and neurotransmitter-related events such as depolarization and calcium influx will induce transcription of IEGs both in cells in culture (Greenberg et al. 1985, 1986; Morgan and Curran 1986) and in the brain in vivo (Morgan et al. 1987; Hunt et al. 1987; Dragunow and Robertson 1987a). Since *c-fos* was originally thought to be involved in cell division, studies demonstrating *c-fos* induction in differentiated PC-12 cells and the discovery of *fos* activation in brain was important in suggesting that IEGs could have a function in postmitotic cells (Greenberg et al. 1986; Morgan et al. 1987; Hunt et al. 1987; Dragunow and Robertson 1987a).

It is now clear from many studies that neurotransmitters and neuronal growth factors in several systems will induce expression of IEGs (table 1). Rapid *c-fos* induction occurs in PC-12 cells following application of elevated K^+, the calcium channel agonist BAY K8644, or external Ba++ (Curran and Morgan 1985; Morgan and Curran 1986; Sheng et al. 1988), suggesting that Ca++, entering through voltage-dependent Ca++ channels and/or L-type Ca++ channels, is the major second messenger regulating IEG expression. There is also evidence that Ca++-calmodulin-dependent protein kinases (CaM kinases) I and II play a role in the transduction of electrical signals to the nucleus, and CREB (the adenosine 3',:5'cyclic phosphate [cAMP] response element-binding protein) may function to integrate Ca++ and cAMP signals (Morgan and Curran 1986; Greenberg et al. 1985, 1986; Sheng et al. 1991). In addition, recent evidence suggests that AP-1 proteins may also have an important part to play

TABLE 1. *Some neurotransmitters involved in activation of IEGs*

Neurotransmitter	Cell or Tissue	Reference
Glutamate	Cerebellar neurons	Szekely et al. 1989
Cholinerglc (nicotinic)	PC-12	Greenberg et al. 1985
Cholinergic (muscarinic)	PC-12	Morgan and Curran 1986
Dopamine (D1, D57)	Striatal neurons	Robertson et al. 1989a, 1989b
Adenosine	Neuron-glia hybrids	Gubits et al. 1990
Nerve growth factor (NGF)	PC-12	Greenberg et al. 1985
Calcitonin gene-related peptide	Rat astrocytes	Haas et al. 1991

in activation and inhibition of gene expression by cAMP. Thus, Jun-D and Jun-B may be important in the regulation of the proenkephalin expression by the cAMP response element (Kobierski et al. 1991), emphasizing the importance of differential regulation of IEGs in control of gene expression.

IEGs: DIFFERENTIAL REGULATION

The situation is clearly more complicated in that many IEGs are simultaneously activated by physiological stimulation, neurotransmitters, and growth factors. It has been suggested that various combinations of IEGs could confer specificity of cell response to different stimuli (Sonnenberg et al. 1989; Sheng and Greenberg 1990; Wisden et al. 1990; Moratalla et al. 1992; Rusak et al., in press), although there is little or no evidence for this idea.

The best known example of regulatory interaction between IEGs is the interactions between the *fos* and *jun* families. Protein products of members of these two IEG families can interact with each other to form heterodimeric transcriptional factor complexes via a conserved dimerization domain called the leucine zipper. Briefly, the protein c-Fos dimerizes with c-Jun to form a c-Fos/c-Jun transcriptional factor that binds with high affinity and specificity to DNA elements of the consensus sequence TGACTCA (the AP-1 site) and, thus, leads to the transcription of nearby promoters by an unknown mechanism (Curran and Franta 1988). However, it is clear that the situation is even more complicated than many IEGs activating transcription because some heterodimeric combinations are transcriptional inhibitors. The heterodimer formed by c-Fos and Jun-B (the protein product of the *jun* family member, Jun-B) represses transcription of genes with AP-1 sites in the promoter region (Chiu et al. 1989; Schutte et al. 1989). Moreover, the situation is made more complicated by the fact that c-Jun is an efficient activator of the *c-jun* promoter, whereas Jun-B inhibits activation of the *c-jun* promoter. Thus,

Jun-B is a negative regulator of *c-jun* (Chiu et al. 1989). Since there are three mammalian Jun proteins (c-Jun, Jun-B, Jun-D) and at least four Fos family members (c-Fos, Fos-B, Fra-1 , Fra-2) to which the Jun proteins can bind, there exist many possible combinations of Fos-Jun proteins. In addition, there is also the possibility of Jun-Jun and Fos-Fos homodimers with unknown potentials. Other members of the two families also probably have unrealized potentials. For example, it has recently been shown that a truncated form of Fos-B inhibits the transcriptional activation of Fos/Jun heterodimers (Nakabeppu and Nathans 1991). Similarly, Fra-1 can combine with Jun and bind to the AP-1 recognition element, raising the possibility that this Fos family member might play a role in regulation at the AP-1 site (Cohen et al. 1989). Finally, it is now becoming apparent that Fos/Jun can regulate other receptors; for example, Fos and Fos/Jun can inhibit the glucocorticoid receptor (Touray et al. 1991), and in turn, other factors, such as retinoic acid (Schule et al. 1991), can regulate AP-1 -responsive genes.

The issue is further confused because in many studies, especially for neural tissue, stimulation leads to activation of a variety of Fos and Jun family members (in addition to other IEGs). For example, stimulation with D1 dopamine agonists can cause rapid expression of *c-fos, c-jun,* and *jun*-B in striatum. Are these IEGs all in the same cell, or more attractively, are c-fos and *c-jun* expressed in one type of cell leading to transcriptional activation while in another cell *c-fos* and *jun*-B expression is producing transcriptional inhibition? It is clear that the answers to such questions are of great importance. However, in some tissues, such as the dentate granule cells after seizure activity, it seems clear that *c-fos, c-jun,* and *jun*-B activation all occur in the same cells (B.J. Chiasson and H.A. Robertson, unpublished observations).

In addition to this complexity, it is now suspected that the so-called flanking sequences to the AP-1 site play an important role in the binding of Jun proteins to the AP-1 site (Ryseck and Bravo 1991). It is important to note that AP-1 -like sites are sometimes common, and it is not immediately obvious which sites might be active (e.g., Hengerer et al. 1990). It is also clear that differential regulation of IEGs does occur, and there are several instances in which *c-fos* is expressed in the absence of *c-jun* or together with *jun*-B rather than *c-jun* (Bartel et al. 1989; Naranjo et al. 1991). There are instances in which the *c-jun* message is expressed following stimulation but no c-Jun protein appears to be expressed, suggesting, among other possibilities, that IEG protein levels might be regulated at the transcription level (Rusak et al., in press).

In summary, it is now clear that the picture of activation of IEGs that encode nuclear proteins that regulate gene expression is misleading. The more accurate picture has been described as regulation by committee, a series of

IEGs, members of various families, all interacting with one another and with other second and third messengers to affect several transcriptional regulating sites and, thus, altering gene expression. What has become clear is that these genes probably play a role in postmitotic tissue, the best example being neurons in the brain. However, it should be kept in mind that some IEGs may perform not as messengers in tightly linked signaling systems but as "housekeeping" genes, regulating the response of cellular metabolism to particular alterations in conditions. For example, it is remarkable to note the similarity between the activation of *c-fos* in cerebral cortex ipsilateral to a lesion (Dragunow and Robertson 1988; Herrera and Robertson 1989, 1990a) and the activation of the heat shock protein, HSP-71 (Gonzalez et al. 1989). However, having raised this caution, it is clear from several examples in the brain that there is good circumstantial evidence that *c-fos* and other IEGs play an important role in the transduction of neuronal stimulation into specific neuronal changes (plasticity); this neuronal stimulation can be elicited by physiological (e.g., light, pain) or pharmacological means.

IEG ACTIVATION: A MAPPING TOOL FOR THE BRAIN?

It has been apparent since the work of Hunt and colleagues (1987) on the effects of pain on the induction of c-fos in spinal cord that, apart from the importance that the IEGs might play in cellular function in brain systems, the robust response of the IEGs might be generally useful as an indication of transsynaptic activation and in the mapping of polysynaptic pathways in the brain. This usefulness has generally been confirmed in many subsequent studies (e.g., Sagar et al. 1988; Rusak et al. 1990, in press; MacDonald et al. 1990). This does not detract from the significance of IEGs as possibly extremely important "third or fourth messengers" in signal transduction. However, it does add an extremely important dimension to the uses of IEG activation.

EXPRESSION OF IEGs IN THE MAMMALIAN BRAIN: KINDLING, LONG-TERM POTENTIATION, AND SEIZURES

Induction of IEGs has now been demonstrated in several systems in the brain and in association with several neurotransmitter systems. The initial studies that suggested an important role for IEGs in the brain described activation of *c-fos* and other IEGs in the brain following drug-induced seizure activity (Morgan et al. 1987; Dragunow and Robertson 1987a; Saffen et al. 1988; Le Gal La Salle 1988; Sonnenberg et al. 1989). A single electrical stimulus to amygdala or hippocampus (at an intensity known to lead to the longlasting effect known as kindling) will also produce an increase in c-Fos protein in the hippocampal formation, with an especially high concentration in the dentate

granule cells (Dragunow and Robertson 1987a; White and Gall 1987; Dragunow and Robertson 1987b). There is a continuing discussion over the possible role of c-*fos* and other IEGs in the phenomenon of long-term potentiation (LTP), which is the persistent (up to several weeks) increase in the excitatory postsynaptic potential elicited at synapses following a high-frequency stimulation of afferent neurons, Early studies suggested that LTP could develop in the absence of c-fos induction (Douglas et al. 1988), and this was confirmed by Cole and colleagues (1989) and Wisden and coworkers (1990) who did, however, demonstrate a correlation between LTP and induction of *NGFI-A.* Surprisingly, LTP induced using "burst" stimulation involves c-fos induction, but LTP induced by the same number of stimulations evenly spaced over the same train duration (i.e., LTP induced by the same number of stimulations but with different spacing) is not associated with increases in Fos protein (Dragunow et al. 1989). However, all earlier studies were done on anesthetized animals, and more recent studies on the development of LTP in unanesthetized animals revealed that, in this situation, *c-fos* induction accompanies LTP induction (Jeffery et al. 1990). From all this work, several points are clear. First, it is already known that LTP develops in milliseconds, whereas *c-fos* mRNA only appears between 10 and 15 minutes later, with c-Fos protein appearing shortly after. The same is true for other IEGs. Thus, although it remains possible that IEGs play a role in maintenance of LTP (but see Jeffery et al. 1990), it seems unlikely that they play any role in induction of LTP. Second, some stimulation parameters produce LTP with *c-fos* activation, but others produce LTP without *c-fos* activation, which suggests (as was already suspected) that there are different types of LTP, differing perhaps in persistence or other properties.

The kindling model is interesting in that, unlike LTP, induction of kindling appears to be a *permanent* change in the brain (for a review of the differences between kindling and LTP, see Cain 1989). A kindling stimulus will activate *c-fos* in the dentate granule cells and in the hippocampal pyramidal neurons (Dragunow and Robertson 1987a; White and Gall 1987). Other IEGs (c-*jun, jun*-B, *NGFI-A)* are also activated by the kindling stimulus (H.A. Robertson, unpublished data). It is known that kindling stimuli lead to synaptic reorganization in the molecular layer of the dentate gyrus of the hippocampus (Sutula et al. 1988). Kindling has also been shown to produce increased synthesis of neurotrophic factors in the dentate gyrus (Ernfors et al. 1991; B.J. Chiasson and H.A. Robertson, unpublished observations), and Funabashi and colleagues (1988) demonstrated that injections of antibody against NGF into the cerebral ventricles during stimulation would prevent kindling. In at least one other system, IEGs appear to play a role in the regulation of NGF synthesis (Hengerer et al. 1990, see below). Thus, it is reasonable to suggest that IEG induction may be the first step in a

sequence of changes involving secretion of neurotrophic factors and synaptic reorganization, culminating in a permanent alteration in the susceptibility of this part of the brain to seizures.

DOPAMINE AND IEG ACTIVATION

The neurotransmitter dopamine appears to play a pivotal role in regulation of movement by the striatum. Loss of dopamine-containing neurons of the substantia nigra leads to a profound loss of motor control as seen in Parkinson's disease. Dopamine is also thought to play a similar role in higher functions, and disorders of dopaminergic neurotransmission are generally assumed to lie behind the schizophrenias and may also contribute in large part to the disturbances of both thought and movement seen in Huntington's chorea. The dopaminergic system also appears to play a central role in cocaine and amphetamine addiction (Roberts and Koob 1982; Koob and Bloom 1988; Di Chiara and lmperato 1988). Activation of *c-fos* in an animal model for Parkinson's disease was first demonstrated using the antiparkinsonian drug L-dopa (the immediate precursor of dopamine) and drugs selective for the D1 -dopamine receptor (Robertson et al. 1989a, 1989b, 1991b; Paul et al., in press). After L-dopa or a D1-dopamine agonist, c-Fos protein was found only in the regions of the brain (striatum) that were experimentally depleted of dopamine and where dopamine receptors were known to be supersensitive. Significantly, activation of IEGs appears to be a D1-dopamine receptor-linked function (Robertson et al. 1989b). Compounds that act indirectly to release dopamine (such as cocaine and amphetamine) also increase expression of *c-fos* and other IEGs in striatum via D1 dopamine receptors (Robertson et al. 1989b; Graybiel et al. 1990; Young et al. 1991; Moratalla et al. 1992). It is most important to note that, whereas amphetamine and cocaine activate IEGs in naive, untreated animals, directly acting D1 dopamine receptor agonists only induce IEG transcription in supersensitive tissue. Thus, even high doses of a D1 agonist or apomorphine will not produce IEG expression in animals that have not been depleted of their dopamine content.

Although D2 dopamine agonists apparently have little effect on IEG activation, it is significant that the combination of a D1 and a D2 dopamine agonist has synergistic effects on induction of *c-fos, c-jun, jun*-B, and *NGFI-A* (Paul et al. 1990, in press; Robertson et al. 1991 b) reminiscent of the synergism seen in studies on locomotion in animal models for Parkinson's disease (Robertson and Robertson 1986, 1989). Moreover, although *c-fos* activation in the striatum following D1 agonist stimulation is spread over the entire striatum, the combination of D1 and D2 agonists produces a pattern that reveals the compartmentalization of this brain region (Paul et al. 1990; Robertson et al.

1991b; Paul et al., in press). This once again illustrates the fact that IEG activation is a potent tool for studying the anatomical organization of neuronal structures.

Dopaminergic and glutamatergic mechanisms interface in the striatum as two control mechanisms central to regulation of striatal function (Clow and Jhamandas 1989). Glutamatergic neurotransmission appears to play some role in dopamine-mediated activation of IEG expression. N-methyl-D-aspartate (NMDA) receptor antagonists reverse amphetamine-induced activation of *c-fos* (Johnson and Robertson 1989; Aronin et al. 1991; Snyder-Keller 1991; Paul et al., in press; Berretta et al., in press), suggesting that glutamatergic and dopaminergic mechanisms also interact at the level of regulation of gene expression in striatum.

It was known that dopamine agonists induce expression of *c-fos,* and it was at the same time surprising and potentially important to note that *c-fos* induction also occurred following administration of D2 dopamine receptor *antagonists* such as haloperidol (Dragunow et al. 1990; Miller 1990) and clozapine (Robertson and Fibiger 1991; Hiroi et al. 1991). These drugs are widely used in the treatment of mental disorders such as schizophrenia. The finding of IEG activation by these drugs in clinically relevant doses suggests the possibility that either the beneficial effects or some of the side effects of these drugs might be mediated by changes in gene expression. Both the symptoms of schizophrenia and the development of some side effects of these drugs have time courses consistent with alterations in gene expression, Thus, selective blockade of dopamine receptors may, like selective activation of dopamine receptors, be associated with long-term changes in neuronal function (table 2). The evidence at this time suggests that, although dopamine D1 agonists exert a stimulatory effect on *c-fos* expression, D2 receptors appear to mediate a tonic inhibitory effect on *c-fos* expression,

TABLE 2. *Some possible associations between drug-induced activation of IEGs and pathophysiological effects of dopaminergic drugs*

Effect	IEG Activator	Drug Class	Reference
Dystonias	L-dopa	D1 dopamine agonists	Robertson et al. 1989a, 1989b
Tardive dyskinesias	Haloperidol	D2 dopamine antagonists	Dragunow et al. 1990
Drug addiction	Amphetamine, cocaine	Psychostimulants	Graybiel et al. 1900; Young et al. 1991

IEGs AND CONTROL OF CIRCADIAN RHYTHMS

In mammals, the suprachiasmatic nucleus (SCN) is the site of the central control mechanism for the regulation of circadian rhythms (Rusak and Zucker 1979). The "clock" in the SCN is "set" (entrained to the environmental lighting) by neurons in the retina that project to the SCN. Exposing an animal to light during its "dark" phase will alter or shift the circadian rhythm. Exposing rats or hamsters to light during the night will result in the induction of *c-fos* and *NGFI-A* (Rusak et al. 1990) and other IEGs (Rusak et al., in press). Most important, light will induce *c-fos* only during the "expected" dark phase in animals kept in total darkness. Such animals continue to maintain the circadian rhythm established before they were placed in total darkness. During the "expected" light phase, exposure to light will not lead to the induction of *c-fos* and other IEGs (Rusak et al. 1990, in press). There is also a good correlation between the intensity of the light required to produce the physiological effect (a shift in the circadian rhythm) and the induction of mRNA production (Kornhauser et al. 1990). There is some evidence that the photic induction of IEGs in the SCN is regulated by glutamate receptors of the NMDA type, at least in part (Abe et al. 1991). The mechanism that prevents light stimulation from inducing the IEGs during the subjective day is pretranscriptional but otherwise unknown, Feasible mechanisms include the possibilities that the retinal ganglion cells do not transmit the information or that the receptors in the SCN exhibit rhythmic sensitivity. It appears that the NMDA type of glutamate receptor is at least involved, but another possibility is that the sensitivity of the NMDA receptor is being regulated via alterations in the depolarization state of the SCN neurons by another endogenous or exogenous factor.

DAMAGE-INDUCED ACTIVATION OF IEGs

Damage to tissues often produces homeostatic responses that include the release of growth factor and induction of cell proliferation. Therefore, it is not surprising that such brain damage will produce widespread activation of *c-fos* (Dragunow and Robertson 1988). Indeed, early experiments attempting to look at the activation of IEG synthesis in slices had been compromised by the fact that the process of preparing the slice produced a maximum activation of IEG expression (S.P. Hunt, personal communication, November 1987). Similarly, when first reported, damage-induced activation of IEGs in brain in vivo was (and is) a serious complication for in vivo experimentation. It meant that introduction of a cannula or an electrode into brain, by itself, produced significant IEG expression, Furthermore, induction of IEGs was seen not only adjacent to the site of damage but also in parts of the cortex remote to the damage (Dragunow and Robertson 1988; Herrera and Robertson 1989, 1990a,

1990b). For c-fos, this activation was confined to the damaged hemisphere and was sensitive to NMDA receptor antagonists (Herrera and Robertson 1989, 1990a, 1990b; Dragunow et al. 1990; Traboulsee et al. 1991). There is at least some circumstantial evidence that damage-induced IEG activation in cerebral cortex is partially the result of spreading neuronal depression (Herrera and Robertson 1990b).

IEGs AND CHANGES IN GENE EXPRESSION

The impetus for studying IEG activation came from the conviction that changes in gene expression lay behind long-term biological change. The excitement associated with the discovery of IEG activation in the instances cited above (and many others as well) is that there is now a key for the problem of how neuronal activity might alter gene expression. However, there is relatively little direct evidence for such changes, and what evidence there is is circumstantial. For example, a kindling stimulus activates c-fos expression, but there is no direct evidence that *c-fos* expression is absolutely required for kindling to occur. Similarly, light exposure during the dark phase leads to IEG expression in the SCN, and in every instance studied so far, there is a relationship between IEG activation and the phase shift in the circadian rhythms. But this cannot be construed as other than circumstantial. On the positive side, there are several situations in which it can be said that IEG expression can lead to changes in gene expression. For example, the availability of mice with a transgenic *c-fos* linked to a metallothionine reporter has been used by Thoenen and colleagues (Hengerer et al. 1990) to demonstrate that activation of *c-fos* in fibroblasts in sciatic nerve precedes NGF synthesis. Furthermore, they were able to show that an AP-1 site in the first intron of the NGF promoter is necessary for NGF synthesis. It IS interesting to note that there are eight AP-1 sites in the promoter region of NGF in the mouse, but only one, apparently, is essential for NGF synthesis. There is also a suggestion that the proenkephalin gene may be up-regulated via an AP-1 site present in its promoter (Sonnenberg et al. 1989). However, such regulation of proenkephalin is probably complex, involving several other control elements (Comb et al. 1988; Kobierski et al. 1991).

Clearly, it is important to define the nature of the late-response genes. Are there any long-term changes that result from activation of IEGs? What are these changes, and how are they carried out? The other task is to show conclusively that the IEGs are involved (or not involved) in long- or short-term changes in function.

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CANADA

Acute Effects of Psychomotor Stimulant Drugs on Gene Expression in the Striatum

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INTRODUCTION

Long-term changes in behavior leading to drug addiction imply concomitant drug-induced changes in central nervous system (CNS) processing. A large body of behavioral work implicates the mesolimbic, mesostriatal, and mesocortical systems as potential sites for such modifiability (Carr et al. 1989; Koob and Goeders 1989; Koob and Bloom 1988; Kuhar et al. 1991). At the cellular level, changes ranging from alterations in receptor expression and receptor interactions with intracellular signaling molecules by pretranslational and posttranslational modification to alterations in expression or activity of such molecules have been demonstrated following repeated drug exposure, Both presynaptic and postsynaptic changes have been suggested (Robinson and Becker 1986; Post and Contel 1983; Graybiel 1990; Terwilliger et al. 1991; Beitner-Johnson and Nestler 1991). Recently, interest has focused also on the possibility that regulation of immediate early gene (IEG) expression in neurons could lie at the basis of some or many of these changes.

Interest in IEG regulation as part of the cellular basis of addiction is based on at least two characteristics of these genes. First, IEGs are positive and negative regulators of the transcription of other genes and can be regulators of their own transcription as well (Sheng and Greenberg 1990; He and Rosenfeld 1991). Thus, affecting IEG transcription could start a cascade of further changes that could have enduring effects on CNS function. Second, IEGs can act as bridges between the short-term effects of transient stimuli impinging on cells and long-term changes resulting from these stimuli. Just such considerations led to the proposal that transcriptional regulation is at the basis of long-term memory in neural circuits (Goelet et al. 1986; Berridge 1986; Morgan and Curran 1989).

What evidence is there that alterations in transcriptional regulation occur following exposure to drugs of abuse? For drugs that affect dopamine and other biogenic amines, including cocaine and amphetamine, there is now direct evidence that acute drug exposure induces expression of IEG mRNAs in the striatum, certain sites in the limbic system, and certain cortical areas (Graybiel et al. 1990; Young et al. 1991; Berretta et al. 1991a, in press; Moratalla et al. 1991, in press, submitted for publication; Cole et al. 1990; Dilts et al. 1990; Grimes et al. 1990; Robertson et al. 1991). There is also evidence that exposure to amphetamine and cocaine elicits expression of nuclear proteins detected with antisera to Fos and related IEG protein products (Graybiel et al. 1990; Young et al. 1991). Thus, transcription and/or mRNA stabilization and translation of IEG protein products are induced by cocaine and amphetamine.

PSYCHOMOTOR STIMULANTS ACTIVATE IEGs IN REGIONALLY SPECIFIC PATTERNS

What characteristics of the IEG induction suggest specificity in the neural circuits showing this response to psychomotor stimulants? First, amphetamine and cocaine acutely activate IEGs in different macroscopic patterns in the rostral caudatoputamen and also in the ventral striatum (Graybiel et al. 1990; Fuxe et al. 1991). Expression of Fos-like protein is concentrated more strongly in the striosomes than in the matrix of the rostral caudatoputamen in rats acutely treated with amphetamine. By contrast, acute cocaine exposure induces Fos-like proteins in both striatal compartments in the rostral caudatoputamen. Combinations of D1-like and D2-like dopamine agonists given in low dose (Paul et al. 1991, in press) and chronic apomorphine (Grimes et al. 1990) also induce Fos-like protein expression in patterns that respect striosome/matrix compartments in at least parts of the dopamine-depleted striatum.

These findings raise the possibility that there is differential neural activation of the striosomal and matrix compartments following exposure to certain amphetamine-like psychomotor stimulants. Such differential activation could contribute to differences in behavioral responses to these drugs, because the striosomes and matrix have different input and output connections (Graybiel 1990). It is not clear how these effects of acute drug treatment relate to the effects of chronic drug exposure. Conceivably, these patterns could be related to the apparently differential toxicity induced in striosome- and matrix-innervating nigrostriatal fibers by continuous high-dose amphetamine (Ryan et al. 1988). It is also interesting that the Fos-like protein induction in the striatum that is elicited by acute psychostimulant exposure is influenced by concomitant morphine treatment and that this drug interaction is also reported to be compartmental (Fuxe et al. 1991). All these lines of evidence suggest that

striosome/matrix compartmentation should be considered in addition to regional subdivisions of the striatum in assessing the neural response to psychomotor stimulants.

The selectivity of these drug effects on the induction of Fos-like proteins extends to the cellular level (Berretta et al. 1991a, 1991b, in press). It has been shown by double immunostaining that amphetamine and cocaine both induce Fos-like proteins in striatal medium-sized neurons expressing the phosphatase inhibitor DARPP-32 (dopamine and cyclic AMP [cAMP]-regulated phosphoprotein) (Berretta et al. 1991a, 1991b, in press). In sharp contrast, neither amphetamine nor cocaine induces detectable Fos-like protein in the enkephalin-containing neurons in the caudatoputamen, even though many of these neurons coexpress DARPP-32. Nor does either stimulant induce Fos in striatal interneurons. These experimental findings establish that when Fos-like protein induction does occur in the striatum in response to psychomotor stimulants, the induction is only in a subset of the neurons present in the regions of activation. As discussed below, the subset of responsive neurons may have special functional characteristics that are related to the behavioral effects of the stimulants.

It seems highly likely that these cell-specific patterns of IEG induction by psychomotor stimulants are related to the differential expression of D1 -like and D2-like dopamine receptors by striatal neurons. The D1-selective dopamine receptor blocker SCH23390 blocks the induction of immunodetectable Fos-like protein by either amphetamine or cocaine (Graybiel et al. 1990; Young et al. 1991; Berretta et al., in press). However, this antagonist blocks 5-HT2 receptors as well as D1-like dopamine receptors so that a role for serotonin in the induction is not yet to be discounted. Control experiments designed to test for this have so far been negative (Graybiel et al. 1990). That the Fos-responsive neurons are immunoreactive for DARPP-32 agrees well with the apparent D1-like dopamine receptor selectivity of the induction, for DARPP-32 is considered to be a marker for cells expressing D1-like dopamine receptors (Walaas et al. 1983). The lack of induction in enkephalinergic striatal neurons also is reasonable because these neurons are known to express D2-like dopamine receptors (Le Moine et al. 1990) and are thought either not to express D1 -like receptors in large numbers (Gerfen et al. 1991) or to include a considerable subpopulation coexpressing D1-like and D2-like receptors (Meador-Woodruff et al. 1991). In the latter case, the D2-like dopamine receptors may block the adenylate cyclase signaling pathway activated by the D1-like receptor activation (Stoof and Kebabian 1981; Berretta et al., in press).

Neurons Expressing Fos-Like Protein Have a Special Functional Status in the Basal Ganglia

The known biochemical anatomy of the striatum suggests that most of the neurons expressing Fos in response to psychomotor stimulants are in the class of striatal output neurons projecting into the movement-releasing pathways of the basal ganglia-the neurons expressing substance P-like tachykinin and dynorphin. Current models of the basal ganglia suggest that when these neurons are activated, thalamocortical pathways are disinhibited, and hence, regions of premotor cortex become active (Albin et al. 1989; Alexander and Crutcher 1990; Graybiel 1991a). Substance P/dynorphin-containing striatal output pathways also lead to the substantia nigra pars reticulata, which in turn results in disinhibition of the superior colliculus. By contrast, the enkephalinergic neurons are striatal output neurons that project into the pathway leading to the subthalamic nucleus, which is thought to decrease movement release by the basal ganglia. Thus, these two sets of neurons-those exhibiting an acute Fos response to psychomotor stimulant exposure and those not doing so-are thought to have antagonistic roles in movement control. The responsive neurons are thought to facilitate the occurrence of movements, whereas the nonresponsive neurons are thought to damp movements. It is not yet known whether the IEG response is related to, or paralleled by, changes in the membrane potential of the responsive neurons. if the transcription factor activation is correlated with neural activation, however, results on induction of Fos-like protein (Graybiel 1991b; Berretta et al., in press) would suggest the following hypothesis: that some of the activating properties of psychomotor stimulants could stem from an imbalance between the activation of movement-releasing and movement-inhibiting pathways of the basal ganglia.

THE IEG RESPONSE ELICITED BY PSYCHOMOTOR STIMULANTS IS SELECTIVE TO SUBSETS OF IEGs

The induction of IEGs in striatal neurons following acute exposure to cocaine and amphetamine is not limited to expression of c-*fos,* but findings to date suggest that the response is nevertheless selective (Moratalla et al. 1990, 1991, submitted for publication; Cole et al. 1990). Judging from Northern blotting and in situ hybridization analysis, mRNAs corresponding to two of the IEGs of the *fos/jun* family so far tested, *c-fos* and jun-*B,* are strongly induced by cocaine and amphetamine. Although c-*jun* mRNA is often coordinately induced with *c-fos,* it is not induced by acute amphetamine or cocaine stimulation. Some other mRNAs of *fos/jun* genes are also unresponsive and are also not detectable by Northern blotting (Moratalla et al., submitted for publication). That other Fos-related antigens may be induced has been suggested by Young and

colleagues (1991). In addition to the *c-fos* and c-jun induction, there is strong induction of the zinc-finger gene NGFI-A *(zif/268, egr*-1) (Moratalla et al., in press). These differential patterns of gene induction are interesting because the DNA-binding proteins coded by these genes function as parts of dimers in conjunction with other binding proteins of the same transcription factor family, and these dimers in turn are parts of binding complexes that influence transcription. As a result, a wide range of control is in principle possible, depending on the particular genes that are coinduced and the levels and kinetics of their induction (Struhl 1991; Sheng and Greenberg 1990; He and Rosenfeld 1991; Morgan and Curran 1991).

ACTIVATION OF INTRACELLULAR SIGNALING PATHWAYS BY PSYCHOMOTOR STIMULANTS: CLUES TO LONG-TERM CHANGES IN NEURAL PROCESSING

The finding of selective patterns of IEG induction by psychomotor stimulants should give clues to the signaling pathways leading to the induction and, ultimately, to the consequences of the particular patterns found. For example, in PC12 cells it has been shown that membrane depolarization activates *c-fos* and *jun-B,* but not *c-jun,* and this pattern has been found in fibroblasts treated with cAMP analogs (Bartel et al. 1989; Mechta et al. 1989). The D1-like receptor selectivity of the psychomotor stimulant activation is probably responsible for the selective patterns of *fos/jun* induction that have been found (Moratalla et al., submitted for publication), because the *c-fos* promoter has a calcium-and-cAMP responsive element (Berkowitz et al. 1989; Sheng et al. 1988, 1990), and the *jun*-B promoter contains a novel repeat element that can be activated by protein kinase A. By contrast, protein kinase A may mediate an inhibition of c*-jun* directly or indirectly (de Groot et al. 1991; Chiu et al. 1989; Schutte et al. 1989; Ryseck and Bravo 1991). Signaling pathways leading from stimulation of adenylate cyclase and cAMP-dependent protein kinase A would then need to be considered as prime targets of the acute effects of psychomotor stimulant drugs. Nestler and colleagues find that molecules in the cAMP/protein kinase A pathway are regulated during prolonged exposure to cocaine and morphine (Guitart and Nestler 1989; Nestler et al. 1990; E. Nestler, personal communication, January 1992). The convergence of evidence for cAMP/protein kinase A mediation of responses to acute and long-term exposure to psychomotor stimulants fits well with the hypothesis that short-term induction of IEGs by these drugs of abuse could be implicated in the long-term responses that follow their repeated use (e.g., Graybiel et al. 1990; Young et al. 1991).

What would be the characteristics of such neural modifications? For first analysis, the problem can be broken down into three parts: the initial signaling paths leading to changes in IEG induction, transcription, and translation; the

effects of the DNA-binding proteins on transcription of other genes; and the downstream effects of such changes (for example, insertion of new receptor proteins into the ceil membrane or regulation of neurotransmitter expression). Some progress has been made in identifying the signaling pathways likely to be involved in the induction of IEGs by acute exposure to psychomotor stimulants, but there is little firm information about the effects of this induction. It is known that neurotransmitters and neuropeptides as well as receptors and messenger molecules undergo altered expression with prolonged exposure to dopamine agonists and antagonists (see Graybiel 1990; Grimes et al. 1990; Gerfen et al. 1991). Several neuropeptide genes have recently been shown to contain AP-1-like DNA binding motifs in the upstream regions of their promoters. Thus, the signaling pathways may be in place for direct effects of Fos-like transcription factors on neuropeptide expression. With transgenic mice it may be possible to test this possibility directly and also to test whether regulatory control is exerted through particular combinations of IEGs on expression of neurotransmitters, receptors, and messenger molecules that could bring about long-term changes in neural function.

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Functional Organization of the Striatum: Relevance to Actions of Psychostimulant Drugs of Abuse

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Recent advances in understanding the functional organization of the basal ganglia may provide some insight into the cellular and molecular action of certain drugs of abuse. For example, the psychomotor stimulant effects of cocaine (e.g., euphoria, locomotion, stereotypy) are thought to be mediated primarily by dopaminergic mechanisms in brain regions such as the striatum, including the nucleus accumbens (DeWit and Wise 1977; Pettit et al. 1984; Roberts et al. 1977). Dopaminergic afferents to the striatum are directed principally to the medium spiny neurons (Freund et al. 1984), which constitute 90 to 95 percent of the striatal neurons and give rise to the major striatal efferent pathways (Somogyi et al. 1981). On the basis of their axonal projections, striatal outputs are divided into two types: those that project to the globus pallidus and those that project to the entopeduncular nucleus/ substantia nigra complex (Kawaguchi et al. 1990). Neurons contributing to both pathways contain glutamic acid decarboxylase (Kita and Kitai 1988) and use γ-aminobutyric acid (GABA) as a transmitter (Chevalier et al. 1985), but each contains different neuropeptides. For example, the majority of striatonigral neurons contain dynorphin and substance P (Brownstein et al. 1977; Get-fen and Young 1988; Vincent et al. 1982), whereas the majority of striatopallidal neurons contain enkephalin (Haber and Nauta 1983). Pharmacological and lesion studies have demonstrated that dopamine differentially modulates the expression of these peptides as determined by changes in immunoreactivity (Hong et al. 1978, 1985; Li et al. 1987, 1988; Voorn et al. 1987) or mRNA levels (Bannon et al. 1986; Gerfen et al. 1991; Morris et al. 1988; Young et al. 1986). The contrasting effects of dopamine on peptide mRNA regulation appear to be dependent on the differential expression of the D1 and D2 dopamine receptors on striatonigral and striatopallidal neurons, respectively (Gerfen et al. 1990). Thus, dopamine-induced alterations in striatal peptide mRNA levels provide a paradigm to study receptor-mediated regulation of specific subtypes of striatal output neurons.

Since cocaine increases dopaminergic transmission in the striatum (Hurd and Ungerstedt 1989; Moore et al. 1977), the effects of this indirect agonist on the mRNA levels of the striatal peptides dynorphin, substance P, and enkephalin using in situ hybridization histochemistry (ISHH) (Hurd et al. 1992) were examined. A treatment schedule was used in which rats were given unlimited access to intravenous cocaine for a period of 7 days to examine effects that may be related to the abuse of this drug. In this study, the self-administration of cocaine at an average daily rate of 58.8 mg/day (approximately 200 mg/kg/day) resulted in significant regionally specific alterations in the levels of mRNA encoding the peptides dynorphin, substance P, and enkephalin in the striatum (table 1). Both dynorphin and substance P mRNA levels show substantial cocaine-induced increases in the dorsal striatum, whereas in the nucleus accumbens, these increases are relatively modest, particularly in the case of substance P (figure 1). Enkephalin mRNA is elevated only in the nucleus accumbens. Since dynorphin and substance P mRNA are expressed by most striatonigral neurons and enkephalin mRNA is expressed predominantly by striatopallidal neurons (Gerfen and Young 1988), cocaine self-administration differentially alters gene regulation in these two striatal output systems. The most pronounced effect of cocaine is an elevation of both dynorphin and substance P mRNA levels in the dorsal striatum, whereas enkephalin mRNA levels are not significantly altered in this region. Thus, in the dorsal striatum there is a difference in gene regulation in striatonigral and striatopallidal

TABLE 1. *Average dynorphin, substance P, and enkephalin ISHH labeling (measured in arbitrary optical density units) in the dorsal striatum and nucleus accumbens in control, cocaine self-administering animals and saline-infused animals that received restricted food to match the weight of cocaine-treated animals*

	Dynorphin ISHH		Substance P ISHH		Enkephalin ISHH	
Area	Control (Food Restricted)	Cocaine (Cocaine vs. Control)	Control (Food Restricted)	Cocaine (Cocaine vs. Control)	Control (Food Restricted)	Cocaine (Cocaine vs. Control)
Dorsal striatum	154.0±1.9 (154.9±2.3)	183.0±2.1 +29.0*	150.4±1.4 (150.2±2.1)	173.6±2.3 +23.2*	161.9±1.1 (164.6±1.4)	162.4±1.4 +0.5
Nucleus accumbens	176.2±2.5 (177.6±2.3)	192.0±1.0 +16.4*	152.4±1.0 (151.0±1.1)	160.92±0.6 +8.6*	159.9±0.9 (161.3±0.9)	164.3±1.1 +4.4*

*p>0.05

SOURCE: Hurd et al. 1992

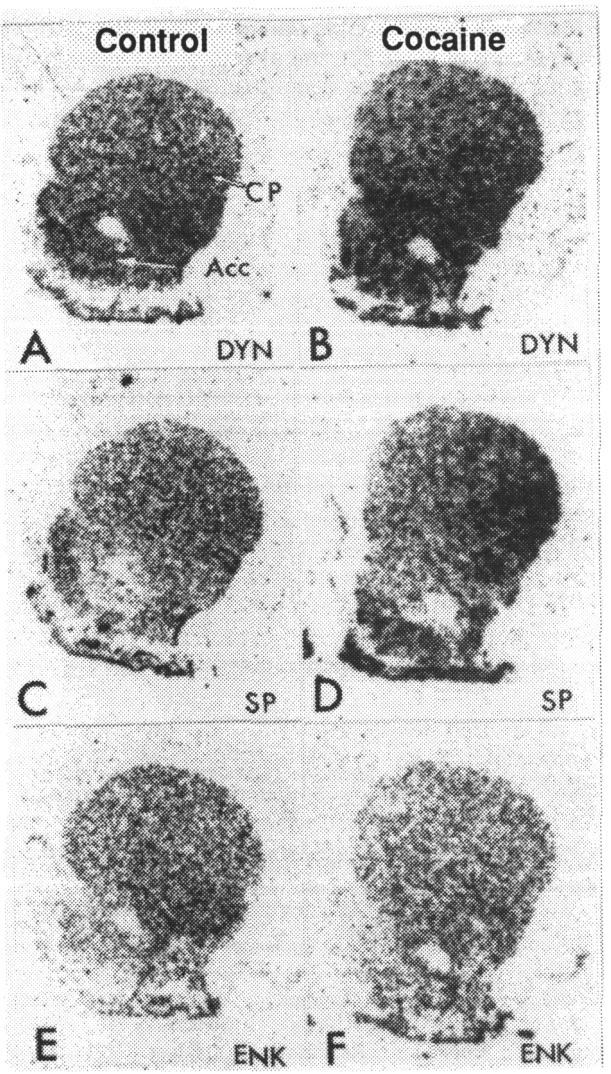

FIGURE 1. *Autoradiographic film images of (A, B) dynorphin (DYN), (C, D) substance P (SP), and (E, F) enkephalin (ENK) ISHH labeling in the striatum in a control (A, B, C) and cocaine self-administration animal (B, D, F). CP=caudate putamen, ACG=nucleus accumbens.*

SOURCE: Hurd et al. 1992

neurons, In the ventral striatum, which includes the nucleus accumbens, the cocaine-induced effects are more subtle and complex. Here dynorphin mRNA levels show the largest increase compared with the other peptide mRNAs, but this increase is considerably less pronounced than in the dorsal striatum. Both substance P and enkephalin mRNA levels show a slight, significant, cocaine-induced elevation in the nucleus accumbens. Ventral striatal neurons that express substance P project to the ventral pallidum (Haber and Nauta 1983); however, it has not been established whether these neurons belong to a set that coexpresses either enkephalin or dynorphin and that provides axon collaterals to both the ventral pallidum and substantia nigra. Nonetheless, the elevation in dynorphin, substance P, and enkephalin mRNA levels suggests that gene regulation is increased, albeit only modestly, in most output neurons in the nucleus accumbens during cocaine self-administration. This is in contrast to the dorsal striatum, where cocaine self-administration results in a rather selective and substantial increase in gene regulation in striatonigral neurons, thus generating a relative imbalance between striatonigral and striatopallidal neurons compared with the control condition.

As has been demonstrated with other pharmacologic manipulations of the striatal dopaminergic system, cocaine appears to alter the relative balance of function of striatopallidal and striatonigral neurons. A model for dopamine's functional effects in the dorsal striatum has been proposed that suggests that this neurotransmitter modulates the balance of activity in the striatopallidal and striatonigral output systems (Albin et al. 1989; Gerfen et al. 1990). The consequences of altering the balance in these output pathways are related to the ultimate effects on the activity of GABAergic neurons in the entopeduncular nucleus and substantia nigra. Increased striatopallidal activity results in an increase in the activity of nigral GABAergic neurons by way of the intervening subthalamic nucleus (Kita and Kitai 1987). Conversely, increased striatonigral activity directly inhibits nigral GABAergic neurons (Chevalier et al. 1985). The behavioral consequences of opposed modulation of GABAergic nigral neurons are generally modeled on the concept that the tonic inhibitory output of these neurons is interrupted by inhibitory inputs to these neurons from the striatum during movements (Chevalier et al. 1985; Deniau and Chevalier 1985). This disinhibitory process has been best characterized for eye movements by Hikosaka and Wurtz (1983a, 1983b, 1983c, 1983d) who, in a series of elegant studies, showed that visual-, memory-, and reward-contingent eye movements are correlated with pauses in the tonic activity of nigral GABAergic neurons and increased activity in superior colliculus neurons. Akinesia that accompanies Parkinson's disease has been related to increased striatopallidal activity and the resultant increase in excitatory subthalamic inputs to nigral GABAergic neurons, which then predominates over the disinhibitory mechanisms required

for the generation of movements (Albin et al. 1989; Mitchell et al. 1989). This model is supported by the report that lesions of the subthalamic nucleus reverse akinesia in monkeys made Parkinsonian with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced lesions (Bergman et al. 1990).

Following cocaine self-administration, there appears to be an increase in the relative function of striatonigral vs. striatopallidal output systems in the dorsal striatum, which, according to the above model, would result in a facilitation of movement-related behavior. Whether such alterations are directly or indirectly related to the effects of cocaine is unclear. A model that relates alterations in the relative activity of ventral striatal output systems to specific behavioral responses is yet to be developed. However, the present results suggest that although cocaine self-administration may not produce as great an imbalance in the output systems of the nucleus accumbens, the fact that both systems show elevated gene regulation indicates that normal activity in this striatal subregion is also altered by cocaine.

Although it has been proposed that the differential effects of dopamine on striatonigral and striatopallidal neurons are mediated by the specific expression of D1 and D2 dopamine receptors by these two types of output neurons, respectively (Gerfen et al. 1990), the cocaine-induced effects reported here do not necessarily indicate a predominant D1 receptor-mediated response. In a previous study, peptide mRNA levels in striatonigral and striatopallidal neurons were shown to be specifically altered by D1- or D2-selective agonists, respectively (Gerfen et al. 1990). However, those drug treatments were given to animals with lesions of the nigrostriatal dopamine pathway and thus examined the consequence of activation mediated by one receptor type in the absence of the other. In animals self-administering cocaine, both types of dopamine receptors would presumably be activated simultaneously. In this circumstance, there is considerable evidence that there are complex synergistic interactions between D1 and D2 dopamine receptors (Carlson et al. 1985; Clark and White 1987; Walters et al. 1987; Wieck and Walters 1987). One such interaction is the synergistic effect of D1 and D2 dopamine receptors, which has been well documented (Carlson et al. 1985; Clark and White 1987; Walters et al. 1987). The cellular basis of interactions between the dopamine receptor subtypes has not been established, but at least three likely mechanisms may be postulated. First, although the majority of striatal projection neurons express only one of the D1 and D2 receptor subtypes, there may be a subset of neurons that express both. Second, there may be interactions between neurons expressing different receptor subtypes through local axon collaterals. Third, striatal interneurons may mediate interactions between D1- and D2-expressing neurons. An example of this type of interaction may involve acetylcholine modulation of D2-containing striatopallidal

neurons, acetylcholine release being induced by substance P (Arenas et al. 1991; Petitet et al. 1991) released from D1-containing striatonigral neurons and acting on substance P receptors that are expressed by cholinergic neurons (Gerfen 1991). These possible mechanisms mediating interactions among striatal neurons are not exhaustive. Thus, the direct action of dopamine on D1- and D2-containing striatonigral and striatopallidal neurons should be considered an important, but not the only, mechanism by which dopamine modulates the balance in the activity of these two striatal output pathways.

Changes in levels of mRNA-encoding peptides in striatopallidal and striatonigral neurons provide an instructive assay of functional changes induced by pharmacologic manipulation of the striatal dopamine system. However, changes in peptide mRNA levels often take several days of repeated treatments to develop to significant levels. The induction of transcription factors such as *c-fos* in striatal neurons following a single pharmacologic treatment provide an assay that supplies information concerning the acute actions of dopamine receptor activation. Such studies have provided data that are consistent with data obtained by using peptide mRNA assays in identifying D1 receptor activation as specifically targeting striatonigral neurons (Robertson et al. 1990). The use of c-fos induction has also proved instructive in studying the acute actions of cocaine and amphetamine on striatal neurons (Graybiel et al. 1990). This approach, when combined with identification of the neurons in which the transcription factors are induced, promises to provide significant new information about the cellular and molecular actions of cocaine and other psychostimulant drugs.

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Regulation of Neural Gene Expression in Opiate and Cocaine Addiction

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INTRODUCTION

Although some aspects of drug addiction can occur relatively rapidly in response to acute administration of a drug of abuse, most changes in brain function associated with addiction occur gradually over time in response to prolonged drug exposure. These gradually developing changes can persist for days, sometimes much longer, after cessation of chronic drug administration. The prominence of such delayed, progressively developing, and persistent adaptations in brain function during drug addiction suggests that long-term changes in neuronal gene expression are likely to be important in mediating addictive phenomena.

Mechanisms Regulating Gene Expression

It is increasingly apparent that the processes controlling the expression of neural genes, even in the fully differentiated adult brain, are extraordinarily complex. However, in recent years, much has been learned about the molecular mechanisms that control the expression of specific genes in the central nervous system (CNS). Attention has focused primarily on a class of DNA-binding proteins, termed "transcription factors," that bind to specific sequences of DNA (response elements) in the regulatory regions of certain genes and thereby increase or decrease the rate at which those genes are transcribed (for review, see Mitchell and Tjian 1989; Goodman 1990; Montminy et al. 1990; Morgan and Curran 1991). The mechanism for this transcriptional activation/repression is not fully understood but likely involves direct or indirect interactions of transcription factors with the RNA polymerase II transcriptional complex (Ptashne and Gann 1990).

A large and increasing number of transcription factors are known, and these exhibit distinct structural, functional, and regulatory properties. A list of some prominent classes of transcription factors in the CNS is shown in table 1. Based on the few neural genes that have been characterized to date, it would

appear that many, and perhaps all, genes contain multiple response elements such that the rate of expression of a given gene at any given point in time is dependent on the integrated effects of many transcription factors.

Transcription factors contain a DNA-binding domain (e.g., zinc finger or homeodomain) that is rich in positively charged amino acids. Many transcription factors, such as the Fos/Jun family, also contain an additional structural motif (e.g., LZ) that enables them to dimerize and thereby form an active DNA-binding protein complex. In fact, different members of the Fos/Jun family have been shown to dimerize with each other, and such heterodimers

TABLE 1. *Examples of transcription factors and their DNA-binding sites*

Transcription Factor	Binding Site	Response Element	Structural Motif
CREB family	CRE	TGACGTCA	LZ
Fos/Jun family	AP-1	TGACTCA	LZ
Zif/268	GC rich	?	ZF
SP1	GC box	GGGCGG	ZF
Steroid receptor family (e.g., GR)	GRE	GGTACAN^{3}TGTTCT	ZF
POU family (e.g., OCT, Plt)	POU	ATTTGCAT	HD
AP-2 (protein unidentified)	AP-2	CCCCAGGC	?
SRF	SRE	GATGTCCATA-TTAGGACATC	?
HSF	HSE	?	?

KEY: LZ=leucine zipper; ZF=zinc finger: HD=homeodomain; GR=glucocorticoid receptor; SRF=serum response factor; HSF=heat shock factor; GC=guanosine and cytosine; GRE=glucocorticoid response element; SRE=serum response element; HSE=heat shock response element

SOURCE: Adapted from Mitchell and Tjlan 1989; Goodman 1990; Morgan and Curran 1991

appear to possess different DNA-binding properties, transcriptional activities, stability, etc. (Morgan and Curran 1991; Ryseck and Bravo 1991). Such formation of heterodimers has significant consequences as it dramatically increases the number of functional transcriptional complexes that can be formed from a finite number of factors. In addition, transcription factors contain an activation domain, which is thought to be the region of the proteins responsible for regulation of transcription (Ptashne and Gann 1990). Each class of transcription factor has been shown to bind to a particular DNA response element. However, these DNA-binding sites, such as those shown in table 1, represent only "consensus sequences" in that the actual sites to which the factors bind in different genes can vary. For example, functional cyclic AMP response elements (CREs) in various neural genes are not all "TGACGTCA"; in fact, only the last five nucleotides appear to be constant among all known CREs (Goodman 1990; Montminy et al. 1990). In addition, it can be seen from table 1 that certain response elements share considerable homology; for example, the CRE and activator protein-1 (AP-1) sites. These observations raise the possibility that the specificity of the cyclic AMP (cAMP) response element binding protein (CREB) and Fos/Jun families for CREs and AP-1 sites, respectively, is not absolute, with Fos/Jun potentially able to bind CRE sites and vice versa. It has even been reported that members of the Fos/Jun and CREB families, both of which contain the LZ motif, can cross-dimerize and thereby form heterodimers with unique DNA-binding and regulatory properties (Hai and Curran 1991). These examples of cross-reactivity between different classes of transcription factors illustrate the enormous complexity of possible mechanisms underlying the regulation of neural gene expression.

Most forms of transcription factors are highly regulated, and it is through such regulatory processes that important aspects of neural plasticity (including drug addiction) would appear to be achieved. Four mechanisms appear to be most prominent. First, the total amounts of certain transcription factors can be dramatically altered via changes in their own transcription. This is the case for the Fos/Jun family; the expression of these proteins is regulated in the adult CNS in response to diverse types of stimuli (Morgan and Curran 1991). Second, the ability of transcription factors to regulate gene expression can be altered via their phosphorylation by diverse types of protein kinases. The prototypical example for this is CREB, whose ability to regulate transcription is controlled primarily through its phosphotylation by cAMP-dependent or calcium-dependent protein kinases (Goodman 1990; Montminy et al. 1990; Sheng et al. 1990, 1991; Van Nguyen et al. 1990). It appears that CREB phosphorylation does not dramatically alter its DNA-binding activity but instead increases the ability of CREB, bound to DNA, to activate the RNA polymerase II transcriptional complex. Third, the ability of transcription factors to bind DNA

can be regulated by their binding to other molecules. An example of this would be inhibitory variants of transcription factors that do not bind DNA but rather dimerize with the active, DNA-binding forms of the protein and thereby inhibit their transcriptional activity. Such variant forms appear to exist for most transcription factors, including Fos/Jun and CREB (Yen et al. 1991; Foulkes et al. 1991). Fourth, the levels of transcription factors in the nucleus can be regulated by altering the rate at which they are translocated from the cytoplasm (where they are synthesized) to the nucleus. The prototypical example of this are the steroid hormone receptors, which are normally cytoplasmic proteins that, on binding their specific hormone, translocate into the nucleus where they act as transcription factors (Evans and Arriza 1989). These four mechanisms are not mutually exclusive; most transcription factors are probably regulated by more than one of them. This is illustrated by the steroid hormone receptors, which are regulated not only by hormone binding and nuclear translocation but also by their phosphorylation by several types of protein kinases and by regulation of their total amount (e.g., Evans and Arriza 1989; Hoeck et al. 1989; Denner et al. 1990).

A Strategy To Identify Changes In Gene Expression That Underlie Drug Addiction

The complexity of genetic regulatory mechanisms suggests that the complete identification of the precise steps by which drugs of abuse alter gene expression in the brain to produce addiction is an extremely difficult task. In an attempt to overcome these obstacles, the authors' laboratory has followed a particular strategy to begin to understand this process, This strategy, summarized in the list below, involves the study of anatomically well-defined, discrete brain regions where behavioral pharmacological studies have indicated an important role in addictive behaviors and where electrophysiological studies have identified some functional changes in specific neurons that underlie the addictive behaviors. In this context, researchers at the authors' laboratory have set out first to identify the specific proteins whose altered expression may underlie the functional changes observed electrophysiologically. These target proteins are then used to study the genetic mechanisms underlying their altered expression. The advantage of this strategy is that it focuses biochemical and molecular studies on phenomena that are directly relevant to drug addiction. The strategy for establishing the role of transcription factors in drug addiction is as follows:

1. Identify proteins whose altered levels of expression contribute to functional aspects of drug addiction in identified neuronal cell types.

2. Study drug regulation of candidate transcription factors that could be involved.

3. Provide direct evidence for a causal role of a specific transcription factor by transfection or viral infection studies.

Together, these studies establish the precise molecular steps through which a drug of abuse alters the expression of some target protein and thereby produces addictive changes in the CNS.

IDENTIFICATION OF BIOCHEMICAL ADAPTATIONS INVOLVED IN DRUG ADDICTION

Studies in the Locus Coeruleus

The locus coeruleus (LC) is the major noradrenergic nucleus in brain, located on the floor of the fourth ventricle in the rostral pons. The electrophysiological effects of opiates in the LC are well established. Acutely, opiates inhibit LC neurons via the activation of a K^+ channel and the inhibition of a slowly depolarizing Na^+ channel (Aghajanian and Wang 1987; North et al. 1987). Both actions are mediated via pertussis toxin-sensitive G proteins (G_1 and/or G_o (Aghajanian and Wang 1986; North et al. 1987), and regulation of the Na_+ channel is mediated by reduced levels of neuronal cAMP and of activated cAMP-dependent protein kinase (Wang and Aghajanian 1987, 1990; Alreja and Aghajanian 1991). Chronically, LC neurons develop tolerance to these acute inhibitory actions as neuronal firing rates recover toward control levels (Aghajanian 1978; Christie et al. 1987). The neurons also become dependent on opiates after chronic exposure in that abrupt cessation of opiate treatment, such as through administration of an opiate receptor antagonist, leads to a severalfold elevation in LC firing rates above control levels in vivo (Aghajanian 1978; Rasmussen et al. 1990). A variety of pharmacological and behavioral studies have indicated that such regulation of LC neuronal excitability contributes to physical aspects of opiate addiction, namely, physical dependence and withdrawal (Rasmussen et al. 1990).

The tolerance and dependence that occur in LC neurons during chronic opiate exposure cannot be explained by changes in opiate receptors (Christie et al. 1987) and this led our laboratory several years ago to consider the possibility that intracellular messenger pathways may be involved. Since that time, it has been demonstrated that chronic administration of opiates leads to a dramatic up-regulation of the cAMP system at every major step between receptor and physiological response. Chronic opiates increase levels of $G_{i\alpha}$ and $G_{o\alpha}$ (Nestler et al. 1989), adenylate cyclase (Duman et al. 1986), cAMP-dependent protein kinase (Nestler and Tallman 1988), and several phosphoprotein substrates for the protein kinase (Guitar-t and Nestler 1989, 1990), including tyrosine hydroxylase (Guitart et al. 1990), the rate-limiting enzyme in the biosynthesis

of catecholamine neurotransmitters. These adaptations require chronic exposure to the opiates and are blocked by concomitant adminstration of an opiate receptor antagonist, indicating that persistent activation of opiate receptors is involved.

The up-regulation of adenylate cyclase and cAMP-dependent protein kinase can be viewed as a compensatory, homeostatic response of LC neurons to persistent opiate inhibition of the cells (figure 1) (Nestler and Tallman 1988). According to this view, up-regulation of the cAMP system increases the intrinsic excitability of LC neurons and thereby accounts, at least in part, for opiate tolerance, dependence, and withdrawal. That is, in the opiate-dependent state, the combined presence of the opiate and the up-regulated cAMP system would return LC firing rates to control levels, whereas removal of the opiates would leave the up-regulated AMP system unopposed, which would lead to withdrawal activation of the neurons, Several lines of evidence support this scheme.

First, cAMP and agents that elevate cAMP levels excite LC neurons via the activation of cAMP-dependent protein kinase and the subsequent activation of the slowly depolarizing Na+ channel (Wang and Aghajanian 1990). In fact, the basal firing rate of LC neurons appears to be entirely dependent on the activity of the cAMP system and of this channel (Alreja and Aghajanian 1991). Second, the time course by which certain components of the up-regulated cAMP system revert to normal during opiate withdrawal parallels the time course by which withdrawal activation of LC neurons and various behavioral signs of withdrawal recover (Rasmussen et al. 1990). Third, recordings from LC neurons in brain slices in vitro, where most synaptic connections of the neurons have been severed, have indicated that LC neurons from dependent animals exhibit basal firing rates more than twofold greater than those from control animals (Kogan et al. 1992). This finding establishes that intrinsic mechanisms contribute to opiate dependence in these cells. Moreover, LC neurons from dependent animals show a dramatically greater maximal responsiveness to cAMP analogs, providing functional evidence for an up-regulated cAMP system during dependence (Kogan et al. 1992). Taken together, these findings demonstrate that up-regulation of the cAMP system represents one mechanism by which opiates induce addiction in these neurons.

Opiate-induced up-regulation of the cAMP system in the LC occurs at both the level of protein and messenger RNA (Nestler et al. 1989; Guitart et al. 1990; Nestler 1992), consistent with the possibility that the changes arise through the regulation of gene expression. As described below, opiate regulation of these proteins offers a model system in which to study the mechanisms by which opiates alter gene expression and produce addictive changes in target neurons.

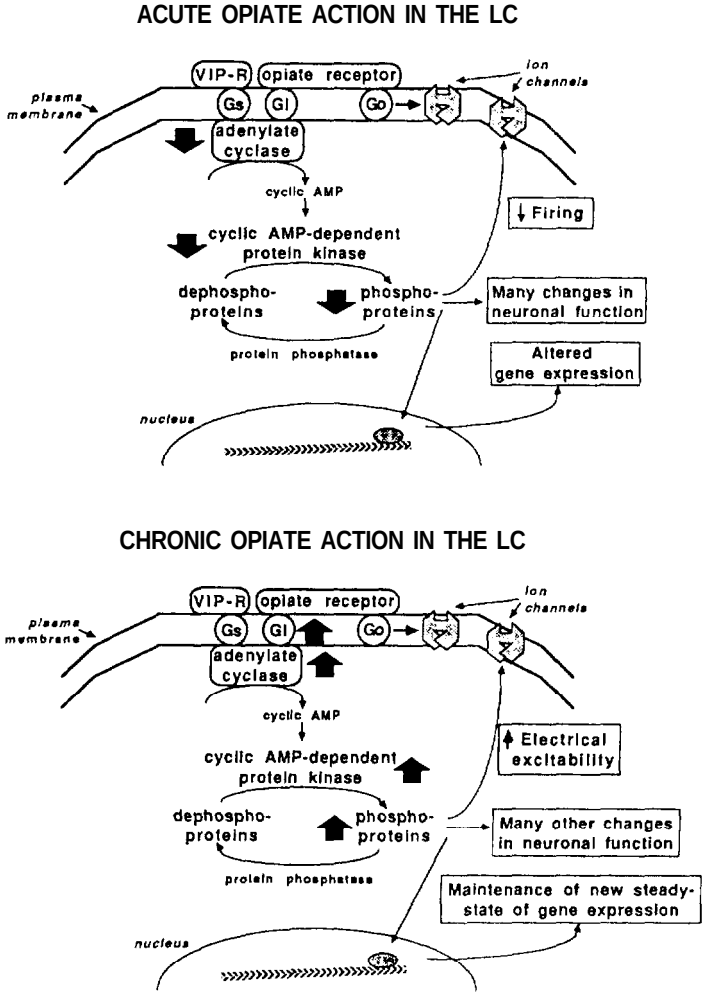

FIGURE 1. *Schematic illustration of the mechanisms of acute and chronic opiate action in the LC. See text for discussion.*

VIP-R=vasoactive intestinal polypeptide receptor

SOURCE: Modified from Nestler 1992. Copyright 1992 by Society for Neuroscience (New York).

A General Role for G Proteins and the cAMP System in Opiate Addiction

Alterations in levels of G proteins and the cAMP system may represent a general mechanism by which certain types of neurons within the CNS respond to opiates and develop addiction (Terwilliger et al. 1991a). Thus, chronic morphine has been shown to increase levels of (1) adenylate cyclase and cAMP-dependent protein kinase in dorsal root ganglion/spinal cord cocultures, amygdala, and nucleus accumbens and of (2) the protein kinase alone in the thalamus (Makman et al. 1988; Terwilliger et al. 1991a). As in the LC, up-regulation of the cAMP system in dorsal root ganglion/spinal cord cocultures could account for some of the electrophysiological changes associated with opiate tolerance, dependence, and withdrawal in these neurons (Crain and Shen 1990).

Opiate regulation of G proteins is more regionally variable, with increased levels of $G_{i\alpha}$ and/or $G_{o\alpha}$ observed in the amygdala (as seen in the LC) and decreased levels observed in dorsal root ganglion/spinal cord cocultures and nucleus accumbens (Attali and Vogel 1989; Terwilliger et al. 1991a). Whether levels of these G proteins increase or decrease in response to chronic morphine may be related to the development of homologous vs. heterologous forms of desensitization, respectively, in the specific neuronal cell types (Terwilliger et al. 1991a).

Studies of Morphine and Cocaine Action in the Mesolimbic Dopamine System

The nucleus accumbens (NAc), together with dopaminergic neurons in the ventral tegmental area (VTA) that innervate the NAc, is implicated in mediating psychological aspects of addiction, namely, drug reinforcement and craving, for opiates and many other drugs of abuse (Wise and Bozarth 1987; Koob and Bloom 1988; Clouet et al. 1988). Thus, it was of particular interest to test whether chronic cocaine might produce changes similar to morphine in G proteins and the cAMP system in the VTA-NAc pathway. Indeed, chronic cocaine was found to decrease levels of $G_{i\alpha}$ and $G_{o\alpha}$ (Nestler et al. 1990) and to increase levels of adenylate cyclase and cAMP-dependent protein kinase in the NAc (Terwilliger et al. 1991 a). Morphine and cocaine regulation of these intracellular messenger proteins, summarized in figure 2, was not observed in the other major dopaminergic system in the brain, the nigrostriatal system; nor was such regulation seen in response to other classes of psychotropic drugs that lack reinforcing properties. These biochemical actions of cocaine could account for the supersensitivity of D1-dopamine receptor function observed in this brain region in response to chronic cocaine in electrophysiological studies (Henry et al. 1989; Henry and White 1991). Although the electrophysiological

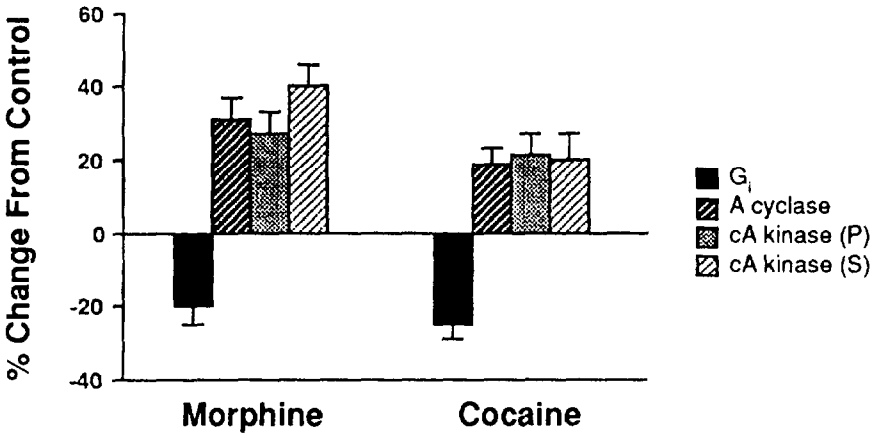

FIGURE 2. *Common adaptations in G proteins and the cAMP system in the NAc in response to chronic morphine and chronic cocaine. G_1 immunoreactivity of $G_{i\alpha}$ subunit; A cyclase, adenylate cyclase activity; cA kinase, cAMP-dependent protein kinase activity in particulate (P) and soluble (S) fractions. Data represent means±SEM (n=8 to 74). All changes shown in the figure are statistical/y significant by χ^2 test.*

SOURCE: Data from Terwilliger et al. 1991a

effects of chronic morphine action on NAc neurons have not yet been studied, similar effects compared with chronic cocaine would be predicted based on the biochemical findings.

Morphine and cocaine were also shown to exert common chronic actions on several phosphoproteins in the VTA-NAc pathway, including increased levels of tyrosine hydroxylase in the VTA, lower levels of active tyrosine hydroxylase in the NAc, and lower levels of several forms of neurofilament proteins in the VTA (Beitner-Johnson and Nestler 1991; Beitner-Johnson et al., in press). Regulation of these morphine- and cocaine-regulated phosphoproteins (MCRPP), like the changes in the G proteins and the cAMP system, showed regional and pharmacological specificity.

In addition to their regional and pharmacological specificity, further evidence to support the idea that morphine and cocaine regulation of G proteins, adenylate cyclase, cAMP-dependent protein kinase, and the various MCRPPs is related to drug reward mechanisms includes the findings that differences in these

intracellular messenger proteins exist in the VTA-NAc pathway of Lewis and Fischer 344 rats. Lewis rats self-administer opiates, cocaine, and alcohol at much higher rates than Fischer rats (George and Goldberg 1989; Suzuki et al. 1988) and also develop greater degrees of conditioned place preference to morphine and to cocaine (Terwilliger et al. 1991b), Interestingly, the NAc of Lewis vs. Fischer rats contains lower levels of G,, higher levels of adenylate cyclase and cAMP-dependent protein kinase, and lower levels of tyrosine hydroxylase, whereas the VTA of Lewis vs. Fischer rats contains higher levels of tyrosine hydroxylase and lower levels of neurofilaments (Beitner-Johnson et al. 1991; Guitart et al. 1991; Terwilliger et al. 1991b), In each case, levels of these specific intracellular signaling proteins in the VTA-NAc pathway of Lewis rats, compared with Fischer rats, resemble morphine- and cocaine-induced changes in these proteins in outbred Sprague-Dawley rats.

The common chronic actions of morphine and cocaine in the mesolimbic dopamine system are particularly striking since the two drugs exert opposite electrophysiological effects on VTA neurons acutely (Henry et al. 1989), yet both are clearly addicting. Therefore, the common chronic actions could represent a general mechanism by which these drugs of abuse exert some of their long-term reinforcing actions in this neural pathway. The findings in Lewis and Fischer rats raise the additional, exciting possibility that similar mechanisms may contribute to individual genetic vulnerability to drug addiction (Nestler, in press).

GENETIC MECHANISMS UNDERLYING BIOCHEMICAL ADAPTATIONS IN THE cAMP SYSTEM

General Scheme

Based on the knowledge of opiate and cocaine action in specific brain regions and of the mechanisms underlying the regulation of gene expression, one can formulate a working scheme to explain how these drugs of abuse might alter the expression of genes for specific target proteins in particular neuronal cell types. Two possible regulatory mechanisms involving the CREB and Fos/Jun families of transcription factors are shown in figure 3. These two mechanisms are not mutually exclusive, nor are they the only types of mechanisms that could be involved, because any of the transcription factors, *or* a combination of factors, shown in table 1 could conceivably play a role. However, evidence for neurotransmitter regulation of transcription factors is best established for the CREB and Fos/Jun families. Moreover, several of the known target genes for opiates and cocaine (e.g., cAMP-dependent protein kinase, tyrosine hydroxylase, neurofilaments) are known to contain cAMP and/or AP-1 response elements (Cambi et al. 1989; Reeben et al. 1991; S. McKnight, personal communication, September 1990).

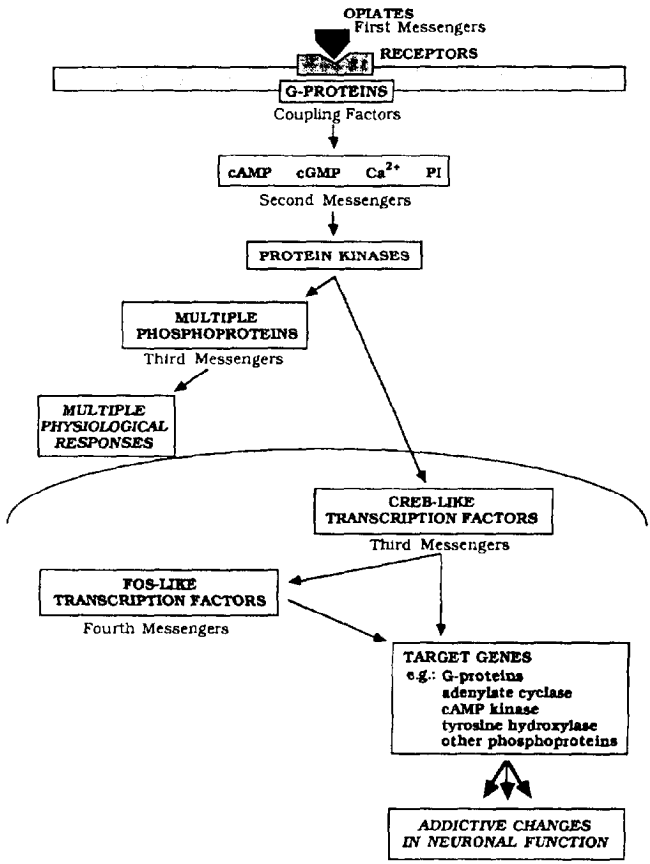

FIGURE 3. *Schematic illustration of the intracellular messenger pathways through which diverse extracellular signals, including drugs of abuse such as opiates, could regulate gene expression in target neurons. The figure illustrates two general types of mechanisms that could be involved. In one mechanism, opiates, through the regulation of second messengers and protein kinases, regulate the phosphorylation of a CREB-like transcription factor. This alters the transcriptional activity of CREB and leads to altered expression of specific target proteins. In the second mechanism, regulation of CREB phosphorylation leads to altered expression of a fos-like transcription factor. Altered levels of fos, in turn, lead to altered expression of specific target proteins.*

SOURCE: Modified from Nestler 1992. Copyright 1992 by the Society for Neuroscience (New York).

As discussed in the introduction, CREB proteins are generally thought to be constitutively expressed in neurons, with their transcriptional activity regulated primarily via their phosphorylation by cAMP- or calcium-dependent protein kinases. According to the scheme shown In figure 3, opiates in the LC, via their initial effects on the cAMP pathway (via inhibition of adenylate cyclase) (Duman et al. 1988) or calcium pathway (via inhibition of neuronal firing) (Aghajanian 1978; Christie et al. 1987) could lead to decreases in the phosphorylation state of CREB and to subsequent changes in gene expression. Because this mechanism involves the regulation of a transcription factor that is already present in the neurons, it could begin to occur very rapidly (i.e., within minutes). The specificity for opiate action in this scheme would reside in (1) the presence of opiate receptors, (2) the ability of the activated receptors to regulate the cAMP and calcium pathways, and (3) the particular genes accessible to CREB, in a given neuronal cell type.

In contrast to CREB, the Fos/Jun family of transcription factors tends to be expressed at low levels in neurons under basal conditions. In response to diverse types of extracellular signals, high levels of expression of these transcription factors are induced via increases in rates of transcription and mRNA translation (for review, see Morgan and Curran 1991). It appears that phosphorylation of CREB, or a CREB-like protein, by cAMP- or calcium-dependent protein kinases mediates the effects of such extracellular signals on Fos expression (Sheng et al. 1990); similar mechanisms may underlie the regulation of the other transcription factors in the Fos/Jun family. Thus, regulation of Fos and Fos-like transcription factors represents an additional mechanism by which opiates could regulate the expression of other genes via alterations in the cAMP or calcium systems (figure 3). Because this mechanism requires protein synthesis, it is slower than the first mechanism described above, with earliest changes in Fos/Jun target gene expression expected perhaps 30 to 60 minutes following the initial stimulus.

The two regulatory schemes presented in figure 3 represent a first attempt to explain the actions of opiates on gene expression in the LC and other target neurons. As more is learned about the regulation of the CREB, Fos/Jun, and other families of transcription factors, more complete hypotheses can be formulated to account for the actions of opiates, cocaine, and other drugs of abuse on neural gene expression.

Regulation of Specific Transcription Factors by Drugs of Abuse

Regulation by Opiates. As an initial attempt to study the transcription factors that mediate opiate action in the LC, Hayward and colleagues (1990) studied regulation of Fos expression in response to acute and chronic opiate

administration and during opiate withdrawal. It was found that acute opiates decrease levels of Fos mRNA and protein in this brain region. This reduced level of Fos expression persists with chronic opiate treatment, whereas precipitation of opiate withdrawal by an opiate receptor antagonist produces a dramatic induction of Fos at the mRNA and protein levels (figure 4). The observed changes in Fos levels are consistent with the possibility that increased levels of expression of $G_{i\alpha}/G_{o\alpha}$ cAMP-dependent protein kinase, and tyrosine hydroxylase in the LC during chronic opiate treatment could be mediated to some extent via decreased levels of Fos and that the return of levels of expression of these intracellular messenger proteins toward control levels during withdrawal could be achieved as a result of elevated Fos expression. Induction of Jun mRNA was also observed during opiate withdrawal (Hayward et al. 1990), indicating that several members of the Fos/Jun family could be involved in these processes.

The induction of Fos and Jun during opiate withdrawal was observed in several brain regions in addition to the LC, including the NAc, VTA, neostriatum, and frontal cortex, but not in other regions, notably hippocampus and whole pons (figure 5) (Hayward et al. 1990). Regulation of Fos expression in the NAc and VTA indicates that this class of transcription factor could contribute to opiate actions on the G proteins and the cAMP system in the mesolimbic dopamine system via a mechanism similar to the one suggested above for the LC.

What does induction of Fos during withdrawal reflect? It has been suggested that induction of Fos can be used as a molecular measure of neural activity (Morgan and Curran 1991), and indeed, depolarizing stimuli would be expected to induce Fos via activation of the calcium pathway. However, such a relationship between Fos expression and neural activity is not absolute, since Fos can be induced by a cAMP signal (Sheng et al. 1990; Morgan and Curran 1991) and cAMP is known to exert negative effects on the firing rate of particular neurons (see below for cocaine). Thus, a rigorous interpretation of these data is that induction of Fos can be used to map those neurons in the brain in which the intracellular cAMP and/or calcium systems become activated during opiate withdrawal or other drug treatments.

The recent development of a back phosphorylation and immunoprecipitation procedure has made it possible to study morphine regulation of CREB phosphorylation in the rat LC (Guitart et al. 1992). This procedure results in the specific immunoprecipitation of a 43-kD phosphoprotein that represents CREB: The protein comigrates with purified, phosphorylated CREB on one- and two-dimensional gel electrophoresis and yields identical phosphopeptide maps as does the purified protein. By use of this procedure, it has been demonstrated that acute morphine administration decreases the phosphorylation state of

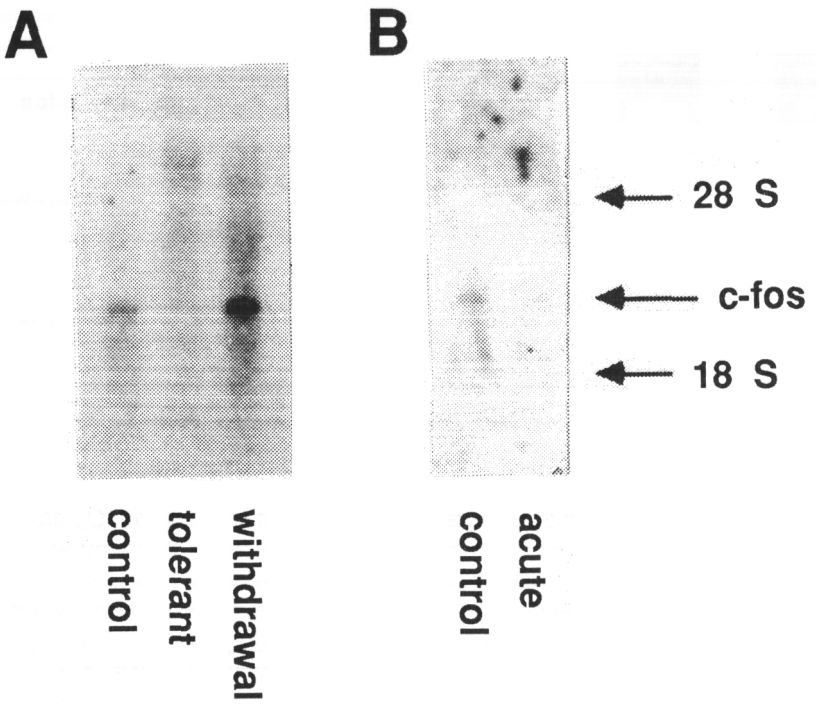

FIGURE 4. *Autoradiograms showing regulation of Fos mRNA in the LC in response to acute and chronic morphine treatment and opiate withdrawal. A: Control, subcutaneous (SC) administration of normal saline to control rats; tolerant, SC administration of saline to morphine-tolerant rats; and withdrawal, SC administration of naltrexone to morphine-tolerant rats. Animals were sacrificed 1 hour after the injections. B: Control, SC administration of normal saline to control rats and acute, SC administration of morphine to control rats. Animals were sacrificed 90 minutes after the injections, and levels of Fos mRNA were analyzed (using 5 mg of total RNA) by Northern blotting.*

SOURCE: Hayward et al. 1990. Copyright 1990 by Elsevier Science Publishers (Amsterdam).

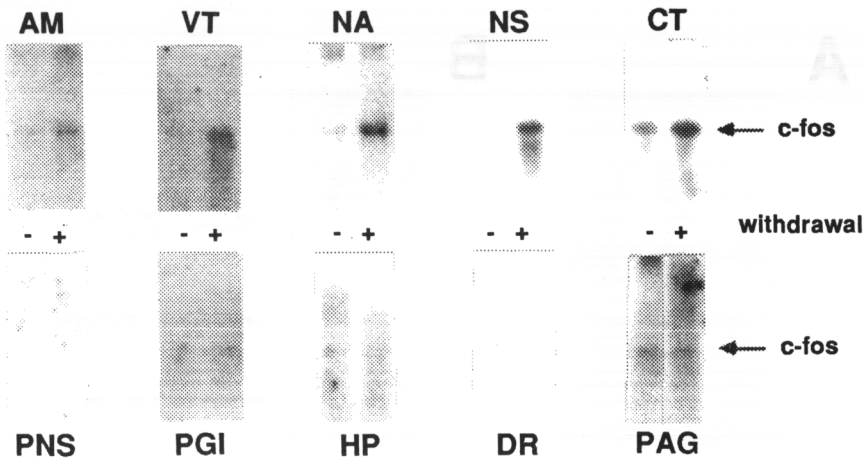

FIGURE 5. *Autoradiograms showing regulation of Fos mRNA in various regions of rat brain in response to opiate withdrawal. Opiate withdrawal was precipitated in morphine-tolerant rats by SC administration of naltrexone. Animals were sacrificed 1 hour after the initiation of withdrawal, and levels of Fos mRNA were analyzed (using 5 to 10 mg of total RNA) by Northern blotting. AM, amygdala; VT, ventral tegmental area; NA, nucleus accumbens; NS, neostriatum (caudate/putamen); CT whole cerebral cortex; PNS, whole cross-sections of anterior pons from which LC nuclei had been excised; PGI, nucleus paragigantocellularis; HP, hippocampus; DR, dorsal raphe; PAG, periaqueductal gray.*

SOURCE: Hayward et al. 1990. Copyright 1990 by Elsevier Science Publishers (Amsterdam).

CREB in the LC, an effect that diminishes after chronic exposure to the opiate. In contrast, precipitation of opiate withdrawal increases CREB phosphorylation in this brain region (Guitart et al. 1992). This regulation of CREB phosphorylation is consistent with the known effects of acute and chronic opiates and opiate withdrawal on the activity of the cAMP system in the LC.

These studies of opiate regulation of transcription factors, although preliminary, highlight the utility of the LC as a model system in which to study transcription factor regulation. The LC is a system in which specific candidate target genes have been identified and in which changes in those genes have been shown to

be physiologically important. Through increasingly mechanistic studies of this system, it will be possible to delineate the precise molecular steps by which opiates regulate the expression of specific intracellular messenger proteins and, as a result, induce aspects of tolerance, dependence, and withdrawal in these neurons.

Regulation by Cocaine. In a series of related experiments, the authors' laboratory has begun to study the effects of acute and chronic cocaine on Fos expression in the mesolimbic dopamine system. Hope and Nestler (1991) and Hope and colleagues (in press) have replicated the findings of other groups (Graybiel et al. 1990; Young et al. 1991) showing that acute administration of cocaine (at a dose of 15 mg/kg intraperitoneal [IP]) induces Fos mRNA levels in the NAc (figure 6) and the caudate/putamen 45 minutes after drug administration (Hope and Nestler 1991; Hope et al., in press). Moreover, it was shown that acute cocaine elicits a similar induction in mRNA levels for Jun and *zif. (Zif* is a transcription factor that, although binding to a distinct response element, is regulated in several circumstances in a fashion similar to Fos and Jun.) As systemic administration of cocaine is known to inhibit a majority of neurons in the NAc (White et al. 1987), cocaine induction of Fos and related transcription factors represents one example, alluded to above, where these proteins can be induced in the absence of an increase in neuronal activity. One possible mechanism of cocaine action, which can be tested directly, is that the drug induces Fos via the indirect activation of D1-dopamine receptors and the subsequent activation of the cAMP pathway, which also exerts inhibitory effects on these cells electrophysiologically. An alternative possibility that the authors do not favor but cannot exclude is that cocaine induces Fos in the minority of neurons in the NAc that are activated by systemic administration of the drug.

Chronic administration of cocaine (15 mg/kg IP b.i.d. for 14 days) almost completely abolishes the ability of a subsequent acute dose of cocaine (administered 18 hours after the previous dose) to induce Fos mRNA levels in these two brain regions (figure 6) (Hope and Nestler 1991; Hope et al., in press). A similar "desensitization" was observed for the ability of cocaine to induce *jun* and *zif.*

To further investigate this apparent desensitization, Hope and Nestler (1991) and Hope and colleagues (in press) studied acute and chronic regulation of AP-1 binding activity in the NAc using the gel shift assay (figure 6). It was found that acute cocaine dramatically induces AP-1 binding activity in this brain region 2 hours following drug administration, indicating that induction of Fos and Jun mRNA is associated with an increase in the functional activity of these or related transcription factors. However, chronic cocaine administration

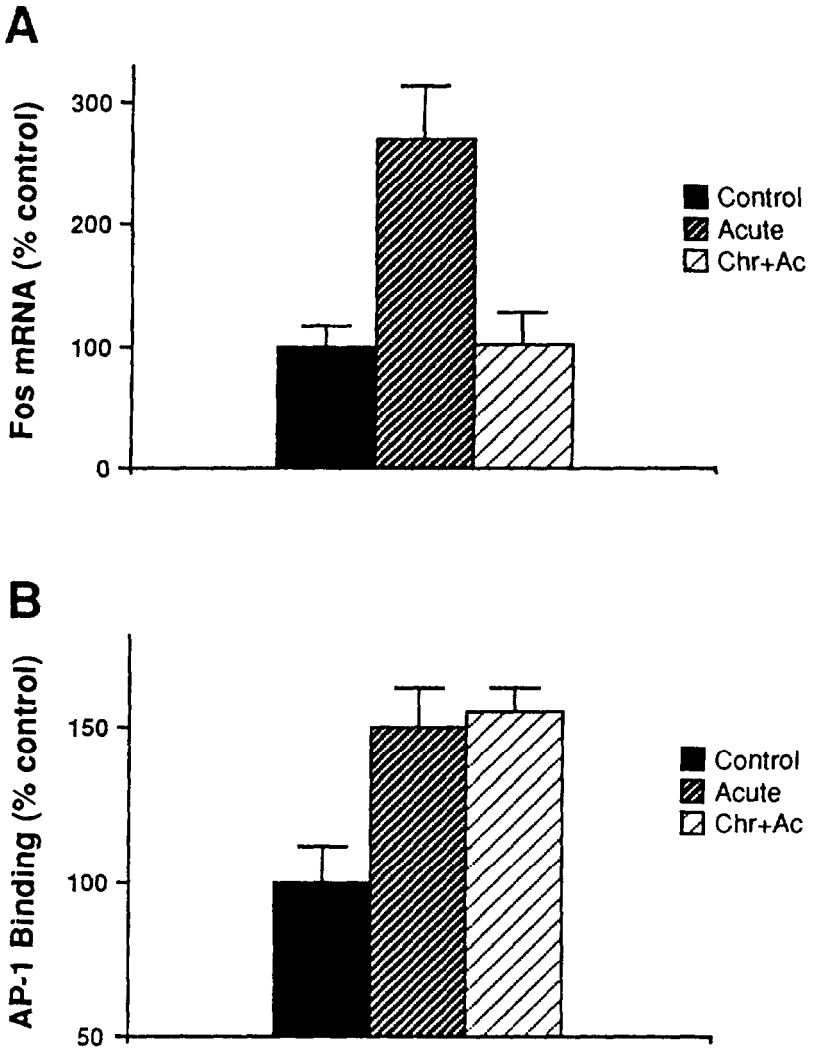

FIGURE 6. *Regulation of Fos mRNA and of AP-1 binding activity by acute and chronic cocaine in the rat NAc. Control rats received twice daily IP injections of saline for 14 days and an additional saline injection on day 15; as with the controls, acute rats received saline injections for 14 days but received a single injection of cocaine hydrochloride (15 mg/kg) on day 15; chr+ac rats received twice daily injections of cocaine for 14 days and an additional cocaine injection on day 15. Animals were sacrificed 45 minutes*

(for analysis of Fos mRNA levels) or 2.5 hours (for analysis of AP-1 binding activity) after their last injection on day 15. A: Levels of Fos mRNA in the NAc of control and treated rats analyzed by Northern blotting. B: Levels of AP-1 binding activity in the NAc of control and treated rats analyzed by gel shift assays. Data represent means±SEM (n=4 to 8).

SOURCE: Data from Hope and Nestler 1991; Hope et al., in press

appears to produce a persistent increase in AP-1 binding activity in the NAc: Levels of AP-1 binding are elevated 18 hours after the last chronic dose of cocaine whether or not an additional acute dose is administered. These observations indicate that chronic cocaine leads to a persistent increase in Fos- and/or Jun-like proteins.

If confirmed, these findings suggest that chronic cocaine produces a gradual change in the protein composition of the AP-1 complex in NAc neurons. For example, chronic cocaine not only could produce a desensitization to Fos responsiveness but also a concomitant accumulation of various Fos- or Jun-related transcription factors. Such a change in the composition of the AP-1 complex could alter its transcriptional activity and/or specificity (e.g., see Ryseck and Bravo 1991) and thereby lead to some of the changes in gene expression seen in response to chronic cocaine in this brain region. In other words, such a change in the composition of the AP-1 complex could represent a “molecular switch” underlying some of the chronic actions of cocaine (Hope et al., in press). To test this possibility directly, it will be necessary to develop antibodies that distinguish the numerous individual members of the Fos and Jun family in immunocytochemical and immunoblotting studies. The immunocytochemical studies offer the additional possibility of identifying subsets of NAc and striatal neurons that exhibit differential immediate early gene responses to acute and chronic cocaine administration.

As a first attempt to identify the specific proteins that account for the increased AP-1 binding activity observed under acute and chronic cocaine treatments, levels of Fos and Jun protein in the NAc by immunocytochemical techniques are now being studied in collaboration with Drs. Barry Kosofsky and Steven Hyman at Harvard. Acute cocaine treatment induces a dramatic increase in levels of Fos-like immunoreactivity, as seen for Fos mRNA, in the NAc and caudate/putamen. However, similar to the observations with northern blotting, preliminary evidence suggests that chronic cocaine decreases the inducibility of Fos-like immunoreactivity (Hope et al., in press). In the striatum, this desensitization in Fos induction shows an interesting medial to lateral gradiant

indicating that subpopulations of neurons respond differentially to chronic cocaine.

Future Studies

The types of studies described above have been and will continue to be useful in identifying specific types of transcription factors that could be involved in opiate and cocaine regulation of gene expression in specific target brain regions, However, at best, these studies can offer only circumstantial evidence for the possible role of a given transcription factor in mediating drug action on the expression of specific target proteins. Ultimately, more direct evidence is required to establish a causal relationship between a given transcription factor and target gene. Such direct evidence could theoretically be obtained by monitoring the transcriptional activity of a target gene under conditions where the activity of a specific transcription factor (or of a second messenger/protein phosphorylation pathway involved in the functioning of that factor) is specifically altered.

Our laboratory is testing the use of a nonpathogenic strain of herpes simplex virus-1 (HSV-1) (Dobson et al. 1990) as a vehicle for introducing DNA into specific neuronal cell types in vivo. In this procedure, the recombinant virus is injected in the vicinity of a brain region of interest, where it appears to be able to infect a certain percentage of the nearby neurons (C.M. Bergson and E.J. Nestler, unpublished observations, June 1991). An example of how this procedure could be used to more directly study possible transcriptional mechanisms involved in drug addiction is described below for the case of opiate regulation of tyrosine hydroxylase gene expression in the LC. A fusion gene subcloned into the recombinant HSV-1, consisting of part of the regulatory region of the tyrosine hydroxylase gene coupled to a reporter gene-any gene whose expression can be easily monitored (e.g., genes for ß-galactosidase, chloramphenicol acetyltransferase, or luciferase)—can be introduced into LC neurons via HSV-1 infection. If chronic morphine regulation of tyrosine hydroxylase in the LC occurs at the level of gene expression as inferred from the mRNA data (see above), treatment of rats with chronic morphine would also be expected to increase expression of the reporter gene. Next, introduction of fusion genes with mutated response elements in the tyrosine hydroxylase promoter could then be used to determine which response element (or combination of response elements) is required for such transcriptional regulation. The direct involvement of a specific transcription factor in this process could also be tested by introducing DNA that, when transcribed, encodes an RNA strand that is antisense for a given transcription factor mRNA. The antisense RNA would hybridize with the sense mRNA and thereby prevent it from being translated into the transcription factor under

investigation. Ultimately, similar types of experiments could be carried out using transgenic animals, where the constructed genes would be introduced into all somatic cells, including the relevant neurons through germ line transmission. At very early stages of investigation, these examples illustrate how the precise mechanisms by which drugs of abuse regulate gene expression can be delineated using molecular biological techniques.

CONCLUSIONS

This chapter described the experimental strategy that the authors' laboratory is using to investigate the role of changes In gene expression in the development of opiate and cocaine addiction. The strategy involves the study of discrete brain regions that are known to contribute to behavioral aspects of drug addiction and in which electrophysiological studies provide insight into the functional changes at the neuronal level that underlie the addictive behaviors, This makes it possible to understand biochemical and molecular actions of the drugs within a functional context. The focus is on identifying intracellular signaling proteins that are regulated by chronic drug treatments at the level of gene expression. These target proteins are then used to investigate the mechanisms by which the drugs of abuse alter the expression of the *genes* encoding them. To date, it has been possible to identify alterations in G proteins and the cAMP system in specific regions of the CNS in response to chronic opiates and/or chronic cocaine. Of particular interest is the up-regulation of the cAMP system observed in the LC in response to chronic opiates (biochemical adaptations shown to contribute to opiate tolerance, dependence, and withdrawal exhibited by these neurons) and a similar up-regulation of the cAMP system observed in the mesolimbic dopamine system in response to chronic opiates and chronic cocaine, which could be related to drug reinforcement or craving mediated via this neural pathway. Current studies are aimed at investigating opiate and cocaine regulation of transcription factors in these discrete brain regions. Ultimately, it will be possible to expand on the correlative data resulting from these studies with more direct analyses that make use of in vivo methods for detecting changes in target gene expression. Together, these studies of opiate and cocaine action will help define the precise mechanisms, at the molecular level, by which these drugs of abuse alter the expression of specific genes in particular neuronal cell types and thereby produce physical and psychological aspects of addiction.

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C-Fos and Fos-Related Antigens as Markers for Neuronal Activity: Perspectives From Neuroendocrine Systems

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INTRODUCTION

The alterations in neuronal function underlying drug abuse are complex, yet important. Several specific neuronal systems have been implicated in drug-seeking behavior, addiction, and withdrawal, but none has emerged as key. What may be needed is an approach to mark neurons throughout the brain that are influenced by drugs of abuse. Once targeted neurons are identified, studies can be extended to define their phenotype and projections and to determine their functions in drug-related processes. The use of immediate early gene (IEG) products may provide an important tool for labeling neurons whose activity has changed as a result of drug treatment. As is detailed below, c-Fos and Fog-related antigens (FRAs) serve as markers for identifying neuronal systems whose activity is increased or decreased in response to a stimulus.

In situ hybridization of IEG messenger RNAs (mRNAs) as well as immunocytochemical localization of protein products are useful for addressing this issue. The use of the protein products in examining specific systems can have advantages over the study of mRNA. First, molecular probes are not available for all IEGs, whereas antisera have been generated that recognize the protein products of some genes not yet cloned. Second, IEG products such as c-Fos, c-Jun, and Zif/268, when present, concentrate within the cell nucleus (Sheng and Greenberg 1990). This feature makes it possible to label these proteins along with cytoplasmic markers for the neurotransmitter (Ceccatelli et al. 1969; Hoffman et al. 1990; Jacobson et al. 1990) and/or retrogradely transported tracers (Menetrey et al. 1969) using standard double immunocytochemical techniques. For heterogenous neuronal populations, or diffusely organized neuronal systems, this capability is imperative. In contrast, mRNA is located in the cytoplasm. Techniques for double labeling of either two

mRNAs or one mRNA and an immunocytochemically identified marker are not yet sufficiently reliable to be easily applied; generally, there is great compromise in the quality of either the mRNA label, the immunocytochemical marker, or both. Another advantage to localizing the proteins is that their appearance is delayed by at least 30 to 45 minutes after the stimulus is delivered, unlike the mRNA, which increases within 5 to 10 minutes after stimulation. This delay allows the investigator to move or manipulate the animal prior to sacrifice without concern that such handling will result in IEG expression. The case is well illustrated for studies of light activation. In this instance, for study of mRNA changes, animals must be sacrificed under safelight conditions, whereas immunocytochemical analysis would allow an animal to be anesthetized and brought into the light for perfusion without allowing sufficient time for the light stimulus to be translated into new IEG proteins. A disadvantage in using IEG products localized by immunocytochemistry rather than by in situ hybridization of their mRNA is the lack of precise quantitation inherent in the immunocytochemical methods. In this chapter, the authors present evidence that, in spite of this problem, localization of IEG products with standard immunohistochemical techniques permits assessment of relative changes in gene expression. The studies presented in this chapter use natural stimuli and document that the expression of the IEGs is not only induced by drug treatment or other experimental manipulation but also is involved in normal homeostatic function.

C-FOS AS A MARKER FOR NEURON ACTIVATION: LUTEINIZING HORMONE-RELEASING HORMONE NEURONS AS THE MODEL

Most of the data presented here focus on the authors' studies of neuroendocrine regulation. In particular, study of the regulation of reproductive function has been most revealing. The study of neuroendocrine-adenohypophyseal function has presented challenges to physiologists in that conventional means of assessing neuronal activity are impractical owing to the small size and scattered distribution of the neuroendocrine neurons regulating the anterior pituitary. The case in point is well illustrated for one neuroendocrine system: the luteinizing hormone-releasing hormone (LHRH) (also known as gonadotropin-releasing hormone or GnRH) neurons, which stimulate the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. LHRH neurons are small, few in number, and widely scattered. The rat brain contains only 1,200 LHRH neurons (Wray and Hoffman 1986a, 1986b), 70 percent of which send their axons to the median eminence to effect LH and FSH release (Merchanthaler et al. 1989). The LHRH cells are scattered over the full rostral-to-caudal extent of the forebrain and generally do not reside within classic cytoarchitectonic boundaries (Hoffman and Gibbs 1982; Silverman et al. 1982), To make matters worse, the LHRH somata typically measure only 8 to 10 μm in diameter: even in sites where the LHRH neurons are most numerous, they are

interspersed among non-LHRH neurons, making the possibility of successfully locating the cells and then recording from them remote.

Means have been devised for monitoring LHRH secretory activity. The most direct method involves cannulation of the tiny portal blood vessels that carry LHRH released at the median eminence of the hypothalamus to the anterior pituitary. With this approach, during an LH surge the output of LHRH increases (Sarkar et al. 1976). However, the necessity for anesthesia in performing these studies precludes accurate quantitative assessment of changes in LHRH that occur in the awake state. In sheep, portal blood collection is possible in the awake animal, and measurements of LHRH reveal increases in LHRH release that accompany LH pulses in the basal state, with dramatic increases during an LH surge (Moenter et al. 1990). Yet the methods employed in sheep cannot be easily adapted to smaller mammals. An alternative has been the use of push-pull cannulae placed either in the median eminence or in the anterior pituitary. LHRH measured by push-pull techniques shows pulsatility during baseline states and moderate, but clear, increases in LHRH output during an LH surge (Levine and Ramirez 1982; Park and Ramirez 1989). Unfortunately, damage to the axons that are under investigation invariably accompanies the collection of samples. Moreover, although data from push-pull experiments convincingly link increases in LHRH secretion with increases in LH secretion, they tell little about the stimulation of LHRH neurons at a cellular level. Measurement of changes in IEG expression provided the first direct evidence that cellular LHRH activity accompanied LH surges (Hoffman et al. 1990; Lee et al. 1990a, 1990b). LHRH neurons normally do not express c-Fos. However, c-Fos expression is induced within 45 minutes following electrochemical stimulation and during induced or spontaneous LH surges (Hoffman et al. 1990; Lee et al. 1990a, 1990b). An analysis of the location of stimulated LHRH neurons expressing c-Fos during the peak of an LH surge identifies a subpopulation of LHRH neurons in the preoptic area in the vicinity of the organum vasculosum of the lamina terminalis (OVLT), below the anterior commissure, which extends into the anterior hypothalamus (figure 1). Interestingly, the LHRH neurons located above the anterior commissure and rostral to the OVLT remain “quiet” during an LH surge. Yet these same cells will express c-Fos after electrochemical stimulation. During an LH surge, the activated LHRH population may have been targeted by selective innervation of LHRH neurons, which, when contacted by catecholamine or neurotensin axons (Hoffman 1985), are distributed in a pattern similar to that observed for c-Fos expression in LHRH neurons during a surge (Hoffman et al. 1990; Lee et al. 1990a, 1990b).

In the authors’ studies, female rats were cannulated on the morning of the proestrus LH surge, and blood samples were monitored throughout the day at 30- to 60-minute intervals until the time of sacrifice. These were assayed by radioimmunoassay for LH. Each animal then was anesthetized and perfused

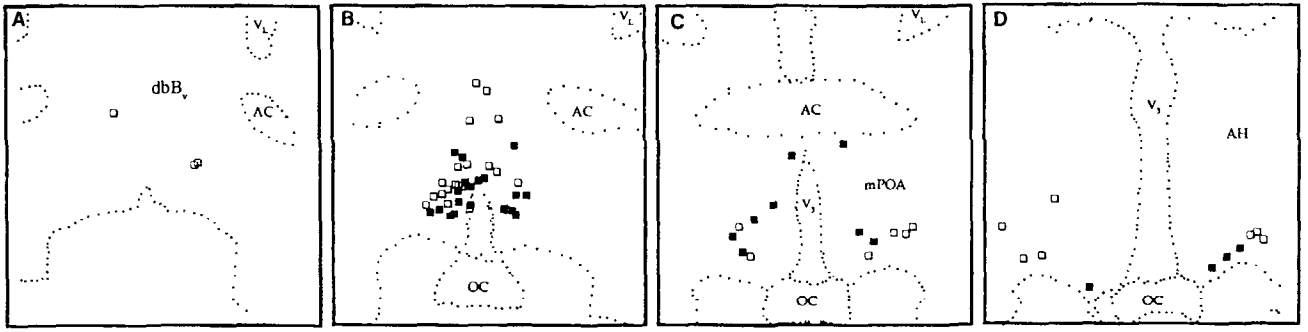

FIGURE 1. *Distribution of c-Fos-activated LHRH cells. The panels illustrate the location of LHRH neutrons that express c-Fos (■) and those that are devoid of c-Fos immunoreactivity (□) throughout the rostra/ forebrain. (A) The components of the LHRH cell field scattered within the vertical limb of the diagonal band of Broca, rostral to the OVLT, do not express c-Fos during an LH surge. (B) At a level close to the OVLT, the LHRH neurons are most numerous, and approximately half express c-Fos. (C) More caudalty, within the preoptic area, LHRH neurons express c-Fos; again, approximate half the cells are stimulated. (D) At the junction of the preoptic area and anterior hypothalamus, c-Fos activation within LHRH neurons continues.*

SOURCE: Modified from Lee et al. 1990b

transaortically first with saline containing 2 percent sodium nitrite and followed by 4 percent buffered paraformaldehyde (pH 6.8) containing 2 to 2.5 percent acrolein (EM grade). The brains were removed, sunk in 25 percent aqueous sucrose solution, and cut into 12 series of 25 µm sections, which were stored in cryoprotectant solution (Watson et al. 1966) until immunocytochemical procedures for c-Fos and LHRH were initiated. Immunocytochemical staining was accomplished with specific antisera for LHRH (LR-1) and antisera generated against the amino acids within the N-terminus of c-Fos (Cambridge Research Biochemicals sheep anti c-Fos, OA11-821; Dr. Tom Curran's anti-*alu* Fos). Sequential staining of first c-Fos followed by LHRH was performed with the ABC "elite" procedure allowing primary antisera concentrations to be 1:44,000-50,000 for the c-Fos antisera and 1:100,000 for anti-LHRH (when double immunoperoxidase methods were used; if immunofluorescence was used instead, the concentration of anti-LHRH was increased to 1:30,000). The c-Fos staining following visualization of peroxidase activity with a nickel diaminobenzidine chromogen appeared blue black; LHRH reactivity was revealed with either diaminobenzidine (immunoperoxidase reactions), which stained golden brown, or Texas Red (immunofluorescence procedure), which fluoroesced red after excitation in the green range. An example of LHRH neurons activated during an LH surge stained with the immunofluorescent procedure is shown in figure 2.

On analysis of plasma LH levels, the authors noted that rats whose plasma LH levels were high 30 to 60 minutes before the time of sacrifice had greater levels of c-Fos expression within their LHRH neurons than did rats whose LH levels were low prior to sacrifice (figure 3). Analysis of plasma LH and c-Fos immunoreactivity in LHRH neurons revealed a highly significant linear relationship (figure 4), indicating that the amplitude of LH secretion reflects the number of LHRH neurons activated. Further investigation of the relationship between LHRH stimulation and LH secretion was aimed at the use of a model in which the amplitude of an LH surge was attenuated by prevention of the actions of progesterone. Intact female rats were treated at 12:30 p.m. on the afternoon of proestrus with the progesterone antagonist RU 486 (5 mg, subcutaneously [SC]) and compared with untreated proestrus rats. In a separate series of experiments, castrated female rats treated with estradiol or with progesterone provided a second similar model. Rats were ovariectomized and 2 weeks later were administered 1 µg estradiol benzoate subcutaneously (SC) at 9:00 a.m. Twenty-four hours later a second injection of estradiol benzoate (50 µg SC) was administered (also at 9:00 a.m.) followed by an SC injection of progesterone (5 mg) or vehicle at 12:30 p.m. Plasma LH was monitored as described above. In animals treated with RU 486 and in castrates receiving only estrogen, the blunting of the LH peak was accompanied by a decrease in the degree of LHRH c-Fos expression (figure 5) (Lee et al. 1990b). Close examination of the LHRH neurons revealed that, in addition to showing fewer

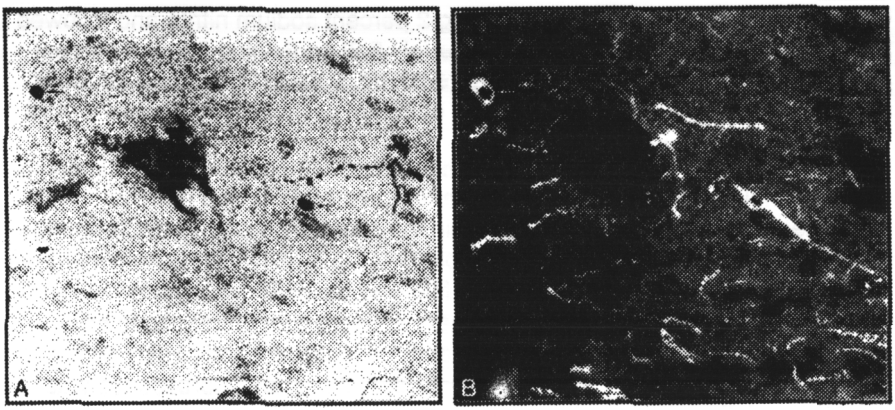

FIGURE 2. *C-Fos expression in LHRH neurons. C-Fos (arrows) is expressed in LHRH neurons during an LH surge. (A) Bright-field image shows that the nuclei of two LHRH neurons in the field contain c-Fos. (B) The same section viewed under fluorescence microscopy reveals immunofluorescent LHRH neurons (arrows) close to the OVLT that express c-Fos in their nuclei.*

LHRH neurons activated when the actions of progesterone were prevented or blocked, progesterone-deprived rats showed a reduced intensity of c-Fos protein immunoreactivity within their nuclei (figure 6). Thus, progesterone appeared not only to recruit more LHRH neurons into the active state but also increased the extent of stimulation of individual cells. More generally, these data indicate that c-Fos expression is graded to the intensity of the stimulus and, as was revealed by progesterone deprivation, is not all or none. Further confirmation of this feature has been obtained for hypovolemic stimulation of magnocellular neurons of the hypothalamus (Roberts et al., submitted for publication), in which, following delivery of increasing hemorrhagic stimuli, c-Fos immunoreactivity within vasopressin and oxytocin neurons increased both in terms of the number of neurons expressing c-Fos and the intensity of the staining within each stimulated neuron. An example showing the general changes in c-Fos expression in the paraventricular nucleus is shown in figure 7. The changes in c-Fos expression paralleled the changes in hormone secretion.

FRAs AS MARKERS FOR BASELINE ACTIVITY: THE TUBEROINFUNDIBULAR DOPAMINE NEURONS AS THE MODEL SYSTEM

The analysis of LHRH, vasopressin, and oxytocin neurons, although demonstrating that c-Fos can effectively serve as a marker for stimulated

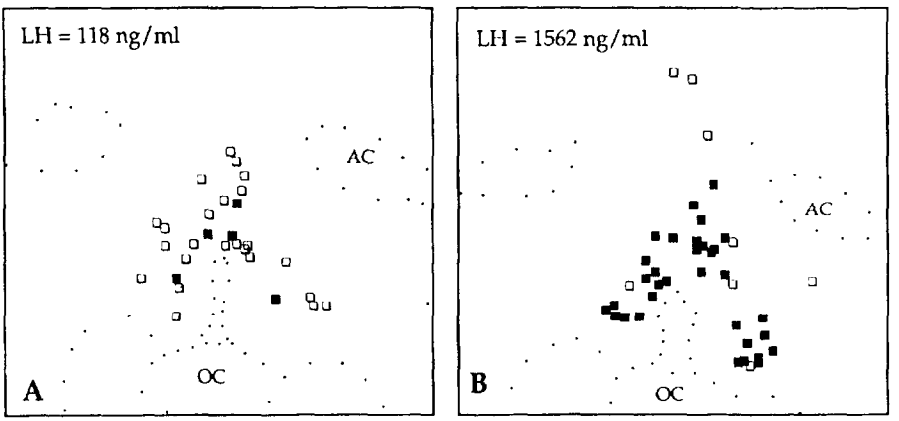

FIGURE 3. *C-Fos activation in LHRH neurons* during *the rising phase of an LH surge. A plot from an* animal *whose plasma LH had only begun* to *rise within the hour prior to sacrifice (A) is compared with a plot from a rat whose plasma LH had nearly reached peak values (B). LHRH neurons expressing c-Fos are depicted as closed squares (■). LHRH neurons devoid of c-Fos immunoreactivity are shown as open squares (□). Note* that *the higher degree of c-Fos activation in LHRH is associated* with a greater *elevation of plasma LH.*

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activity, did not allow the authors to analyze changes in baseline activity. Screening antisera that might allow a broader picture of IEG expression and examining other neuroendocrine systems provided some insight into this problem. Recently, Dragunow and Faull (1990) as well as Jacobsen and colleagues (1990) observed that staining with antisera generated against the M-peptide region of c-Fos revealed many nuclei in the cerebral cortex of control animals, whereas staining with an N-terminally directed c-Fos antiserum (or in situ hybridization of c-Fos mRNA) showed little or no baseline c-Fos expression. Since Western blots of the M-peptide antiserum indicated recognition of multiple proteins, it was reasoned that, in baseline conditions, some FRAs, but not c-Fos, are expressed. Consequently, the authors sought to determine if the basal FRA expression could be manipulated in a fashion to allow the examination of *decreases* in activity following stimulus delivery.

The test system was the tuberoinfundibular dopamine system. This neuroendocrine system, located within the arcuate nucleus of the

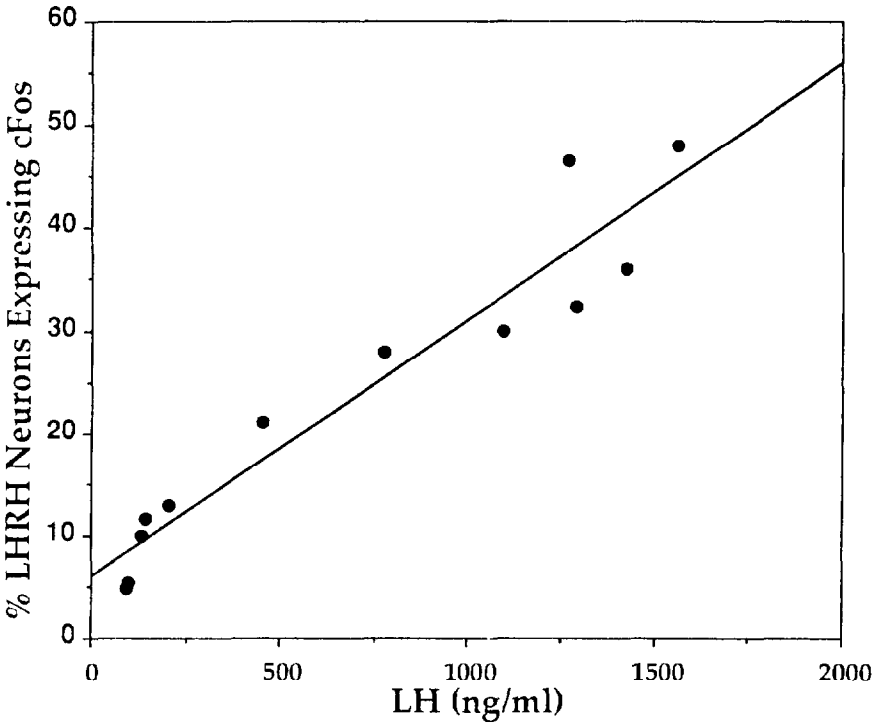

FIGURE 4. *Relationship between the percent of LHRH neurons that expressed c-Fos and plasma LH values 30 to 60 minutes prior to sacrifice on the afternoon of proestrus. Each point represents one animal. R=0.957; p<0.001.*

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hypothalamus, tonically inhibits prolactin release. Following the onset of suckling, prolactin release markedly increases, due at least in part to an inhibition of the dopamine's inhibitory tone (Moore 1987). Groups of lactating rats (postpartum day 12) continually suckling their pups and diestrus rats were compared for the presence or absence of FRA proteins within dopamine neurons of the arcuate nucleus. An antiserum generated against the M-peptide region of c-Fos (generated by Dr. Michael Iadarola) served as the FRA marker and was used at a concentration of 1:15,000. Staining for the enzyme tyrosine hydroxylase (TH) with an anti-TH serum served as a marker for dopamine in the hypothalamic neurons. Staining

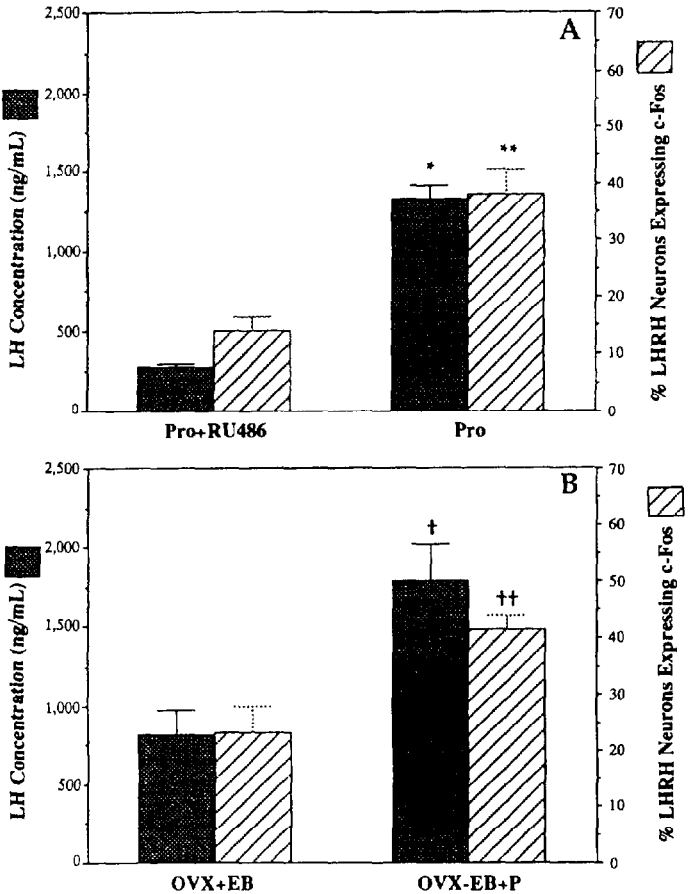

FIGURE 5. *Effects of progesterone on the magnitude of the LH surge and activation of LHRH neurons. (A) Effect of administration of the progesterone antagonist RU 486 on the amplitude of the LH surge (shaded bar) and degree of c-Fos expression within LHRH neurons (striped bar) at the time of the peak of the LH surge. For comparison, the values of the RU 486-treated rats are depicted along with untreated proestrus rats. (B) Effect of administration of estrogen only or estrogen plus progesterone to ovariectomized rats on the magnitude of the LH surge (shaded bar) and degree of c-Fos expression (striped bar) in LHRH neurons at the time of the peak of the LH surge.*

SOURCE: Lee et al. 1990a

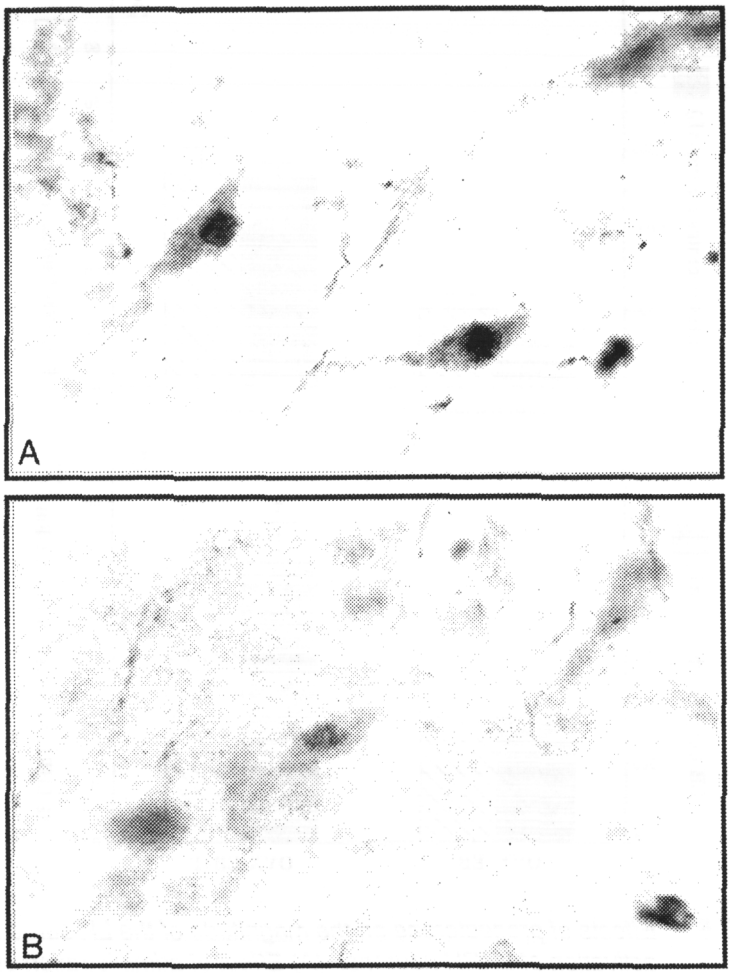

FIGURE 6. *Effect of removal of progesterone's influence on intensity of c-Fos protein in activated LHRH neurons. In addition to reducing the number of neurons activated during an LH surge, ovariectomized rats replaced with estrogen only showed a marked reduction in the intensity of c-Fos staining in LHRH neurons. (A) Ovariectomized rat treated with estrogen and progesterone; (B) ovariectomized rat treated only with estrogen. Similar results were obtained following treatment of intact cycling rats with RU 486.*

SOURCE: Lee et al. 1990a

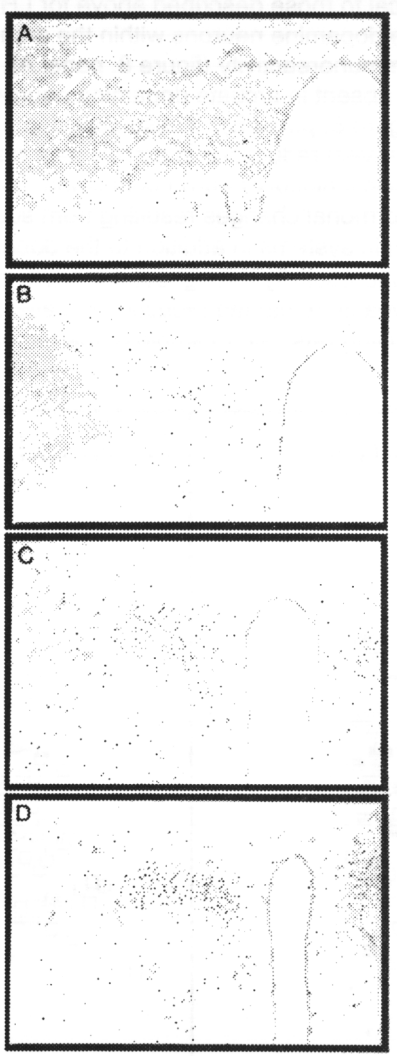

FIGURE 7. *Responses of neurons of the paraventricular nuclei to graded hemorrhage, Increasing stimuli to* the *magnocellular neurons of the paraventricular nuclei resulted in increasing levels of c-Fos expression. (A) Control, (B) 2 cc blood removed, (C) 4 cc blood removed, (D) 6 cc blood removed.*

strategies were identical to those described above for LHRH and c-Fos. Approximately half the dopamine neurons within the arcuate nucleus of diestrus rats expressed FRA immunoreactivity (figure 8, panel A). In contrast, FRA immunoreactivity was absent in arcuate dopamine neurons from lactating rats suckling their pups (figure 8, panel B). These data demonstrate that FRA immunoreactivity can reveal reductions in activity as a result of the suckling stimulus within a specific inhibitory neuron population. The suckling stimulus, or consequences of hormonal changes resulting from suckling, appears to affect a great many brain systems in addition to the dopamine neurons. Analysis of FRA immunoreactivity throughout the brain, including the neocortex and hippocampus, revealed a marked reduction in baseline levels in lactating rats compared with cycling rats. An example of the pattern of FRA staining in

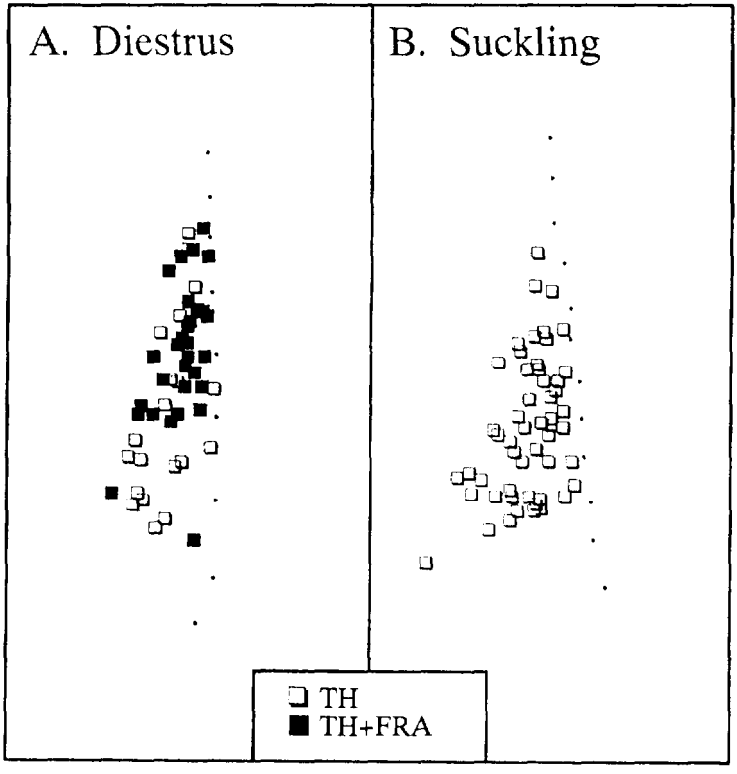

FIGURE 8. *FRA expression in the dopamine neurons of the arcuate nucleus in (A) a diestrus and (B) a lactating female rat. FRA proteins, normally expressed within the dopamine neurons, are no longer expressed in suckling rats.*

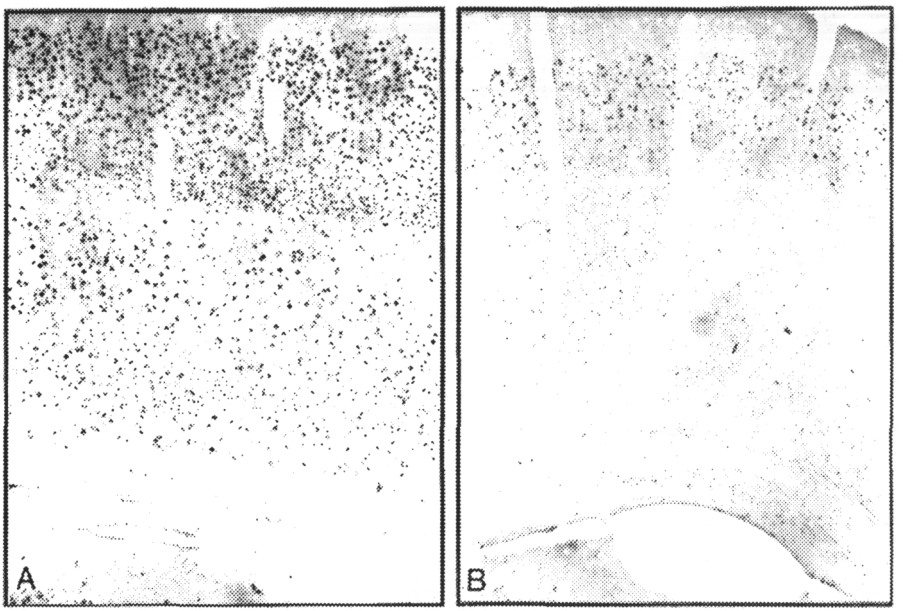

FIGURE 9. *Baseline FRA expression in the somatomotor cortex. (A) In the diestrus rat many neurons thoughout all cell layers express FRAs. (B) In the* lactating *rat the baseline FRA expression in the* cerebral *cortex is markedly reduced.*

the neocortex of a diestrus rat and a lactating rat is shown in figure 9. These changes are consistent with the attenuation of many responses to stress observed in lactating rats and offer another example of the use of FRA staining as a means of identifying neuronal populations whose activity diminishes in response to neuronal stimuli and/or hormonal changes,

REPRODUCTIVE STATE AS A VARIABLE IN STUDYING BRAIN ACTIVATION: LACTATION AND STIMULATION BY NMA

Lactating rats, in addition to showing blunted behavioral changes to stress, fail to possess spontaneous pulses of LH release. In investigating the possible mechanisms of this reproductive quiescence, the authors treated lactating animals with the excitatory amino acid N-methyl-aspartate (NMA) at doses that stimulate LH secretion in cycling rats (Smith and Lee 1990). Attention in these studies was returned to c-Fos as a marker for stimulated activity. Lactating rats and a group of diestrus female rats were fitted with jugular cannulae, and four

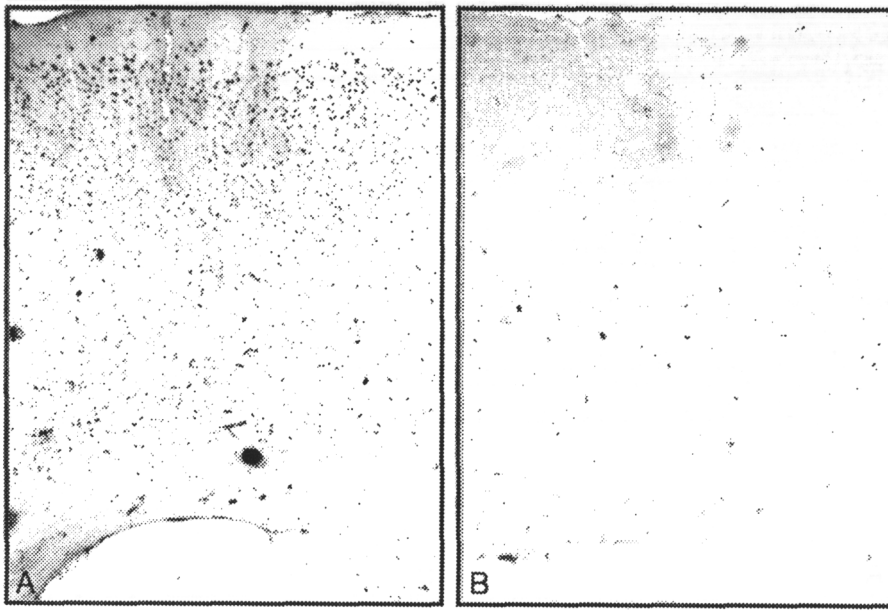

FIGURE 10. *Induction of c-Fos in the cerebral cortex of an NMA-treated rat (A) Diestrus female rats show strong induction of c-Fos in the somatomotor cortex following subconvulsive doses of NMA. (B) Similar treatment of lactating rats fails to induce c-Fos in the cerebral cortex. The patterns of c-Fos expression appeared to mirror the behavioral effects of NMA.*

pulses of 40 mg/kg NMA were administered at 10-minute intervals. Ninety minutes following the last dose of NMA, the rats were anesthetized and perfused for localization of c-Fos. In the diestrus rats, administration of NMA elicited signs of hyperactivity, but no seizures. In contrast, administration of NMA to lactating rats did not induce behavioral changes. Examination of c-Fos revealed marked induction of c-Fos in neurons throughout the neural axis of the diestrus rats, whereas the same treatment of lactating rats did not induce c-Fos immunoreactivity. Examples of c-Fos staining within the neocortex of a diestrus and lactating rat treated with NMA are shown in figure 10. Initial studies exploring the recovery of excitability in lactating rats reveal that both the suckling stimulus as well as progesterone participate in the dampening of NMA responsiveness (Abbud et al. 1991). These studies not only provide another example of IEG products as effective tools for studying neuronal activation but also illustrate the importance of the animal's endocrine state in determining the effects of drugs on the nervous system.

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Mechanisms of Opioid-Mediated Antinociception: Correlation of Fos Expression and Behavior

Kathleen R. Gogas, Jon D. Levine, and Allan I. Basbaum

INTRODUCTION

Recent studies have emphasized the utility of monitoring the expression of immediate early genes (IEGs) to map functionally relevant pathways in the central nervous system (CNS) (Hunt et al. 1987; Morgan et al. 1987; Sager et al. 1988). The authors' laboratory has been studying the expression of the *c-fos* gene to characterize the circuitry underlying the transmission of noxious (i.e., painful) information into the spinal cord (Menétrey et al. 1989; Presley et al. 1990; Gogas et al. 1991). Despite the rapid growth of this new field, many questions are still unsettled as to the appropriateness of *c-fos* message or Fos protein as a marker of neuronal activity and as to the stimulus specificity of Fos expression. Thus, the goals of this chapter are twofold. First, it will be discussed how the expression of *c-fos* can be used in the author's system to identify which populations of spinal cord neurons are activated by noxious stimulation and to evaluate the effects of known analgesics on noxious stimulus-evoked Fos expression. Second, the question of stimulus specificity will be addressed by comparing the pattern of *c-fos* expression produced by nonnoxious as well as noxious stimuli.

SPINAL CORD NOCICEPTIVE CIRCUITRY

The authors' analyses of the expression of the *c-fos* gene has been done primarily in the spinal cord dorsal horn. This region is the first CNS site for the transfer of nociceptive (i.e., pain) messages from the periphery and, thus, plays a key role in nociception. The dorsal horn has been divided on cytoarchitectural grounds into several discrete laminae, numbered I through VI from dorsal to ventral. High threshold (i.e., nociceptive) myelinated A-S and unmyelinated C primary afferent fibers carry nociceptive information from the periphery to the dorsal horn; A-o fibers synapse on neurons in lamina I, the outer portion of lamina II, and lamina V; C-fibers predominantly synapse on neurons in laminae

I and outer II (Light and Perl 1979; Sugiura et al. 1987). Consistent with the anatomical projection of the primary afferent fibers, electrophysiological studies have established that neurons in laminae I and IIo respond predominantly to noxious input; lamina V neurons respond to both noxious and nonnoxious stimuli and are thus referred to as wide-dynamic range neurons (for a review, see Besson and Chaouch 1987). Cells in the nucleus proprius (laminae III, IV) and in lamina VI are predominantly driven by nonnoxious stimulation. Finally, many neurons in laminae VII and VIII of the ventral horn and in lamina X, around the central canal, also respond to noxious stimulation.

Although they provide important information about the functional properties of spinal neurons, electrophysiological techniques have limitations. Specifically, since only one cell is analyzed at a time, it is not possible to monitor the responses of large populations of neurons to noxious stimulation. Thus, sample size is typically small. Furthermore, since the animals are almost always anesthetized and/or spinalized, it is not possible to correlate physiology with behavioral responses to stimulation. Even when recording is done in awake animals (e.g., Collins 1987), one is still limited by the number of cells that can be recorded.

THE USE OF FOS EXPRESSION TO ASSESS THE ACTIVITY OF NEURONS IN THE CNS

Hunt and colleagues (1987) were the first to show that immunocytochemical localization of the Fos protein product of the c-*fos* protooncogene can be used to monitor the "activity" of large populations of neurons in the spinal cord dorsal horn following either noxious or nonnoxious stimulation. As discussed in detail elsewhere in this monograph, c-*fos* is the cellular homolog of a retroviral transforming factor that induces osteosarcoma in susceptible strains of mice. Transcription of the c-*fos* gene is among the earliest nuclear events that occur when a cell is stimulated (Curran and Morgan 1985). Activation of transcription can be produced by a variety of external stimuli, including neurotransmitters and membrane depolarization (Curran and Morgan 1985; Greenberg et al. 1986; Morgan and Curran 1986). The latter stimuli in turn increase intracellular calcium, cyclic AMP (cAMP), diacylglycerol, and other second messengers, which, either directly or indirectly, stimulate transcription of the *c-fos* gene. Although increased Fos expression is associated with cellular proliferation and differentiation, its function in postmitotic neurons has not been established. However, it is clear that the expression of c-fos as well as other IEGs (e.g., c-*jun)* acts as a link between the excitation of neurons and long-term adaptive responses in the cell. Since the c-*fos* message appears within 5 to 10 minutes of stimulation and the Fos protein within another 10 to 20 minutes, it has been

proposed that the expression of IEGs, either by immunocytochemistry or in situ hybridization, provides a sensitive assay for assessing the activity of neurons in the CNS.

Since the basal levels of Fos protein in the spinal cord are low, stimulus-induced increases in Fos expression are readily detected. In studies of c-*fos* expression after noxious peripheral stimulation, it was first demonstrated that the pattern of Fos immunoreactivity is consistent with the anatomical location of nociresponsive neurons in the dorsal and ventral horn. Thus, following noxious stimulation in anesthetized rats, Hunt and colleagues (1987) reported that Fos immunoreactivity was concentrated in regions that contain neurons that respond to noxious stimulation, including the superficial laminae (I and Ilo) and in lamina V of the dorsal horn, Two important questions arose from that study. First, would the pattern of Fos expression be similar in awake animals? Second, would the magnitude of the behavioral responses to noxious stimuli correlate with the numbers of Fos-immunoreactive neurons? To address these questions, the authors developed a model whereby they could administer a noxious stimulus to a rat, record its behavioral response to the stimulus, and, most important, correlate the behavior with the levels of Fos expression in the cord.

FOS EXPRESSION AFTER SOMATIC NOXIOUS STIMULATION

In the authors' initial studies, Gogas and colleagues (1991) used the formalin pain model in which a dilute concentration of formalin is injected into the hindpaw of the rat. The formalin model has been well characterized both electrophysiologically and pharmacologically (Dickenson and Sullivan 1987a, 1987b; Hunskaar et al. 1986; Drower et al. 1987). Formalin injection produces a characteristic behavioral response that lasts for as much as 4 hours and can be readily quantified in terms of intensity (Dubuisson and Dennis 1977). The basic protocol was to inject the formalin into the plantar surface of the hindpaw. The rats were then placed into an observation chamber so that their behavior could be observed and recorded over a 1-hour period (Gogas et al. 1991). The animals were then anesthetized and perfused, with the spinal cord tissue removed and postfixed as previously described (Presley et al. 1990; Gogas et al. 1991). The tissue was processed immunocytochemically with the avidin-biotin procedure of Hsu and colleagues (1981) using commercially available kits (Vectastain ABC; Vectorlabs, Burlingame, CA). We have used a rabbit polyclonal antiserum directed against an in vitro translated product of *c-fos* (provided by Dr. Dennis Slamon of the University of California, Los Angeles).

An important advantage of Fos immunocytochemistry is that the results can be studied quantitatively. To this end, the outlines of three sections through the

lumbar enlargement were made with a camera lucida attachment under dark-field illumination; then under bright-field conditions the distribution of Fos-positive cells was plotted with a 4X objective and a camera lucida. To quantitate the anatomical results, the spinal cord drawings were divided into four segments: (1) the superficial laminae (laminae I, Ilo, and Iii), (2) the nucleus proprius (laminae Ill and IV), (3) the base of the dorsal horn (laminae V and VI), and (4) the intermediate zone and the ventral horn (laminae VII, VIII, IX, and X). The number of Fos-positive cells in each of these regions was counted and averaged so that each animal had a mean value for regional Fos expression. In addition, the sum of the mean regional values (i.e., superficial Fostnucleus proprius Fos+neck Fos+ventral Fos) was used as a measure of the total number of Fos-immunoreactive neurons in each animal. The investigator responsible for plotting and counting the Fos neurons had no knowledge of the drug treatment of each animal.

In light of the controversy concerning the factors that induce Fos expression, a significant finding using the formalin stimulus is that the evoked Fos immunoreactivity is largely found ipsilateral to the stimulated hindpaw (figure 1). This finding reinforced the idea that the increased number of Fos-positive cells was not related to handling or stressing of the animal during the protocol. In addition, the rostral-caudal distribution of the formalin-evoked Fos immunoreactivity was restricted to the lumbar cord (figure 2) with maximal staining in the lumbar enlargement (L4-L5), the area that receives the densest primary afferent innervation from the portion of the sciatic nerve territory at the site of hindpaw injection. The greatest number of Fos-immunoreactive neurons was seen in the superficial laminae of the cord (laminae I and Ilo), in the neck of the cord (lamina V and VI), and in lamina VII and around the central canal. Each of these areas has previously been shown to contribute to the processing of nociceptive information in the cord (Besson and Chaouch 1987).

The time course of the Fos expression also closely mirrored the time course of the behavioral response (figure 3). Thus, the maximal levels of Fos immunoreactivity were seen at 1 to 2 hours post-formalin injection, with decreasing activity over the next 6 hours; by 24 hours, almost no Fos staining remained in the cord. Taken together with the finding that the level of basal Fos expression in the cord is low, these results led to the conclusion that the Fos immunoreactivity in the cord is related to the noxious nature of the formalin stimulus and not to nonspecific factors secondary to the animal's perception of the stimulus (e.g., stress).

FOS EXPRESSION AFTER VISCERAL NOXIOUS STIMULATION

Further evidence in support of this hypothesis is derived from studies of the pattern of Fos immunoreactivity evoked by a noxious visceral stimulus

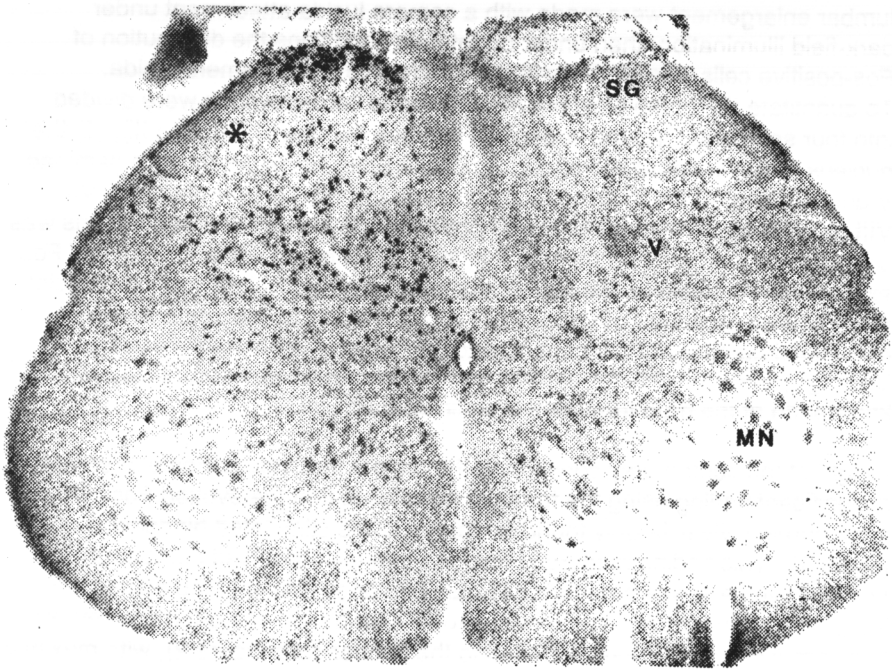

FIGURE 1. *This photomicrograph illustrates the pattern of hindpaw formalin-evoked Fos immunoreactivity in neurons of the L4-L5 spinal cord of a rat that received an intraperitoneal (P) injection of saline. There is dense labeling in the superficial layers (laminae I and II) and in the neck of the dorsal horn (V). There are no Fos-immunoreactive neurons in the motoneuron (MN) pool. The densest labeling is in the medial part of the superficial dorsal horn; the lateral part (asterisk) does nor receive primary afferent input from the plantar hindpaw. SG=substantia gelatinosa.*

SOURCE: Presley et al. 1990. Copyright 1990 by the Society for Neuroscience (New York).

(Menétrey et al. 1989). Since the pattern of visceral primary afferent termination in the cord is different from that of somatic afferents (Cervero 1985; Sugiura et al. 1989), it was expected that the pattern of Fos expression following visceral stimulation with formalin would differ. For this set of experiments, rats were anesthetized and injected (IP) with acetic acid in the right lower quadrant of the abdomen, which provides a model of visceral stimulation (Taber et al. 1969). Several important differences were found

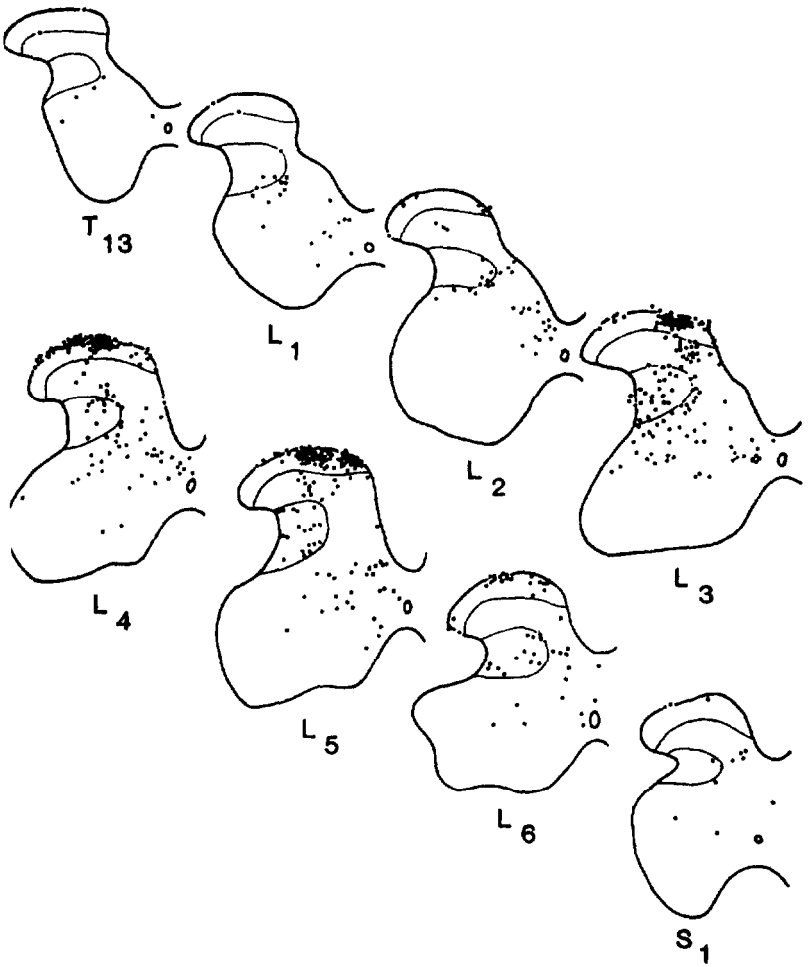

FIGURE 2. *Camera lucida drawings showing the rostrocaudal distribution of Fos-immunoreactive neurons 2 hours after formalin injection into the ipsilateral plantar hindpaw. The most intense immunoreactivity is noted at the L3-L5 segments, the major site of termination of afferents from the plantar hindpaw. Labeling in the superficial dorsal horn is concentrated in the L3-L5 segments, whereas labeling in the neck of the dorsal horn and ventromedial gray is present throughout the lumbar enlargement.*

SOURCE: Presley et al. 1990. Copyright 1990 by the Society for Neuroscience (New York).

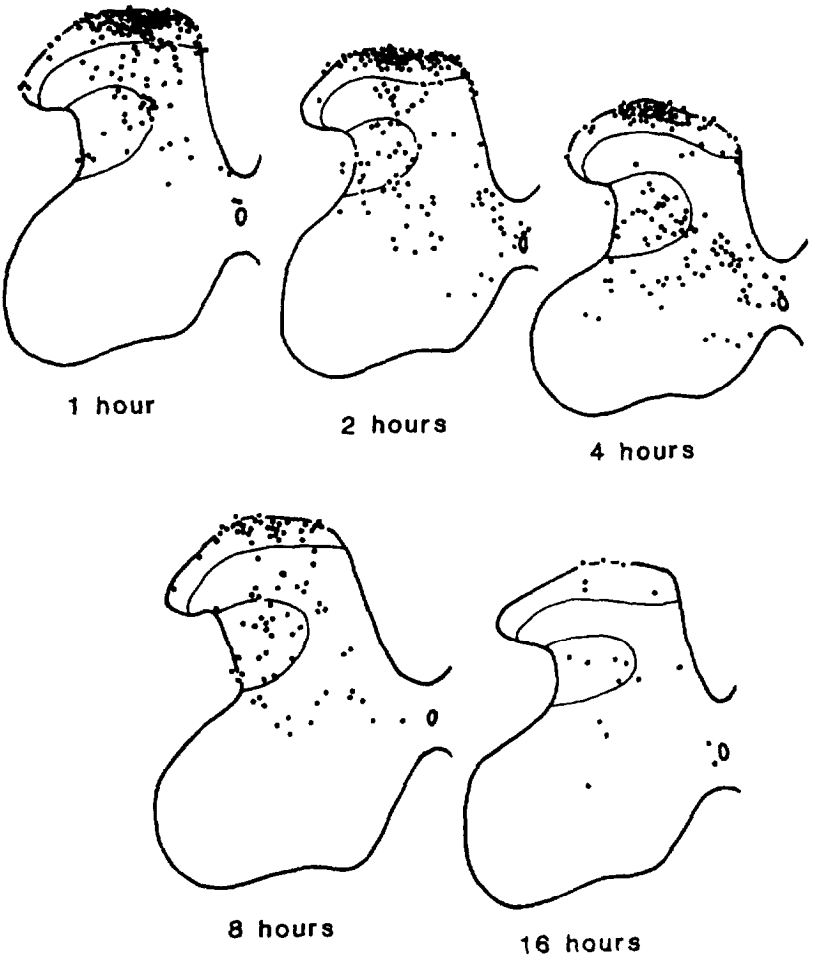

FIGURE 3. *Camera lucida drawings taken from the L4-L5 level of rats killed at various times after plantar hindpaw formalin injection. Maximal Fos immunoreactivity is present at 2 to 4 hours postinjection and then it disappears slowly. Staining is barely above control by 16 hours postinjection.*

SOURCE: Presley et al. 1990. Copyright 1990 by the Society for Neuroscience (New York).

between the pattern of Fos immunoreactivity evoked by the visceral stimulus and that observed in response to hindpaw formalin stimulation (figure 4). First, the densest staining was in the caudal thoracic rather than the lumbar cord, as was seen following formalin. Second, the staining following visceral stimulation was largely restricted to laminae I and V of the dorsal horn and to the area around the central canal. This different topographic distribution further argues that the induction of Fos immunoreactivity in spinal neurons is not secondary to stress but is related directly to the particular stimulus. Third, there was a far more extensive rostral caudal distribution of the Fos immunoreactivity, particularly in lamina I, where staining from the upper cervical to the sacral cord was found. This is consistent with the known anatomy of the primary afferent fibers that carry noxious visceral information; these fibers terminate over many segments rostral and caudal to their major termination point in the cord (Sugiura et al. 1989). The absence of Fos immunoreactivity in lamina II is also remarkably consistent with the central termination pattern of visceral afferents (Cervero 1985; Sugiura et al. 1989).

FOS EXPRESSION AFTER NONNOXIOUS STIMULATION

To further validate the use of Fos immunocytochemistry to monitor activity in pain pathways in the CNS, it was important to show that the pattern of Fos immunoreactivity in the cord following a nonnoxious stimulus was different from that seen following a noxious stimulus. Initial work by Hunt and colleagues (1987) suggested that this would be difficult to document. In their study, nonnoxious stimuli produced only minimal increases in Fos immunoreactivity, albeit in appropriate regions of the dorsal horn. However, Gogas and colleagues (1990) found that allowing an animal to walk on a slow-turning rotarod device for 1 hour evokes widespread Fos immunoreactivity in the cord. Fos expression was notably lacking in laminae I and Ilo of the cervical and lumbar segments. However, the inner portion of lamina II as well as laminae III and IV, areas that contain neurons that respond exclusively to nonnoxious stimulation (Bennett et al. 1980; Light et al. 1979), contained many labeled cells. Additional staining was found in the medial portion of laminae V and VI, an area that receives inputs from joint afferents (Brown and Fyffe 1978, 1979), and in lamina IX, where many Fos-immunoreactive MNs were found. These results are important since they provide considerable support for the hypothesis that Fos protein expression is stimulus specific and can, therefore, be used to study those populations of neurons in the cord that are activated by noxious stimulation. Furthermore, the modulation of noxious stimulus-evoked Fos expression by pain-relieving drugs such as opioids can also be studied, which can provide information about the mechanisms by which these compounds produce analgesia.

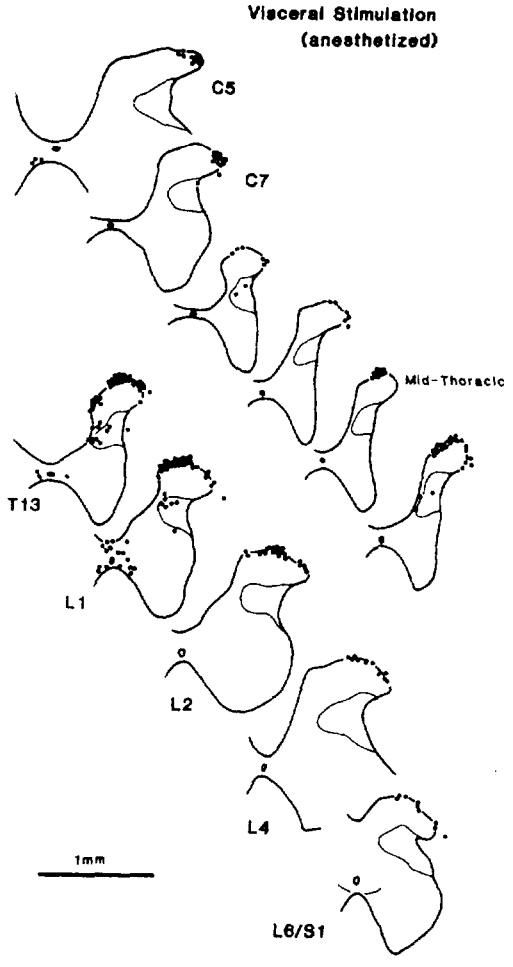

FIGURE 4. *These schematics illustrate the cervical (C5) through lumbrosacral (L6-S1) distribution of Fos-immunoreactive cells in response to noxious visceral stimulation. Note that the densest staining is at the thoracolumbar junction (T13-L1), and the most extensive rostrocaudal distribution of labeled cells was found in the superficial dorsal horn.*

SOURCE: Menérey, D.; Gannon, A.; Levine, J.D.; and Basbaum, A.I. 1989. *Journal of Comparative Neurology.* Copyright © 1989 by Wiley-Liss. Reprinted by permission of Wiley-Liss, a division of John Wiley and Sons, Inc.

THE EFFECTS OF ANALGESICS ON NOXIOUS STIMULUS-EVOKED FOS EXPRESSION

Opioids produce their analgesic effects by acting at μ-, δ-, or κ-opioid receptors (for a review, see Martin 1983) that are located at both supraspinal and spinal levels (Basbaum and Fields 1978, 1984). At supraspinal sites, opioids are believed to activate neurons in the midbrain periaqueductal gray, which in turn excite neurons of the medullary nucleus raphe magnus. The latter neurons project to and inhibit spinal cord nociresponslve neurons. Opioids also act at opioid receptors in the spinal cord where they can directly inhibit incoming nociceptive transmission in the dorsal horn (Yaksh and Noueihed 1985). If formalin-evoked Fos immunoreactivity in the spinal cord is, as proposed, related to the activity of nociresponsive neurons, then doses of an opioid, such as morphine, that produces behavioral analgesia should also inhibit noxious stimulus-evoked Fos expression.

THE EFFECTS OF SYSTEMIC MORPHINE ON FORMALIN-EVOKED FOS EXPRESSION

Presley and colleagues' (1990) initial studies using systemic morphine injection supported the authors' hypothesis. Systemic administration of morphine, 10 minutes prior to formalin injection, produced a dose-related inhibition of both the pain behaviors and Fos immunoreactivity evoked by the formalin (figure 5). At the highest dose tested (10 mg/kg, IP), morphine produced a complete suppression of the formalin-evoked behaviors and greatly attenuated the levels of formalin-evoked Fos immunoreactivity; however, labeled cells could still be detected in the superficial dorsal horn. The effects of this dose of morphine were reversed by the opiate receptor antagonist, naloxone, consistent with activity of morphine at an opiate receptor. The finding that morphine could produce complete behavioral analgesia without completely blocking noxious stimulus-evoked Fos expression at the level of the cord is important. Apparently, analgesia can be achieved without completely "shutting off the activity of dorsal horn neurons in the spinal cord.

As described above, it is important to show not only that the pattern of Fos immunoreactivity is stimulus specific but also that the levels of Fos protein in the cord are related to the expression of an overt, recordable behavior. In fact, the number of Fos-immunoreactive neurons and the magnitude of the animal's behavioral response to formalin are closely related; animals that exhibit the highest levels of pain-related behaviors have many Fos-immunoreactive neurons in the spinal cord (figure 6). Conversely, animals that are completely analgesic exhibit much lower levels of Fos immunoreactivity. This finding provides further evidence that Fos expression is a valid measure of neuronal activity.

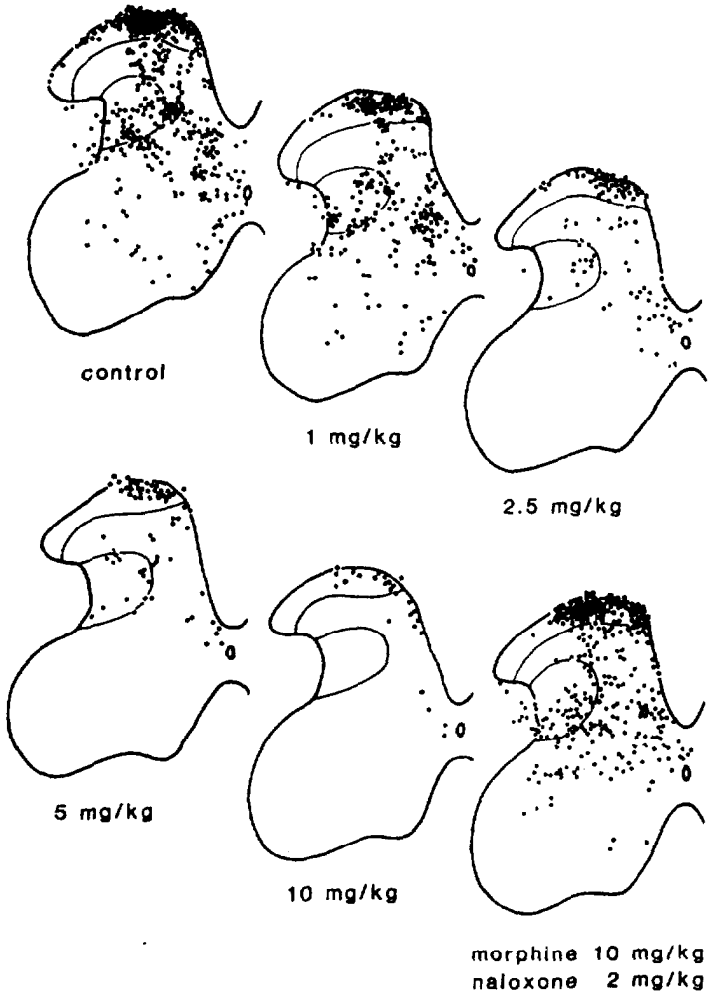

FIGURE 5. *Camera lucida drawings of representative L4-L5 sections showing a dose-related, naloxone-reversible inhibition of formalin-evoked Fos-like immunoreactivity (FLI) by morphine. The labeling in the ventral gray matter is profoundly suppressed by 2.5 mg/kg; at any given dose, the region with the most residual staining is the superficial dorsal horn.*

SOURCE: Presley et al. 1990. Copyright 1990 by the Society for Neuroscience (New York).

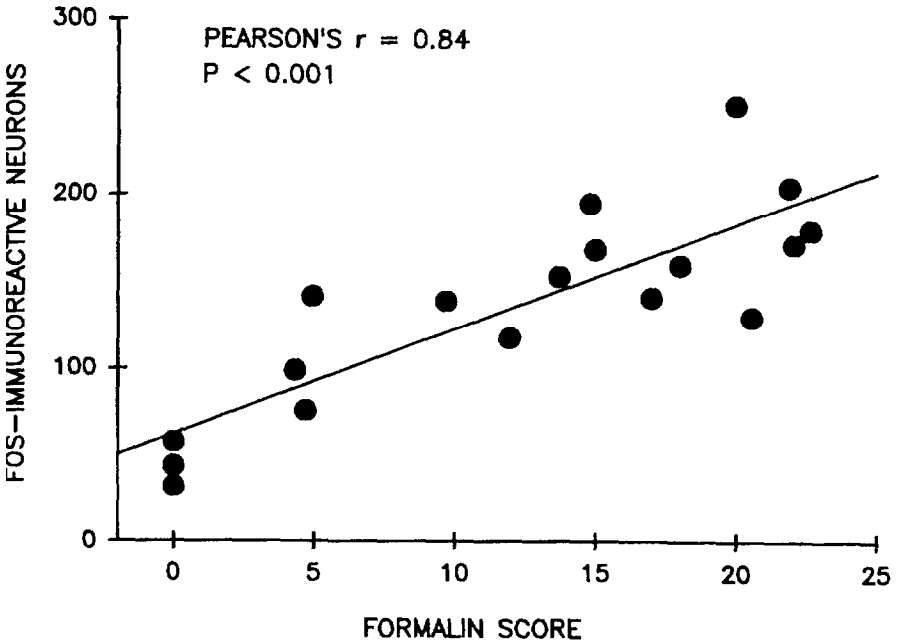

FIGURE 6. *Correlation between noxious stimulus-evoked "pain" behavior and spinal cord FLI. The mean area-under-the-curve (AUC) scores for behavior (abscissa) are plotted against the overall mean FLI values (i.e., the mean sum of the superficial+nucleus proprius +neck+ventral FLI; ordinate) for each of the six intracerebroventricular (ICV) drug treatments used (0.006, 0.02, 0.06, 0.20, 0.60 µg DAMGO (D-Ala^2 NMe—Phe^4, Gly-OL^5-enkephalin) or saline; n=3 per group). The expression of pain behavior was significantly correlated with the number of neurons expressing FLI (Pearson's r=0.84, p<0.001).*

SOURCE: Reprinted with permission from *Neuroscience* 42, Gogas, K.R.; Presley, R.W.; Levine, J.D.; and Basbaum, A.I. The antinociceptive action of supraspinal opioids results from an increase in descending, inhibitory control: Correlation of nociceptive behavior and c-fos expression, 1991, Pergamon Press, plc.

That the number of Fos-immunoreactive neurons in individual regions of the cord can be quantified further allows the differential regulation by morphine of the activity of neurons in functionally distinct areas of the cord to be determined (figure 7). Importantly, the dose-response curve for the effects of morphine

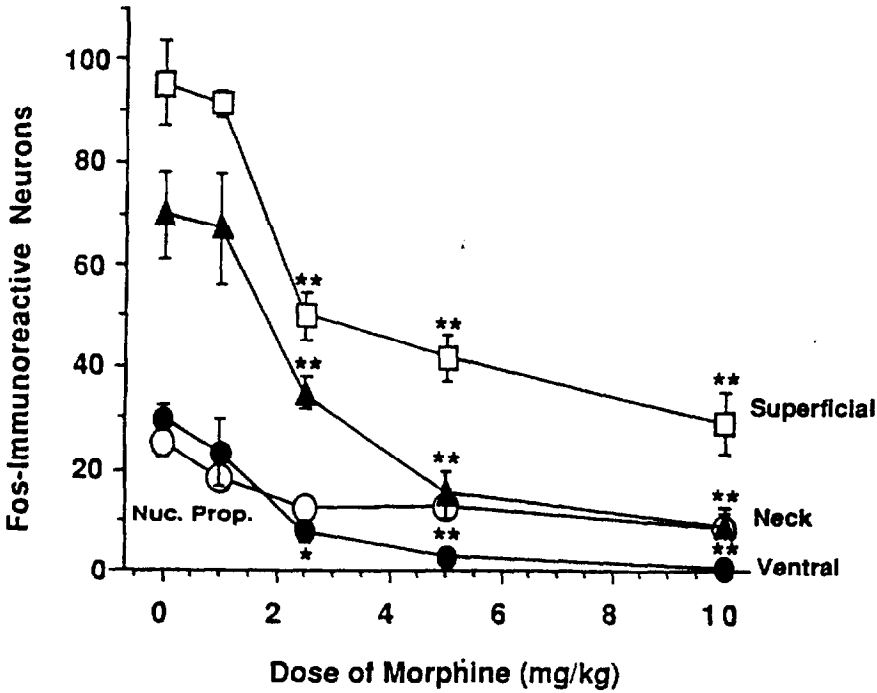

FIGURE 7. *Dose of morphine vs. the number of Fos-immunoreactive neurons in different laminar regions of the spinal gray matter (meant SEM), including the supeficial dorsal horn (laminae I and II), the nucleus proprius (laminae III and IV), the neck of the dorsal horn (laminae V and VI), and the ventral gray (laminae VII, VIII, and X). There was a statistically significant suppression of Fos-immunoreactive neurons after doses of morphine 2.5-10 mg/kg (*p<0.05, **p<0.01) in the superficial dorsal horn, in the neck of the dorsal horn, and in the ventral gray. The superficial dorsal horn consistently contained the greatest number of residual labeled cells; this was particularly evident at higher doses of morphine (5.0 and 10.0 mg/kg). In contrast, labeling in the ventral gray was eliminated by these doses. The dose-response curve for the nucleus proprius was flat, with no statistically significant suppression of labeling.*

SOURCE: Presley et al. 1990. Copyright 1990 by the Society for Neuroscience (New York).

on Fos expression in the nucleus proprius, the region that contains primarily nonnociresponsive neurons, was flat; that is, no significant inhibition of Fos expression at any of the morphine doses tested was found. This is consistent with the fact that morphine predominantly inhibits nociresponsive neurons (Le Bars et al. 1976a, 1976b). By contrast, the inhibition of Fos immunoreactivity in the superficial dorsal horn, laminae V and VI, and the ventral horn of the cord was dose related. The dose-response curves in laminae V and VI and the ventral horn were steeper than in the superficial laminae. However, even at the highest dose tested, which produced 100 percent inhibition of behavioral analgesia, there was still significant residual "activity" in all areas, particularly the superficial cord. Since a major component of the residual staining was found in the marginal layer (lamina I) of the superficial dorsal horn, a region that contains neurons that respond almost exclusively to noxious stimulation, this finding is of particular interest. However, lamina I contains a mixture of projection and interneurons. In a double labeling study, Menétrey and colleagues (1969) found that approximately 45 percent of marginal layer neurons that express Fos following noxious stimulation project to regions of the brain that have been implicated in the rostral transmission of nociceptive information. Conceivably, opioids preferentially inhibit the population of lamina I projection neurons; that is, the residual labeling may be concentrated in a population of interneurons that do not contribute to the “pain”-related response. That retrograde labeling and Fos immunocytochemistry can be combined (figure 8) makes it possible to determine whether opioids produce a selective reduction in the number of retrogradely labeled marginal cells that express Fos immunoreactivity. This possibility is being tested. Again, it is important to emphasize that this son of experiment could not be done without using Fos immunoreactivity as a marker for populations of neurons activated by noxious stimulation.

THE EFFECTS OF SUPRASPINALLY ADMINISTERED OPIOIDS ON NOXIOUS STIMULUS-EVOKED FOS EXPRESSION

Since systemically administered morphine produces analgesia via an action at both spinal and supraspinal sites (Yaksh and Noueihed 1985; Basbaum and Fields 1984), we could not distinguish the relative contribution of either site to the inhibitory effects found. One approach to addressing this issue was to separately test the effects of supraspinal and intrathecal (IT) administration of morphine on formalin-evoked Fos expression in the cord. It was found that administration of morphine into the third ventricle (ICV) or directly onto the spinal cord (IT) inhibits both formalin-evoked behaviors and Fos immunoreactivity (K.R. Gogas, G.I. Batchkine, H.J. Cho, and A.I. Basbaum, manuscript in preparation). Since morphine is relatively nonselective for μ-, δ-, or κ-receptors (Martin 1983), it is still not clear which opioid receptor subtype

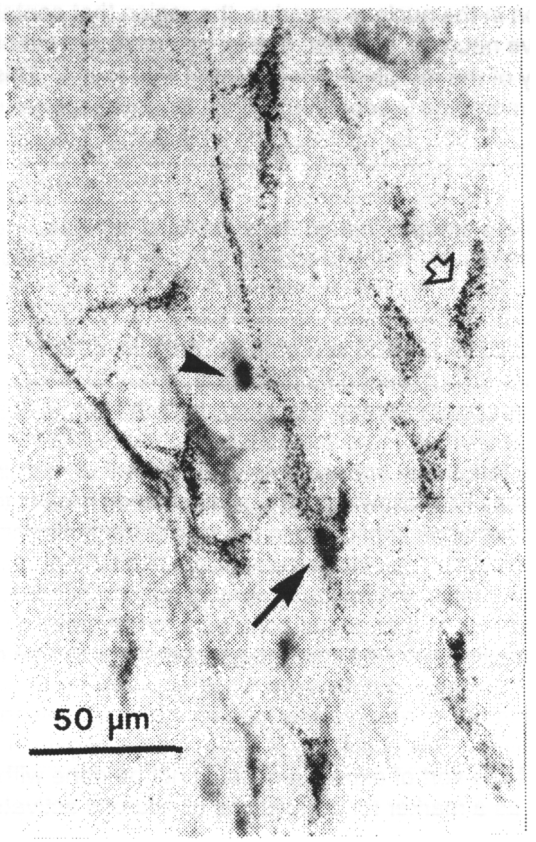

FIGURE 8. *This bright-field photomicrograph illustrates Fos-immunoreactive and retrogradely labeled neurons in a 40-µm thick horizontal section through laminal of the lumbar dorsal horn. The retrograde tracer WGAapoHRP-AU was injected into the contralateral brain stem and thalamus. Fos-positive cells* have *a uniformly stained nucleus (➤). Projection neurons contain punctate cytoplasmic label that results from silver intensification of the colloidal gold in the tracer; the nuclei of single-labeled projection neurons are unstained (⇨). Double-labeled cells (→) have a densely stained nucleus and cytoplasmic silver precipitate.*

SOURCE: Menétrey, D.; Gannon, A.; Levine, J.D.; and Basbaum, A.I. 1989. *Journal of Comparative* Neurology. Copyright © 1989 by Wiley-Liss. Reprinted by permission of Wiley-Liss, a division of John Wiley and Sons, Inc.

morphine is acting on to produce its effects. There is considerable controversy concerning the specific circuitry by which oploids such as morphine produce antinociception (e.g., Fang et al. 1986; Roerig and Fujimoto 1989; Heyman et al. 1987). In addition, it is not clear whether activation of μ-, δ-, or κ·receptors produces analgesia.

This pharmacological question is ideally suited to analysis with Fos immunocytochemistry. Specifically, by studying the effects of selective agonists for either μ-, δ-, or κ- opioid receptors on both formalin-evoked pain behavior and on spinal cord Fos immunoreactivity, not only can it be determined whether these compounds are analgesic, but also the circuitry through which they produce their analgesic effects can be examined. We started this series of experiments by studying the effects of supraspinal administration of the μ-selective agonist DAMGO on pain behaviors and on spinal cord Fos expression evoked by noxious stimulation in rats. For ICV drug infusions, guide cannulae were stereotaxically implanted in the third ventricle of rats. After surgery, the animals were allowed to recover for 7 to 10 days prior to behavioral testing. For dose-response studies, ICV injections were made 10 minutes prior to injection of formalin into the hindpaw. Immediately thereafter, the animal was placed into a Plexiglas chamber so that the animal's movements could be viewed and its behavior recorded over a 1-hour period as discussed above.

The laminar distribution of formalin-evoked Fos expression in the spinal cord was similar to that seen previously (figure 9, panel A). DAMGO (ICV) produced a dose-related, naloxone-reversible suppression of both the nociceptive behaviors and spinal cord Fos immunoreactivity evoked by formalin (figure 9). Since the dose-response curve for ICV DAMGO spanned two orders of magnitude (0.006 to 0.60 μg), the authors were able to calculate estimates of the dose required to produce a 50-percent reduction (i.e., ED_{50}) in both pain behaviors and Fos immunoreactivity. It was found that ICV DAMGO exhibits similar potencies for inhibition of pain behavior ($ED5_0$ =0.06 μg) and overall Fos immunoreactivity (ED_{50}=0.05 μg); this suggests that there is a similar mechanism of action for the two measures. There was a significant correlation between the expression of behavioral nociception and overall Fos immunoreactivity produced by formalin (figure 6), again supporting the contention that noxious stimulus-evoked Fos immunoreactivity can be used as a marker for nociceptive neurons.

Although the potencies for DAMGO-mediated inhibition of formalin-evoked pain behaviors and Fos immunoreactivity were similar, the maximal levels of inhibition (i.e., Emax) for each measure were different. Specifically, a dose of DAMGO that produced 100-percent inhibition of behavioral nociception produced only 81 percent overall inhibition of Fos immunoreactivity. When the

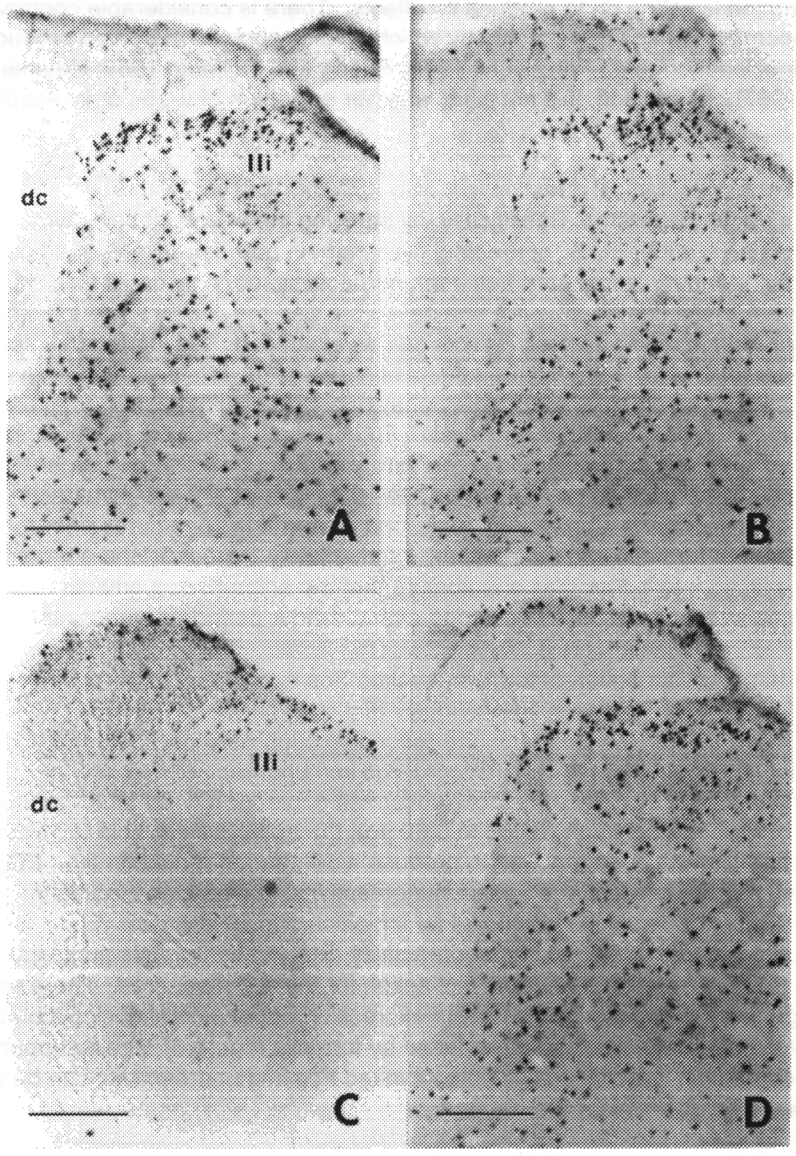

FIGURE 9. *Photomicrographs showing the pattern of formalin-evoked FLI in neurons from sections of the lumbar enlargement of rats that received ICV injections of saline (A), 0.06 µg DAMGO (B), 0.60 µg DAMGO (C), or 0.60 µg DAMGO plus 1.0 µg naloxone (D).*

In saline-treated rats (A) there is dense labeling in laminae I and II as well as *in the neck of the dorsal horn and the ventral horn. Pretreatment with 0.06 µg ICV DAMGO (B) produced 50-percent inhibition of overall FLI (i.e., superficial FLI+nucleus proprius FLI+neck FLI+ventral FLI) A quantitative analysis of the number of neurons expressing FLI in the superficial, nucleus proprius, neck, or ventral regions of the* cord showed that *this dose produced close to 50-percent inhibition in the neck and ventral regions but had minimal effect on the level of FLI in the superficial layers. Pretreatment with 0.80 µg DAMGO (C) produced approximately 84-percent inhibition of the FLI in the neck and ventral regions but only 64-percent inhibition of the FLI in the superficial dorsal horn. The effects of 0.60 µg DAMGO were completely reversed by naloxone (D). Calibration bars=250 µm; dc=dorsal column; Ili=inner laminae II.*

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potencies of ICV DAMGO were considered in different laminae (figure 10), it was found that there was approximately a fourfold difference in the estimated ED,, values for the laminae V/VI or ventral horn vs. the superficial laminae; thus, at doses of 0.04 to 0.06 µg ICV there was an approximate 50-percent decrease in both the behavioral response and the Fos expression in laminae V-X. By contrast, the dose-response curve for DAMGO-mediated inhibition of Fos immunoreactivity in the superficial cord was shallow. In fact, at the ED,, doses for inhibition of Fos expression in the neck and ventral regions of the cord, there was less than 1 O-percent inhibition of Fos in the superficial dorsal horn.

These data are consistent with previous findings with systemic morphine (Presley et al. 1990), which also showed that complete behavioral analgesia could be produced without eliminating the Fos immunoreactivity in the superficial cord. On the other hand, our studies with both systemic and ICV morphine showed that a dose of morphine that produced approximately 50 percent inhibition of Fos immunoreactivity in the neck and ventral cord produced a comparable suppression in the superficial dorsal horn Fos immunoreactivity. This contrasts with our findings using DAMGO in which doses that produced a 50-percent reduction in Fos immunoreactivity in the

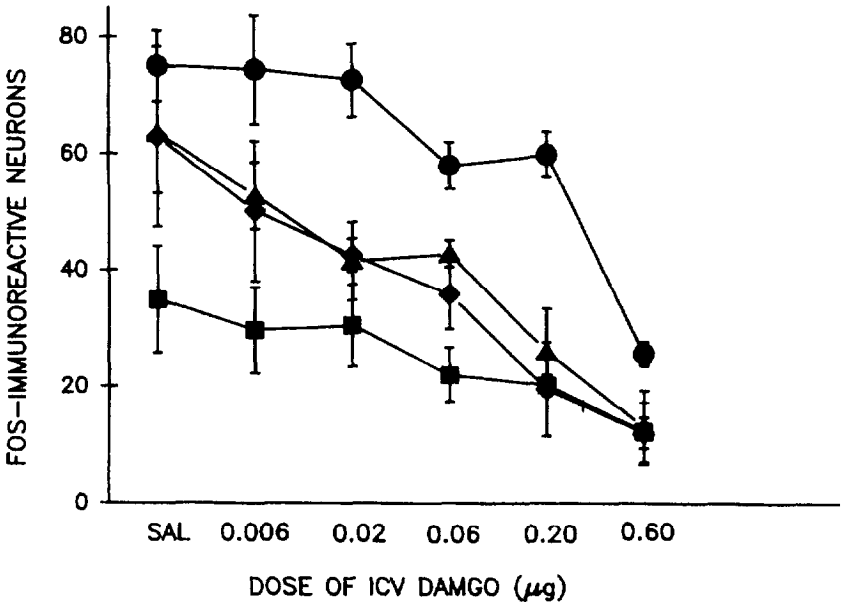

FIGURE 10. *Effects of ICV DAMGO on noxious stimulus-evoked FLI in the spinal cord, Animals (n=3 per dose) were treated and the tissue processed as described in the text Results are expressed as mean (±SEM) number of neurons per 50-μm section expressing FLI in either the superficial (•), nucleus proprius (■), neck (▲), or ventral (◆) portions of the ipsilateral spinal cord. Drug treatments are indicated on the abscissa. The potency for CV DAMGO-mediated inhibition of FLI in the superficial region (ED_{50}=0.18 μg) was approximately four times less than for either the neck or ventral regions (ED_{50}=0.04 and 0.05 μg, respectively). The curve for inhibition of the nucleus proprius was flatter than for the other regions of the cord, with significant inhibition only at 0.60 μg ICV DAMGO. Note that the highest dose of ICV DAMGO tested (0.60 μg) produced 100-percent inhibition of pain-related behaviors without producing comparable levels of inhibition in FLI at the level of the cord.*

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neck and ventral cord had almost no effect in the superficial dorsal horn. We hypothesize that the more profound suppression of Fos immunoreactivity in the superficial dorsal horn seen following systemic doses of morphine results from the fact that after systemic administration there Is both a supraspinal and spinal contribution to analgesia.

Since DAMGO is a potent and selective μ-opioid receptor agonist (Handa et al. 1981), and since morphine, when given supraspinally, is also thought to act preferentially at the F-receptor (Fang et al. 1986; Roerig and Fujimoto 1989), the contrast between the levels of superficial suppression following ICV morphine and DAMGO is particularly interesting. Thus, morphine (ICV) produced a maximal inhibition (i.e., 65 percent) of superficial dorsal horn Fos immunoreactivity at the lowest dose tested (2.5 μg); there was no greater inhibition when higher doses were tested. By contrast, the dose-response curve for ICV DAMGO-mediated Fos suppression in the superficial cord was shallow. In fact, significant inhibition occurred only at the highest dose tested (0.60 μg), If one considers this difference in terms of the relationship between inhibition of superficial dorsal horn neuronal activity and behavioral antinociception, it can be seen that doses of ICV morphine and DAMGO that produce 50-percent inhibition of behavioral nociception produce 65-percent and cl O-percent inhibition of superficial Fos immunoreactivity, respectively. Since DAMGO is thought to be a much more efficacious agonist than morphine, it is highly unlikely that the differences between the two compounds relate to differences in activity at the v-receptor. Rather, these differences suggest that ICV morphine acts at another site in addition to the p-receptor, possibly the δ-receptor. Alternatively, since some researchers have provided evidence for the existence of subpopulations of μ-opioid receptors in brain (Wolozin and Pasternak 1981; Rothman et al. 1983), it is possible that the μ-receptor populations with which morphine and DAMGO interact are different. Regardless of the receptor(s) through which morphine acts to produce its effects, it is clear that by monitoring the regulation of Fos protein expression by these compounds, in addition to monitoring behavioral effects, much new information on the mechanisms through which these drugs work can be obtained.

THE EFFECT OF DORSOLATERAL FUNICULUS LESIONS ON OPIOID-MEDIATED INHIBITION OF NOXIOUS STIMULUS-EVOKED FOS IMMUNOREACTIVITY

It is commonly believed that analgesic compounds, such as morphine, activate descending systems within the brain stem, which in turn inhibit spinal cord nociresponsive neurons (Basbaum and Fields 1978, 1984). For example, supraspinal microinjection of morphine attenuates behavioral responses in

nociceptive tests, such as the tail-flick or hot-plate test (Jensen 1988; Tung and Yaksh 1982), and intracerebral injection of morphine inhibits the firing of dorsal horn neurons that respond selectively to noxious stimuli (Bennett and Mayer 1979; Du et al. 1984; Gebhart et al. 1989). Furthermore, the antinociceptive effects of systemic or intracerebral injection of morphine are attenuated by lesions of the brain stem or spinal cord (Barton et al. 1980; Basbaum et al. 1976, 1977) and can be antagonized by IT injection of monoamine antagonists (Proudfit and Hammond 1981; Jensen 1986). However, there is contradictory neurophysiological evidence that indicates that intracerebral and ICV morphine decreases, rather than increases, descending controls to produce analgesia (Bouhassira et al. 1988; Dickenson and Le Bars 1987; Duggan et al. 1980). The authors' results provide strong evidence in support of the hypothesis that both ICV morphine and DAMGO produce analgesia and suppression of spinal cord Fos immunoreactivity by increasing descending controls.

To specifically test this hypothesis, Gogas and colleagues (1991) studied the effects of the administration of supraspinal opioids in animals that underwent bilateral lesions of the spinal cord pathway through which opioids exert their effect (see the dorsolateral funiculus [DLF]). They predicted that DLF lesions would totally block the opioid-mediated behavioral analgesia and the suppression of spinal cord Fos immunoreactivity. In fact, the dose of ICV DAMGO that produced 100-percent behavioral analgesia in normal animals (0.60 µg) had no effect on either behavior or Fos immunoreactivity in animals with DLF lesions (figure 11). This finding supports the conclusion that supraspinal opioids produce their analgesic effects by increasing, rather than decreasing, the activity of descending control systems. This experiment again illustrates the advantage of monitoring large populations of neurons with Fos immunocytochemistry to answer questions that previously could be addressed only at the single-cell level.

CONCLUSION

The examples discussed in this report demonstrate the usefulness and validity of monitoring Fos expression to assess the activity of populations of CNS neurons. However, several important caveats need to be underlined when interpreting the results of these experiments. First, there is no unequivocal way of knowing whether a Fos-expressing neuron is, in fact, nociresponsive. This assumption is based on electrophysiological characterization of neurons in the different spinal cord laminae. Similarly, if a neuron does not express the Fos protein, one cannot conclude that the unlabeled neuron is not nociceptive. Also, since the function of the Fos protein in spinal cord neurons is not presently known, no conclusions can be drawn regarding the biological significance of a neuron expressing Fos immunoreactivity. In spite of these

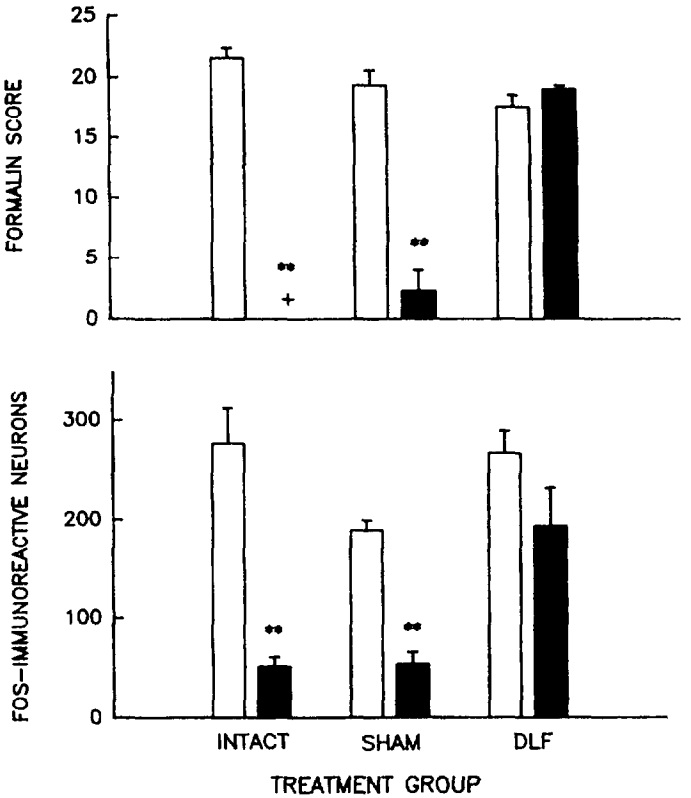

FIGURE 11. *Effects of DLF lesions on noxious stimulus-evoked "pain" behaviors and on overall FLI. Results are expresseb as mean (±SEM, ordinate) behavioral AUC scores (top) or number of neurons per 50-µm section expressing FLI (bottom) in rats receiving either ICV saline (open bars) or DAMGO (0.60 µg; filled bars). DAMGO significantly inhibited both the pain-response and FLI expression in both intact and sham rats. These inhibitory effects were reversed in rats with DLF lesions. The mean response for DAMGO-treated intact animals was zero; this is indicated by + (top). **Indicates groups significantly different from saline controls by either Dunnett's (top) or Neuman-Keuls (bottom) post-hoc tests (p<0.01).*

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limitations, Fos expression can be used as a marker to tell whether and when something happens to a particular neuron. Importantly, the findings (1) that the distribution of noxious stimulus-evoked Fos immunoreactivity closely parallels the known distribution of spinal nociceptors, (2) that there is a significant correlation between the expression of noxious stimulus-evoked pain behaviors and spinal cord Fos expression, and (3) that noxious stimulus-evoked Fos immunoreactivity is suppressed by systemic as well as supraspinal administration of opioid analgesics, such as morphine and DAMGO, strongly suggest that this approach can be used to study the mechanisms by which drugs produce analgesia.

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The Ontogeny of Immediate Early Gene Response to Cocaine: A Molecular Analysis of the Effects of Cocaine on Developing Rat Brain

Barry E. Kosofsky and Sfeven E. Hyman

EPIDEMIOLOGY

Current estimates suggest that between 13 and 17 percent of infants born in urban hospitals in the United States have been exposed to cocaine in utero (Frank et al. 1988). Multivariate analysis has shown that mothers who abuse cocaine during gestation have infants with a 93-g lower birth weight, a .17-centimeter decrement in length, and a .43-centimeter smaller head circumference (Zuckerman et al. 1989). The dramatic decrease in head size is a further indication of in utero effects of maternal cocaine on the developing brain. Drug-induced changes in brain development are likely to account for the hyperreflexia, increased irritability, alterations in tone and coordination, and developmental delay, including fine motor and visual motor skills, seen in some infants exposed to cocaine in utero (Kosofsky 1991).

CENTRAL NERVOUS SYSTEM MATURATION

Clinical data suggest that infants exposed to cocaine only during the first trimester of gestation are normal size at birth but show a statistically significant compromise of the Neonatal Behavioral Assessment Scale (Brazelton), with poor orienting, poor motor ability, and abnormal reflexes (Chasnoff et al. 1989). The implications are that the window of vulnerability to cocaine for developing brain may begin early in gestation and that the toxicity ultimately manifested may be dependent on the time of fetal exposure. The protracted timetable of central nervous system (CNS) maturation confers a continuum of biologic vulnerability to developing brain. Moreover, the developmental consequences of a toxic CNS insult relate critically to the period during which the fetus is exposed to drugs (Evrard et al. 1989).

CNS development requires a complex coordination of genetic factors and environmental forces that direct brain maturation and shape infant development in a reproduceable, yet individualized manner. The cellular events underlying these processes require differential gene transcription. The authors hypothesize that the toxic developmental effects of substances of abuse may be mediated via perturbations of normal patterns of gene expression. To address this question, we have conducted experiments in an animal model, exposing developing rat brain to cocaine, permitting identification and characterization of some of the earliest cellular events (and targets) mediating drug effects. This analysis focuses on a class of immediate early genes (IEGs) that act as transcription factors (IEGs c-*fos,* c-*jun,* and *zif/268)* that may subserve one mechanism by which substances of abuse alter genetic programs during development.

IMMEDIATE EARLY GENES

The molecular mechanisms underlying the neuronal response to cocaine exposure remain elusive. Recently, IEG response has been used as a marker of neuronal activation in various CNS stimulation paradigms, including models of seizure (Morgan et al. 1987; Sonnenberg et al. 1989), nociceptive stimulation (Draisci and ladarola 1989; Naranjo et al. 1991), and circadian neurobiology (Aronin et al. 1990). IEGs are called proto-oncogenes because they represent the normal cellular homoiogues of transforming viral oncogenes (Morgan and Curran 1991). Nuclear proto-oncogenes function in cellular signal transduction as transcription factors binding to the promoters of target genes, thereby influencing transcription of specific gene products. Drug-induced alterations in IEG expression can modify gene expression during development and thereby alter cellular identity and neuronal repertoire. The IEGs *c-fos,* c-*jun,* and *zif/268* are of particular interest because they demonstrate rapid and dramatic induction in specific brain areas of adult rats exposed to cocaine or amphetamine (see other chapters in this volume). In most cell types, basal levels of c-fos and *c-jun* mRNA and of c-Fos and c-Jun protein are relatively low (Morgan and Curran 1991). in contrast, certain cells maintain relatively high basal levels of *zif/268* mRNA and *Zif/268* protein (Worley et al. 1991). The induction of c-*fos,* c-*jun,* and *zif/268* in response to novel stimuli in neurons follows a similar pattern; mRNA transcription occurs within 5 minutes and continues for approximately 20 minutes, with peak steady-state mRNA levels at 30 to 45 minutes and peak protein synthesis at about 2 hours poststimulation (not shown).

Activation of IEGs by Cocaine

The constraints on drug-induced IEG repertoire imposed by cell phenotype in adult rats were addressed by Young and colleagues (1991) and Graybiel and colleagues (1990), who reported that cocaine-stimulated *fos* induction in striatum could be completely blocked by pretreatment with a D1 antagonist (SCH 23390). Interpretation of experiments employing the D2 antagonist (sulpiride) was complex because D2 antagonists independently activate *c-fos* expression. Coupled with the knowledge that In striatum different (though not mutually exclusive) populations of neurons have D1 vs. D2 receptors (Gerfen et al. 1990), one can conclude that receptor phenotype of striatal neurons is one of the cellular determinants conferring cocaine inducibility of IEG expression. This idea is supported by anatomic studies (Berretta et al. 1991) that have identified the subset of neurons in adult rat striatum that express c-Fos after cocaine administration as containing DARP-32, a marker for neurons expressing D1 receptors. By analogy, the susceptibility of the developing brain to be affected by cocaine may relate to the maturation of presynaptic (e.g., aminergic projections), postsynaptic (e.g., D1 receptors), and/or intrinsic (e.g., cellular signal transduction pathways) features specific for a given cell type in a given brain region at a particular point in development.

EXPERIMENTAL RESULTS

The authors have performed northern gel analyses to characterize the acute induction of the IEGs c-*fos,* c-*jun,* and *zif/*268 in three areas of rat brain: dorsolateral neocortex, striatum, and cerebellum. Rats were examined at four different ages: postnatal (P) day 8 (P8, roughly equivalent to term for a human fetus), 2 weeks of age (P15), 4 weeks of age (P28), and at maturity. Animals were injected with cocaine (40 mg/kg in .9 percent saline) and sacrificed 45 minutes later, when regional brain dissections were performed on ice. Total cellular RNA was prepared by a modification of the method from Berger and Chirgwin (1989). Samples were homogenized in 4M guanidinium isothiocyanate followed by centrifugation through a 5.7M cesium chloride pad. RNA was quantitated, and 30-µpg samples were run on a formaldehyde gel. RNA was transferred to a nylon membrane, cross-linked, and hybridized with nick-translated probes for c-*fos,* c-*jun,* or *zif/*268 and for cyclophilin (unregulated internal reference gene). Autoradiograms *were* quantitated with densitometry and represented graphically. Figure 1 (P8), figure 2 (P15), figure 3 (P28), and figure 4 (adult) depict severalfold induction (vs. saline-injected controls) of IEGs by region, representing an average from two experiments for each plot (three to four animals pooled for each condition, for each experiment). There is considerable specificity evident with regard to

which IEG is activated in which brain area at what point in development. At the earliest age examined (figure 1, P8), IEG activation is limited to the striatum, where *c*-fos and *zif/*268 are Induced. By P15 (figure 2) and thereafter (P28, figure 3; adult, figure 4) all three IEGs are induced in striatum and cortex. The elevation of c-jun and *zif/*268 in cortex and striatum at these ages was modest (1.2 to 2.2x) but consistent. C-*fos* induction was more significant in cortex (1.8 to 2.1x) and was dramatically increased in striatum (4.6 to 6.6x). At all ages examined, striatal c-fos demonstrated the most significant cocaine-induced IEG elevation. In cerebellum, *c-jun* and *zif/*268 were not induced at any age examined. At this dose, but not at 30 mg/kg, c-*fos* was induced in cerebellum, but only on or after P15 (data not shown).

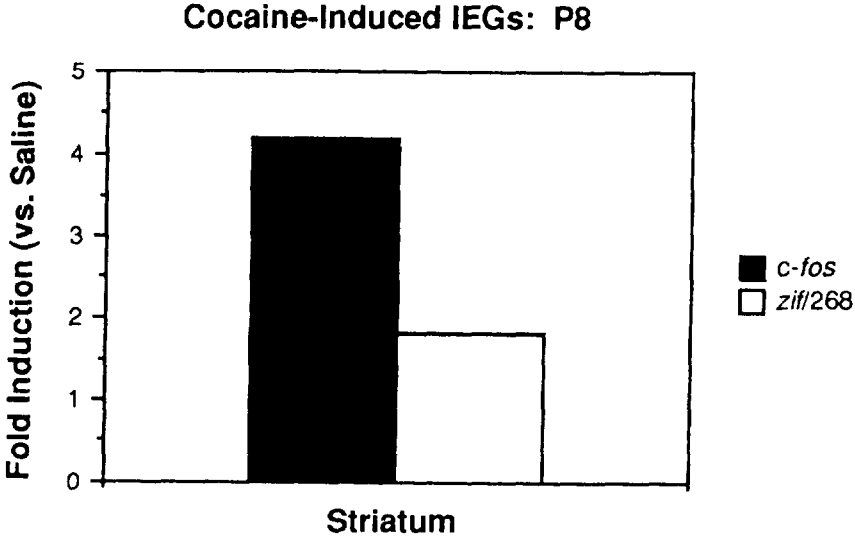

FIGURE 1. *Induction of c-fos mRNA (4.2-fold) and zif/268 mRNA (1.8/fold) in striatum of P8 rats treated with cocaine, 40 mg/kg/intraperitoneal (IP), vs. saline. Rats were sacrificed 45 minutes after injection, At this age, changes in striatal c-jun were not evident, nor was induction of c-fos, c-jun, or zif/268 evident in neocortex or cerebellum.*

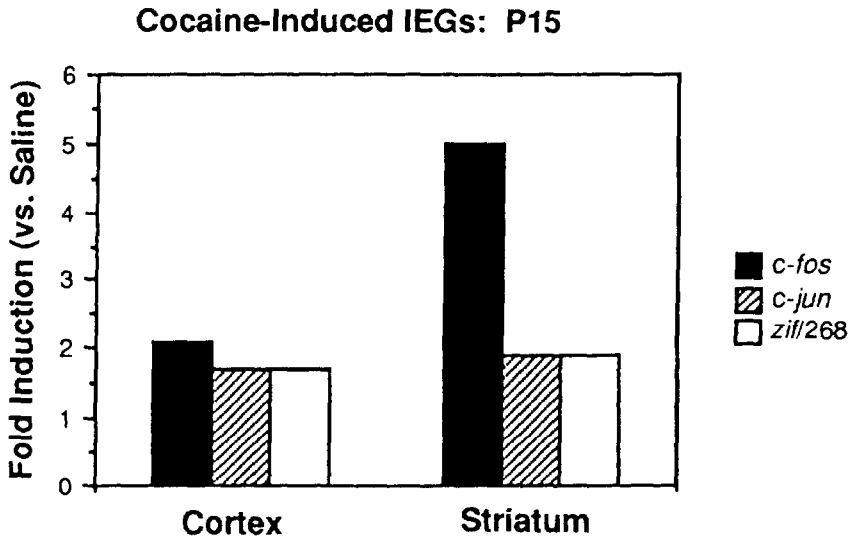

FIGURE 2. *Graph depicting induction (vs. saline) of c-fos, c-jun, and zif/268 mRNA in cortex and striatum of 15-day-old rat pups sacrificed 45 minutes after IP injection of cocaine, 40 mg/kg. All three IEGs are induced in cortex and striatum.*

To characterize the consequences of cocaine-induced striatal IEG activation, the authors first performed gel-shift assays. We examined changes in the level of AP-1 DNA binding activity evident in striata of animals at different ages (P28 and adults) 2 hours after injection with cocaine, 30 mg/kg/IP. Striatal tissue suspended in buffer was homogenized, and protein was isolated as previously described (Nguyen et al., in press). Protein was quantitated and samples preincubated on ice for 15 minutes with poly (dl-dC), with or without cold competitor (100 ng). Samples were then incubated at room temperature for 15 minutes with 1 to 1.5 ng of P^{32}-end-labeled double-stranded oligonucleotide probe and electrophoresed through a 4-percent nondenaturing polyacrylamide gel. On day P28 (figure 5) and in the adult (figure 6), extracts prepared from saline-treated animals demonstrated basal levels of binding activity to the AP-1 DNA sequence. However, cocaine administration augmented AP-1 DNA binding activity 2- to 2.5-fold on day 28 and 3- to 3.5-fold in the adult (representing the average of two samples per condition).

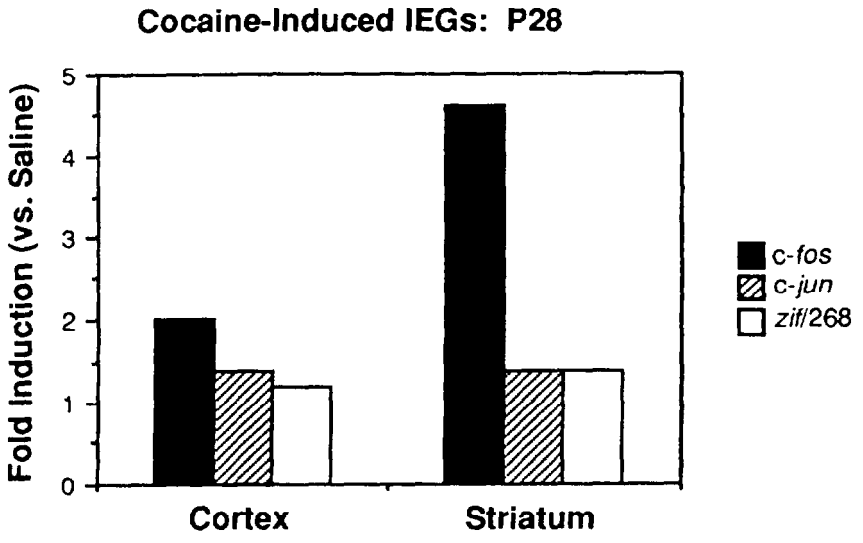

FIGURE 3. *Graph depicting the striatal and cortical induction (vs. saline) of c-fos, c-jun, and zif/268 mRNA in 28-day-old rat pups. Cocaine, 40 mg/kg/IP, was injected 45 minutes prior to sacrifice. C-fos elevation in cortex (2-fold) and striatum (4.6-fold) was greater than that of c-jun or zif/268.*

The specificity of cocaine-induced increases in AP-1 DNA binding activity is demonstrated by competition with unlabeled double-stranded DNA oligonucleotide containing the AP-1 sequence (figure 5, lanes 5 and 6; figure 6, lanes 3 and 7), but absence of competition is demonstrated by an oligonucleotide containing the AP-2 sequence (figure 6, lane 4).

CONCLUSIONS

The authors have demonstrated cocaine-induced changes in gene expression during rat brain development at two levels of analysis: (1) regional and temporal specificity of mRNA induction for the IEGs c-fos, c-jun, and *zif/*268 from northern gel experiments and (2) functional confirmation that in striatum AP-1 DNA binding activity is increased in parallel with the elevation in IEG mRNA described above. These experiments provide evidence that mRNA changes occur within 45 minutes, and AP-1 DNA binding changes occur

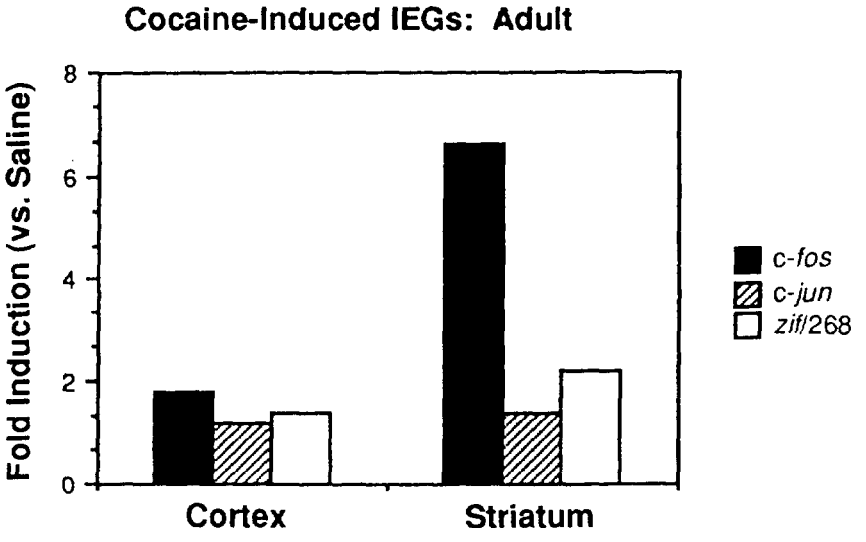

FIGURE 4. *Graph depicting induction (vs. saline) by cocaine (40 mg/kg/IP injected 45 minutes prior to sacrifice) of c-fos , c-jun, and zif/268 in adult cortex and striafum. The most significant response is the induction of c-fos mRNA (6.6-fold) in sfriatum.*

within 2 hours after exposing developing brain to cocaine. One central question concerns the identification of target genes that, by virtue of having AP-1 (or AP-1 -like) DNA binding sequences in their regulatory regions, may be altered in their expression. Candidates include synthetic enzymes for neurotransmitters (e.g., tyrosine hydroxylase), peptide neurotransmitter precursors (e.g., substance P), and growth factors (e.g., nerve growth factor) among many other genes. The regional and temporal specificity of IEG induction by cocaine identifies a mechanism by which selective alteration of target gene expression can be altered during development consequent to drug exposure.

Significance

Exposure of the developing brain to cocaine altered the expression of three IEG transcription factors, which as third messengers may influence the expression of cascades of genes. The implications of the regional and

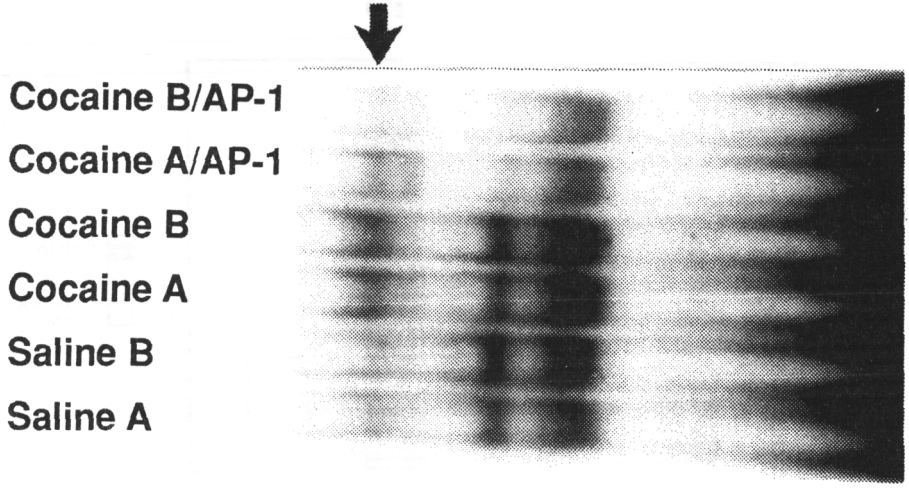

FIGURE 5. *Gel retardation assay performed on striatal extracts (20 µg) from P28 rats prepared from two sets (A and B) of three animals injected IP with saline (lanes 1 and 2) or cocaine 30 mg/kg (lanes 3, 4, 5, and 6) 2 hours before sacrifice. The arrow designates the position of the specific band induced by cocaine administration. Cocaine induces a 2- to 2.5.fold increase in specific AP-1 binding acfivify (compare lane 1 vs. lane 2, and lane 3 vs. lane 4). The specific band is competed by cold AP-1 oligonucleotide at 100-fold excess over labeled probe (lanes 5 and 6).*

temporal specificity of altered IEG expression by cocaine for nervous system development are profound. Normal brain development requires complex and finely tuned genetic coordination, where individual neurons undergo phenotypic differentiation to assume unique chemical, physiologic, and connectional features. The maturation of synaptic circuitry, chemical neurotransmission, and neurophysiologic repertoires that subserve the normal acquisition of developmental skills requires a complex orchestration of genetic regulation and environmental influences. Drug-induced alterations during such critical periods may irrevocably alter CNS form and function. The experiments outlined characterize some of the phenomenology of IEG activation by drugs of abuse in developing rat brain. The significance of this work relates to the hypothesis that (1) as transcription factors, IEGs may play a critical role in the genetic events that shape human behavior and (2) drug-induced IEG dysregulation may have long-lasting structural and functional consequences when perturbed during brain development.

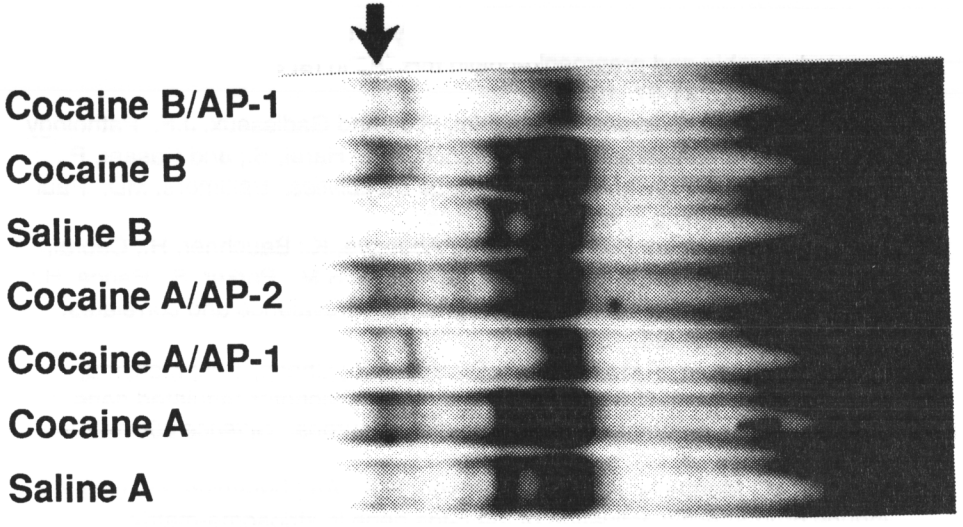

FIGURE 6. *P^{32}-end-labeled AP-1 oligonucleotide binding of striatal extracts (12 µg) prepared 2 hours after IP injection of saline (lanes 1 and 5) or cocaine, 30 mg/kg (lanes 2, 3, 4, 6, and 7) in two sets of adult animals (A and B). The arrow designates the position of the specific band induced by cocaine administration. Basal levels of AP-1 DNA binding activity (lanes 1 and 5) were induced 3- to 3.5-fold by cocaine (lanes 2 and 6). The specificity of binding is demonstrated by competition with cold AP-1 oligonucleotide (lanes 3 and 7), but not by cold AP-2 oligonucleotide (lane 4), both used at 65-fold excess over labeled probe.*

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NMDA Receptor Blockade Prevents Translation, but Not Transcription, of the C-*fos* Gene Following Stimulation With Multiple Extracellular Signals in Cultured Cortical Neurons: Implications for Plasticity and Molecular Memory

Frank R. Sharp, Kinya Hisanaga, and Stephen M. Sagar

INTRODUCTION

Morgan and Curran (1985) first showed that ionic depolarization of neural crest-derived PC12 cells induced the c-*fos* proto-oncogene and that massive brain depolarization produced by metrazole-induced seizures induces c-fos in mouse brain neurons (Morgan et al. 1987). Although metrazole induced Fos-like immunoreactivity (FLI) in cortex, hippocampus, amygdala, and many other structures, it did not induce Fos in all brain structures, nor did it induce Fos in all neurons in each structure.

Hunt and colleagues (1987) showed that noxious sensory stimulation induced FLI in spinal cord neurons. Dragunow and Robertson (1987) showed that kindling induced c-*fos* in granule cells of the rat dentate gyrus. Sagar and colleagues (1988) found that osmotic stimuli induced FLI in hypothalamic paraventricular neurons (Sharp et al. 1991), cortical stimulation induced FLI in cerebellar Purkinje cell neurons (Sharp et al. 1989a), and seizures induced FLI in hippocampus. Sagar and coworkers (1988) suggested that *c-fos* expression could be used to map activated neurons much like the 2-deoxyglucose method has been used to map the central nervous system response to physiological and pharmacological stimuli. Since then, multiple studies have confirmed some of the initial promise of the *c-fos* "mapping" method (Cole et al. 1989). However, several practical and theoretical problems with Fos have become apparent.

First, it is not clear that c-fos can be induced in all neural cells. For example, tactile whisker sensory stimulation does not induce c-fos in any whisker-related pathway. However, section of the infraorbital nerve to the whiskers induces c-*fos* in the spinal trigeminal nucleus pars caudalis and decreases c-*fos* expression in whisker barrel cortex contralateral to the lesion (Sharp et al. 1989b). Therefore, depolarization of neurons in vivo may be necessary, but it is not a sufficient stimulus to induce c-*fos.* This is supported by the finding that light stimulation, which should activate most retinal neurons, induces Fos in a small subpopulation of retinal neurons (Sagar and Sharp 1990). Since depolarization alone cannot account for induction of c-fos, either specific transmitters or the concurrent actions of several extracellular signals might be required for the induction of c-*fos* in neurons. The concurrent activation of two extracellular receptors has been shown to be important for induction of the *c-fos* gene (Curran and Morgan 1985).

Mechanisms of *c-fos* induction in glia appear to be different. In most in vivo studies, *c-fos* appears to be induced in neurons and not in glia. However, Dragunow and Robertson (1988) showed that FLI was induced in glial-like cells at the sites of cortical injury. This was confirmed by Sharp and colleagues (1990). To better define the stimuli responsible for *c-fos* induction in glia following injury, several groups have studied *c-fos* in cultured glia. Arenander and coworkers (1989) found that mitogens and stellation agents induced immediate early genes in cultured rat astrocytes. Hisanaga and coworkers (1990a) showed that c-*fos* is induced in astrocytes with agents that promote differentiation or proliferation, but *c-fos* is not induced in astrocytes with depolarizing agents. Sagar and colleagues (1991) have shown that epidermal growth factor (EGF) and transforming growth factor alpha induce c-*fos* mRNA and Fos protein in identified Muller glial cells of the rabbit retina in vivo. Therefore, it is possible that induction of c-*fos* in glial cells at sites of injury is related to local release of trophic factors that act directly on glia.

Induction of *c-fos* in cortical neurons has been linked to activation of glutamate receptors, Sonnenberg and colleagues (1989) showed that various glutamate agonists, including N-methyl-D-aspartate (NMDA) and kainate, induce Fos, Fos-related antigen, and activator protein-l DNA-binding activity in the mouse brain. Dragunow and Robertson (1988) and Sharp and coworkers (1989b) showed that cortical injury induced c-fos in neurons throughout ipsilateral cortex. Sharp and coworkers (1989b) showed that injection of quinolinic acid, an NMDA agonist, into cortex markedly induced Fos throughout the ipsilateral hemisphere compared with saline-injected subjects. Prior injection of the noncompetitive, NMDA receptor blocker, MK801, into the ventricle before local injury or quinolinic acid injections prevented Fos induction in the ipsilateral hemisphere. These data suggested that the NMDA receptor mediated cortical induction of c-*fos* in neurons following cortical injury.

It is possible that the NMDA receptor plays an important role in c-*fos* induction in cortical neurons via a variety of stimuli. For example, section of the infraorbital nerve induced Fos in brain stem but decreased Fos in cortex (Sharp et al. 1989c). The explanation for the decreases of Fos expression in cortex was unclear. It is hypothesized that cortical induction of Fos in most rats handled in the laboratory is related to stress of handling, injections, and surgery (Sharp et al. 1991). If true, then the decreases in Fos expression in cortex following infraorbital section could be explained by the fact that concurrent activation of the following two receptors is required: (1) activation of stress receptors via unknown pathways and transmitters and (2) concurrent activation of NMDA receptors by thalamocortical fibers. To test the idea that induction of c-*fos* in cortical neurons might require the concurrent action of two receptors, studies of cultured cortical neurons were undertaken (Hisanaga et al., in press).

METHODS

Dissociated neocortical cells were prepared from 16- or 17-day-old Sprague-Dawley rats using methods previously described by Hisanaga and Sharp (1990). Brains were removed and neocortex was dissected in L15 medium. Following incubation for 40 minutes at 37 °C in 10 mL phosphate-buffered saline (PBS) containing 100 U papain, 0.02 percent DNAase, 25 mM glucose, and 1 mM cysteine, the tissue was rinsed in 30 percent heat-inactivated horse serum and triturated with a plastic pipette in 8 mL of medium. Cells were centrifuged at 600 g for 5 minutes. Following aspiration of medium, cells were suspended in minimal essential medium (MEM) containing 0.5 mM glutamine, 20 mM glucose, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 percent fetal calf serum. The cells were passed through an 80 µm mesh, counted, and plated on poly-D-lysine (20 µg/mL)-coated plates at a density of $1x10^6$ cells per cm^2. Cells were maintained at 37°C in 5 percent CO_2/95 percent air, The medium was changed to serum-free MEM or serum- and magnesium-free MEM 4 to 5 hours following seeding. Cultured neurons were studied 3 days after seeding.

Cultured cortical neurons were treated with (1) glutamate (10 µM), (2) zinc chloride (100 µM), (3) carbachol (1, 10, 50 mM), (4) dibutyryl-cyclic AMP (db-cAMP) (1 mM), (5) 12-O-tetra-decanoylphorbol-13-acetate (TPA) (50 nM), (6) potassium chloride (46 mM), (7) vasoactive intestinal polypeptide (VIP) (1 µM), (8) somatostatin (1 µM), (9) neuropeptide Y (NPY) (1 µM), (10) basic fibroblast growth factor (bFGF) (1 µg/mL), (11) nerve growth factor (NGF) (5 ng/mL), (12) EGF (20 ng/mL), and (13) platelet-derived growth factor (PDGF) (1 U/mL). NMDA receptor blockers (MK801, 0.1 µM; CPP, 10 µM; and APH, 30 to 100 µM) were added 15 minutes prior to each stimulant.

C-fos mRNA Northern blotting was performed according to Hisanaga and coworkers' (1990b) method, which is a modification of the method of Auffray and Rougeon (1980). Following isolation of the RNA and electrophoresis, the RNA was transferred to nylon membranes by capillary blotting. C-*fos* riboprobe was prepared from full-length *c-fos* cDNA using SP6 RNA polymerase and ^{32}P-CTP. Radiolabeled RNA was purified over a Nensorb column. The nylon membranes were hybridized with riboprobe in 1.5X SSC, 50 percent formamide, 1 percent SDS gel, 0.5 percent nonfat dry milk, 0.5 mg/mL denatured salmon sperm DNA, and 0.2 mg/mL yeast tRNA at 65 °C for 24 to 36 hours. Membranes were washed twice at 65 °C for 30 minutes in 2X SSC 0.1 percent SDS, 0.5X SSC 0.1 percent SDS, 0.2X SSC 0.1 percent SSC. They were exposed to film at -70 °C with an intensifying screen.

For detection of Fos protein and Fos-related antigen proteins, immunocytochemistry was performed. Neurons were fixed with pacaformaldehyde/lysine/polylysine fixative 2 hours after each stimulation. After two PBS washes, cultures were incubated for 20 minutes in PBS containing 2 percent goat serum, 0.2 percent Triton X-100, and 0.1 percent bovine serum albumin. Cultures were incubated with polyclonal antibody to Fos and Fos-like antigens diluted 1:50 for 2 to 3 days at 4 °C. The first antibody was detected using a biotin-conjugated secondary antiserum (diluted 1:200), followed by the avidin-peroxidase complex reacted with diaminobenzidine and hydrogen peroxide as previously described (Sharp et al. 1990; Sagar et al. 1988).

Cultures stained for glial fibrillary acidic protein using monoclonal antibodies were negative, whereas 1 to 2 percent of the cells in each well were immunoreactive for vimentin (presumed glioblasts). The remaining 98 to 99 percent of the cells stained for alpha-tubulin and neuron-specific enolase, demonstrating that the cultures were nearly purely neuronal.

RESULTS

Control cultures treated with saline, PBS, or vehicle showed that only a few neurons (0.5 to 1.5 percent) stained for FLI, which was induced in a much greater percentage of neurons (6 to 10 percent) following treatment with glutamate, bFGF, zinc, VIP, K+, db-cAMP, and TPA. This occurred in magnesium-containing and magnesium-free medium. NGF, EGF, PDGF, somatostatin, NPY, and carbachol did not induce FLI in the cultured neurons,

Glutamate, bFGF, zinc, VIP, K+, db-cAMP, and TPA induced c-*fos* mRNA in the cultured neurons. Pretreatment with NMDA receptor blockers prevented glutamate and db-cAMP induction of c-*fos* mRNA. Pretreatment with NMDA

receptor blockers did not affect fibroblast growth factor (FGF) induction of *c-fos* mRNA and only partially blocked K+, TPA, zinc, and VIP induction of c-fos mRNA.

However, the most novel aspect of this study was the observation that pretreatment of cultured cortical neurons with noncompetitive (MK801) and competitive (CPP and APH) NMDA antagonists completely blocked the induction of FLI by all agents tested. Treatment of cultured cortical neurons with NMDA antagonists alone had no effect on *c-fos* mRNA or FLI. In addition, treatment of cultures with NMDA antagonists had no effect on total protein synthesis as measured by ^{3}H leucine into trichloroacetic acid-precipitated proteins. Since the polyclonal antibody used in this study detects both Fos protein and two lower molecular weight Fos-related antigens (Aronin et al. 1990, 1991), the data demonstrate that the NMDA antagonists prevent induction of Fos protein and Fos-related antigens.

DISCUSSION

Glutamate induces c-fos mRNA and Fos protein in a small subpopulation (8 to 10 percent) of cultured cortical neurons. This induction appears to occur via the NMDA receptor since both competitive and noncompetitive NMDA receptor blockers prevent glutamate induction of c-*fos* mRNA and Fos protein, Similar results have been observed in cultured cerebellar granule cell neurons (Szekely et al. 1989).

The data also show that K+, TPA, bFGF, zinc, VIP, and db-cAMP induce c-*fos* mRNA and Fos protein in a similar small subpopulation (8 to 10 percent) of cultured cortical neurons. Moreover, although NMDA receptor blockers prevent c-*fos* mRNA induction by glutamate and db-cAMP, other agents like K+, TPA, bFGF, zinc, and VIP all induce *c-fos* mRNA in the presence of NMDA receptor blockers. These observations prove that induction of c-*fos* mRNA via these signaling pathways is not linked to release of glutamate and/or activation of the NMDA receptor. The data also show that several agents (including NGF, PDGF, EGF, NPY, somatostatin, NMDA antagonists, and carbachol) do not induce the c-*fos* gene in cultured cortical neurons.

The unexpected finding of this study was that NMDA receptor blockers do prevent the induction of FLI by all agents tested, including K+, TPA, bFGF, zinc, VIP, and db-cAMP as well as glutamate. The authors believe that the results are not artifactual since (1) the agents did induce c-*fos* mRNA; (2) three chemically different NMDA antagonists blocked induction of Fos protein, but not c-*fos* mRNA; (3) total protein synthesis was not affected by the NMDA antagonists; and (4) graded doses of NMDA antagonists produced graded

decreases in FLI when glutamate was used as the stimulus. However, it is possible that the observed effect might be unique to cultured, fetal cortical neurons and not be a general phenomenon of developing or mature cortical neurons in vivo. However, preliminary data in the authors' laboratory suggest that NMDA antagonists can block translation, but not transcription, of the c-*fos* gene in the intact mammalian brain.

Nevertheless, the mechanism by which NMDA receptors might inhibit induction of Fos protein, but not *c-fos* mRNA, by VIP, FGF, zinc, and other stimuli is potentially very interesting. There are at least three possible mechanisms. First, it is conceivable that activation of the NMDA receptor is necessary for posttranslational processing of Fos or for phosphorylation of Fos. If the authors' polyclonal antibody does not recognize unprocessed or unphosphorylated Fos protein, then the data would show that the NMDA receptor is essential for the posttranslational processing or phosphorylation that would make it possible to immunostain the protein in culture. This seems unlikely since other polyclonal antibodies to Fos detect unphosphorylated and phosphorylated Fos (Sonnenberg et al. 1989). Second, it is possible that the NMDA receptor might downregulate protease activity in some undescribed fashion, For example, NMDA receptor blockade could lead to overactivity of a protease that leads to rapid degradation of the Fos protein so that it was not detectable immunocytochemically. There is little precedent for this. Third, and most likely, is the possibility that the NMDA receptor is somehow involved in translation of c-*fos* mRNA to Fos protein. This could occur via calcium entry through the NMDA channel. Regulation of translation does occur in other systems, Expression of yeast transcription activator GCN4 (whose DNA binding site is related to the Fos-Jun binding site) is regulated at the translational level. External stimuli appear to activate a protein kinase that mediates translational regulation of GCN4 mRNA (Hinnebusch 1990). In addition, one calcium-calmodulin kinase affects translation by phosphorylating the elongation factor 2 (Ryazanov et al. 1988).

If NMDA antagonists are shown to block induction of Fos protein, but not *c-fos* mRNA, from a variety of stimuli, these results could have important implications. NMDA antagonists cause learning impairment (Morris et al. 1986) and prevent plasticity in cortex and other brain regions (McDonald and Johnston 1990). Fos could play a role in NMDA-mediated plasticity by producing long-term changes in gene expression related to a variety of extracellular signals. The authors' results suggest that if neurotrophic factors and neurotransmitters mediate plasticity by induction of *c-fos* mRNA, the expression of Fos protein by these agents requires concurrent activation of the NMDA receptor. Therefore, blockade of NMDA receptors not only prevents direct NMDA-mediated events but also prevents induction of Fos protein from a variety of other extracellular signals.

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Induction and Suppression of Proto-Oncogenes in Rat Striatum After Single or Multiple Treatments With Cocaine or GBR-12909

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INTRODUCTION

Dopamine is well known as a neurotransmitter, but its concurrent role as a regulator of gene expression in postsynaptic neurons is just beginning to be fully appreciated. Alterations in gene expression are one way a neuron can modify its complement of expressed proteins in response to an alteration in synaptic input. Increases or decreases in gene expression can lead to corresponding changes in cellular concentrations of neuropeptides, neurotransmitter synthesizing enzymes, transmitter receptors, or intracellular targets of second messengers. Plastic modifications in the transmitter signaling apparatus in a key set of neurons subsequently may alter the functional status of entire circuits of interconnected neuronal populations. Theoretically, permanent or quasi-permanent alterations in behavior can result, be they adaptive or maladaptive. The molecular mechanisms that effect a change in the expression of a gene are not fully understood. However, understanding events at the molecular level will be an essential component for determining the basis of postnatal neuronal plasticity. How does the brain react to novel or prolonged changes in stimuli? Certainly the changes induced by chronic ingestion of central nervous system (CNS)-active drugs, be they abused or not, can be conceptualized in terms of plastic or adaptive changes. Thus, investigating changes in neuronal gene expression and the mechanisms governing gene expression following acute and chronic administration of abused drugs is a powerful new approach to understanding the neuromolecular basis for the initiation and maintenance of drug addiction.

A longstanding precedent for studying gene expression in the context of the dopamine system is found in studies of chronic dopamine D2 receptor blockade with the antipsychotic drug haloperidol. In animals, chronic treatment with

haloperidol increases striatal proenkephalin gene expression as determined by an elevation in striatal content of proenkephalin-derived peptides (Hong et al. 1978) and preproenkephalin mRNA (Tang et al. 1983). Furthermore, the increase in preproenkephalin gene expression seen in animals appears to apply also to humans. Examination of proenkephalin-derived peptides in cerebrospinal fluid (CSF) of human subjects treated chronically with haloperidol shows that they are significantly elevated (Iadarola et al. 1988a), suggesting that the increase seen in striatal tissue in animals is reflected in an increase in CSF content in humans. The relevance of the striatum to CSF peptides is also supported by the observation of decreased CSF levels following striatal lesions (Iadarola and Mouradian 1989). In humans, acute and, in some cases, permanent changes in behavior (e.g., parkinsonian-like side effects and tardive dyskinesia, respectively) can occur following antipsychotic drug therapy. Although a causal relationship between the increase in enkephalin biosynthesis and the development of parkinsonian-like side effects has not been established, the example illustrates that (1) chronic drug treatment can modify neuronal gene expression and (2) results obtained in dopaminergic neural circuits in animals may be applicable to humans. Thus, molecular studies of the dopamine system and the effects of cocaine in animal models may be particularly relevant to the human situation.

Generally, an increase in mRNA content reflects a change in gene transcription and/or a modification of mRNA stability. Gene transcription is regulated, in part, by interactions of sequence-specific DNA-binding proteins (Pans-acting factors or transcription factors) with binding sites on DNA *(cis*-acting elements or enhancer). It has recently been hypothesized that one class of transcription factors, called cellular immediate early genes (IEGs), serves to couple synaptic transmission at the plasma membrane to alterations in transcription in the cell nucleus (for review, see Morgan and Curran 1991). It is hypothesized that signals transduced by receptors, via second messengers, initially activate transcription and translation of IEGs, which then translocate to the nucleus, acting there as “third messengers” to modify the expression of a set or network of secondary target genes. Thus, these genes may represent a requisite first step in long-term modification of neuronal function. In vivo and in vitro experiments have demonstrated that expression of IEGs is very malleable. Expression of c-*fos,* the prototypical IEG, can be altered by several types of highly specific stimuli in several different neuronal circuits (see other chapters, this volume).

Initial experiments in spinal cord (Draisci and Iadarola 1989) with c-fos and the prodynorphin opioid peptide gene first suggested that dopamine might be an activator of c-fos gene expression in striatum. In the spinal cord, a marked, prolonged increase in prodynorphin gene expression was observed in dorsal

horn neurons following transsynaptic activation during peripheral inflammation (ladarola et al. 1986, 1988b). The dynorphin gene codes for the protein precursor of the dynorphin family of opioid peptides. In an initial attempt to understand the transcriptional processes regulating dynorphin gene expression, our attention was drawn to c-*fos.* Fos-immunoreactivity was shown to be rapidly induced by nociceptive stimuli in the same regions of the spinal cord as was dynorphin (Hunt et al. 1987). The authors' laboratory showed that an increase in dynorphin gene expression was preceded by an increase in c-*fos* mRNA (Draisci and ladarola 1989) and its protein product Fos, as well as several other Fos-immunoreactive proteins seen in Western blots of spinal cord nuclear extracts (ladarola et al. 1989). Furthermore, we also showed that both Fos-immunoreactive proteins and dynorphin peptide immunoreactivity or preprodynorphin mRNA were colocalized within the same spinal cord dorsal horn neurons (Noguchi et al. 1991). Given this suggestive, albeit circumstantial, evidence, the authors and colleagues hypothesized that Fos, a known transcription factor, might be involved in the regulation of dynorphin gene expression. These data provided the basis for our prediction (Young et al. 1991) that, in striatum, the increase in prodynorphin gene expression seen following dopaminergic activation (Li et al. 1988; Sivam 1989; Gerfen et al. 1991) would be preceded by an increase in c-fos expression (despite the disparate location and innervation of the two CNS regions). We observed that a single injection of cocaine induced a rapid (within 30 minutes), fivefold to eightfold elevation in striatal *c-fos* mRNA. GBR-12909, a longer acting dopaminergic uptake blocker, also increased *c-fos* mRNA and striatal Fos protein content. Similarly, but to a proportionately lesser extent, expression of the NGFI-A gene (Milbrandt 1987), which encodes a zinc finger-containing transcription factor, is elevated following cocaine injection.

Single-dose studies are undeniably informative but do not provide an accurate model of human cocaine use in addicts (Gawin and Ellinwood 1988; Gawin 1991; Spotts and Schontz 1980). These individuals generally administer cocaine by intravenous injection or by smoking "crack," a preparation of the free base form of cocaine. Both routes of administration provide nearly equivalent blood levels (Foltin and Fischman 1991) and yield a rapid-onset euphoria-of relatively short duration (less than 10 minutes) but very intense—called a "rush" or "flash" (see case studies in Spotts and Shontz 1980). The cocaine user administers the drug multiple times over the course of a drug use session (during an evening or longer, up to several days in some cases) to experience the rush numerous times. Although this is a very characteristic human usage pattern, neurochemical or molecular studies of the effects of cocaine in animals generally do not try to model this pattern. The authors have employed a multiple administration schedule as a first approximation for the multiple administrations seen in human cocaine abuse. We find that

cocaine, administered multiple times in a single day, has a dual effect on the expression of IEGs: Cocaine administration can either stimulate or suppress the expression of c-fos. A single injection stimulates c-*fos* expression but at the same time triggers a process that inhibits c-*fos* expression. We refer to the latter effect as suppression or refractoriness to induction. A single injection produces a short-lived suppression of approximately 1 to 2 hours. However, multiple injections produce a suppression that can last for days. Thus, the duration of suppression is greatly magnified with repeated administration. The net effect of the prolonged suppression of IEG expression is to functionally uncouple dopaminergic neurotransmission from dopaminergic regulation of gene expression.

MATERIALS AND METHODS

Treatments

Male Sprague-Dawley rats (300 g) were housed in groups of three, given access to food and water ad libitum, and kept on a 12-hour light/dark cycle. Cocaine hydrochloride (HCI) was dissolved in saline and administered intraperitoneally (IP) unless specified otherwise, in a volume of 0.2 or 0.3 mL/g of body weight. Rats were sacrificed at various times after injection by exposure to CO,. The brain was rapidly removed, and the frontal cortex, caudate-putamen, hippocampus, and cerebellum were dissected out on an ice-cold plate. GBR-12909 HCI was dissolved as follows: 20 mg was added to 40 µL of glacial acetic acid, followed by 60 µL of H_2O, and allowed to dissolve completely; next, 1.7 mL of 200 mM sodium carbonate was added and mixed, followed by 200 µL of Tween 20. Rats were injected either IP or intravenously (0.2 mL/100 g body weight). In some cases animals were pretreated IP 30 minutes prior to cocaine with various doses of SCH-23390, a dopamine D1 receptor antagonist, or sulpiride, a dopamine D2 receptor antagonist (Young et al. 1991).

Analyses

RNA was extracted from caudate-putamen, frontal pole or cerebral cortex, hippocampus, and cerebellum by the guanidine thiocyanate/cesium chloride centrifugation method as described previously (Draisci and ladarola 1989). RNA blots were prepared and probed for transcripts with [^{32}P]-labeled, cloned rat cDNA c-*fos* insert (Curran et al. 1987) or oligonucleotide probes to c-fos, NGFI-A, prodynorphin, cyclophilin, or ß-actin, the latter two being used for standardization.

Western blots were prepared with proteins extracted from isolated striatal cell nuclei by a modification of the method of Tata (1974). After electrophoresis in a lo-percent polyacrylamide gel and transfer to polyvinyldifluoride (PVDF) membranes, the membranes were blocked with 1 percent gelatin, 0.25 percent Tween 20, 10 mM HEPES buffer pH 7.4, and 0.02 percent sodium azide for 60 minutes: incubated for 1 hour with rabbit polyclonal antisera; washed 2X times; incubated with [^{125}I]-protein A for 1 hour; and washed several times at room temperature. Autoradiographs were then obtained.

Gel shift assays *were* performed on proteins extracted from the isolated nuclei. A synthetic double-stranded 21 nucleotide oligomer from the gibbon ape leukemia virus (GALV) enhancer containing an AP-1 (TGAGTCA) consensus sequence at its core was used as a probe.

Immunocytochemical analysis of Fos-positive neuronal cell nuclei was performed with standard peroxidase-antiperoxidase method on 30-µ frozen sections after perfusion of rats (under deep pentobarbital anesthesia) with 4 percent paraformaldehyde. Visualization of immune complexes involved using diaminobenzidine as the chromogenic substrate. The anti-Fos antibody was employed at a dilution of 1:10,000 or 1:20,000.

RESULTS

A single injection of cocaine has a powerful effect on c-*fos* gene expression. A dose of 30 mg/kg IP produced a marked, fivefold to sevenfold increase in striatal c-*fos* mRNA within 30 minutes (figure 1). The increase in mRNA content was relatively short lived and did not substantially outlast the 30-minute time point. The elevation in mRNA content was both dose dependent and regionally specific, occurring in regions receiving a strong dopaminergic innervation (e.g., striatum) but not in hippocampus (figure 2). Immunocytochemical staining for Fos-immunoreactive proteins disclosed nuclear staining in neurons in striatum, nucleus accumbens, olfactory tubercle, and the islands of Calleja that was weak in saline-injected controls (figure 3) and very intense following treatment with cocaine (figure 4) or GBR-12909 (figures 5 and 6). The effect of cocaine was dose related in terms of the number of cells that could be counted and in terms of the intensity of staining. We quantitated the number of stained cells for the dose response at 2 hours postcocaine, a time at which the protein staining was at a peak (figure 7, upper panel), using doses of 0.3 to 30 mg/kg (Young et al. 1991). There was some light staining in control sections, but this varied between rats and generally was not of an intensity to be easily quantitated. The number of cells was increased significantly at the 3.0 mg/kg dose and tended to reach a plateau between 10 and 30 mg/kg (figure 7, lower panel). What is not apparent in the cell counts is that at higher doses, although the

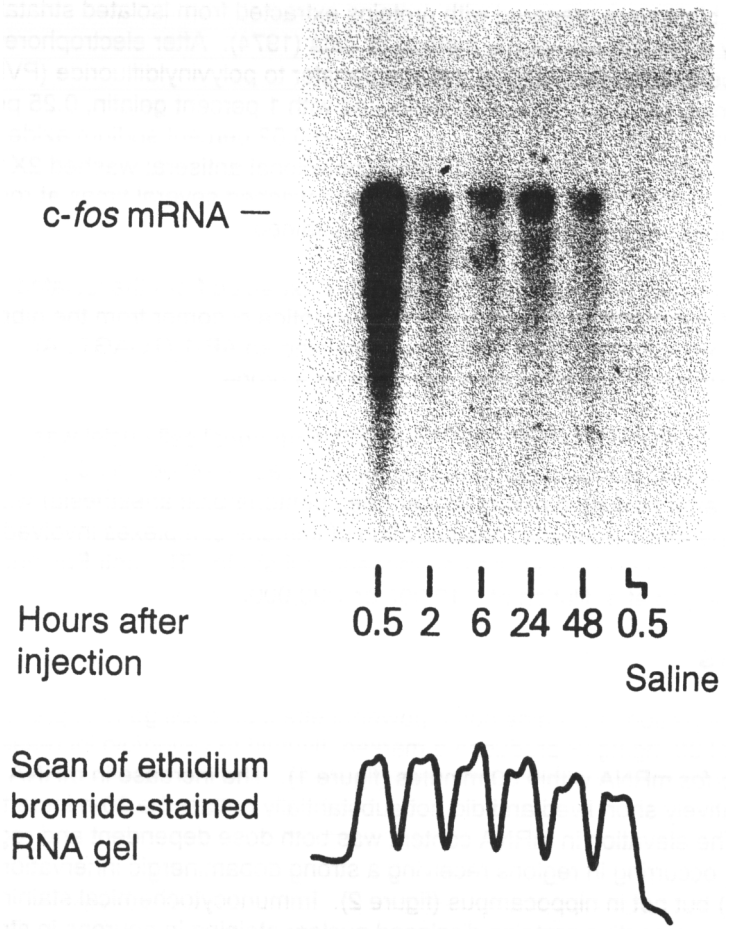

FIGURE 1. *Time course of increase in striatal c-fos mRNA. Rats were administered 40 mg/kg of cocaine IP. The main increase (eightfold to tenfold) occurred at 30 minutes. The autoradiogram is overexposed to show that only minor elevations of c-fos transcript were detected between 2 and 48 hours compared with saline (last lane). Densitometric scan of the corresponding ethidium bromide-stained RNA gel is placed at the bottom and indicates that the amount of RNA loaded in each lane is similar.*

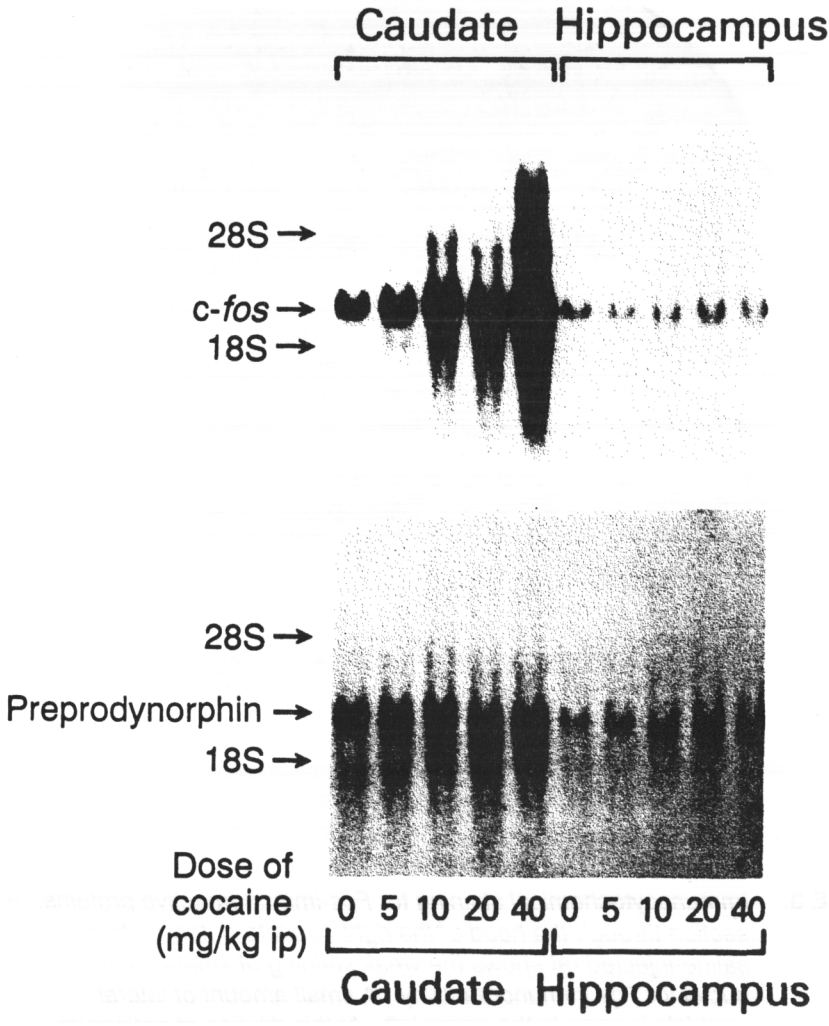

FIGURE 2. *Upper panel: Dose-response of c-fos mRNA increase assessed 30 minutes after IP injection of cocaine at doses between 5 and 40 mg/kg. The increase is regionally selective, occurring in striatum but not in hippocampus. Increases over control were detected at all doses of cocaine. Lower panel: The blot was reprobed for preprodynorphin mRNA. No change was seen in the amount of this transcript at 30 minutes after cocaine in either caudate or hippocampus. The blot was probed a third time for ß-actin (not shown), which demonstrated that the amount loaded was very similar across the 10 lanes.*

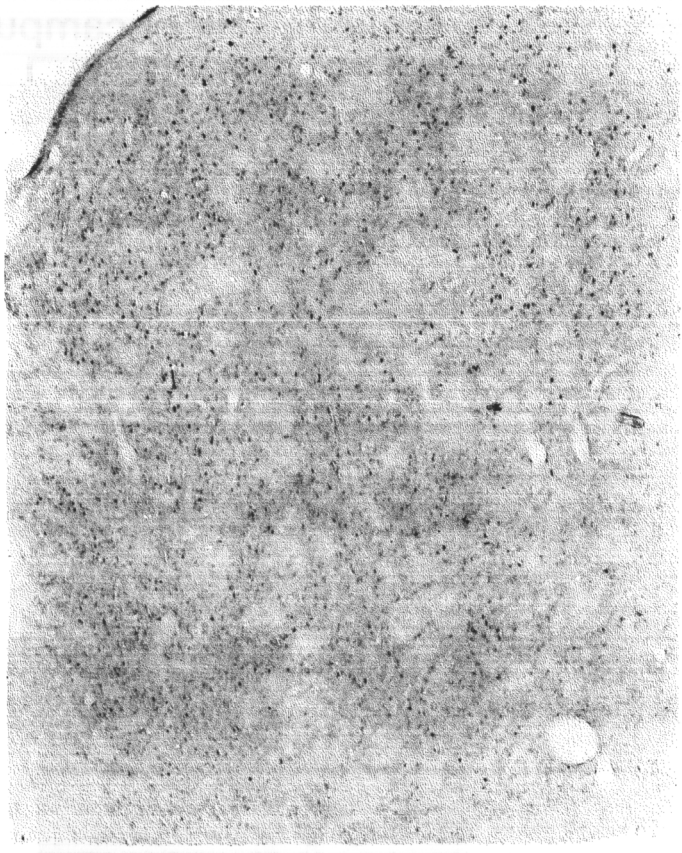

FIGURE 3. *Immunocytochemical staining for Fos-immunoreactive proteins. A section through the head of the right caudate-putamen from a saline-injected rat shows the weak staining of striatal neuronal nuclei for Fos immunoreactivity. A small amount of lateral ventricle is seen in the upper left. At this dilution of antiserum (1:10,000) control sections were generally blank except for scattered darkly stained neurons in pyriform cortex and olfactory tubercle. (64X magnification)*

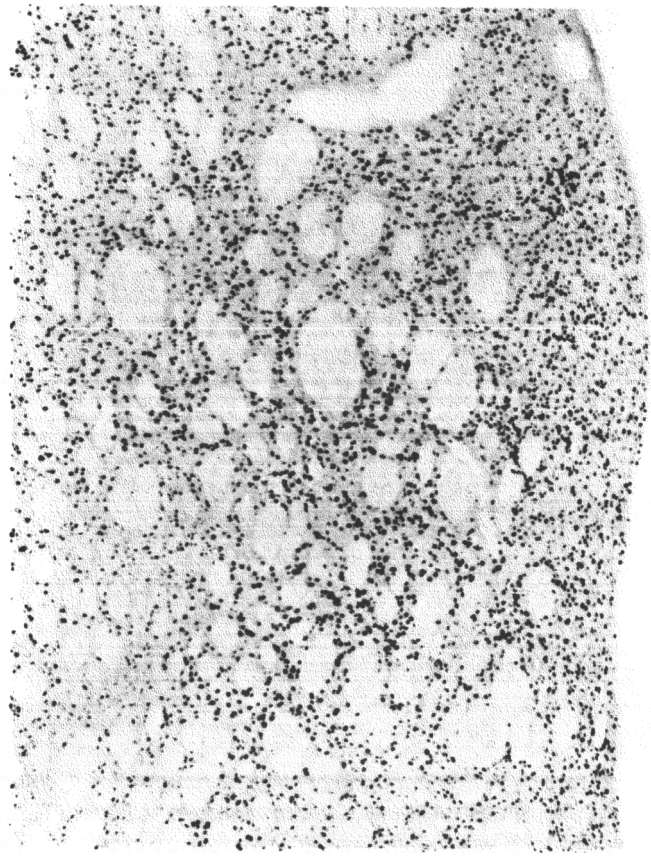

FIGURE 4. *Increase in striatal Fos immunoreactivity after cocaine treatment. Dense nuclear staining can be seen for Fos-immunoreactive proteins at 2 hours after IP injection of 30 mg/kg cocaine. The lateral ventricle is on the right. (64X magnification; antibody dilution, 1:10,000)*

number of cells recruited was not that much greater, the *intensity* of staining increased dramatically. The degree of staining intensity throughout the affected regions went from light brown to darker brown to nearly black in many cells as the dose of cocaine or GBR-12909 increased. The intensity aspect was not quantitated and does not reproduce well in the black and white photomicrographs but can be readily appreciated in the tissue sections. GBR-12909 was also capable of increasing Fos protein content in caudate nucleus

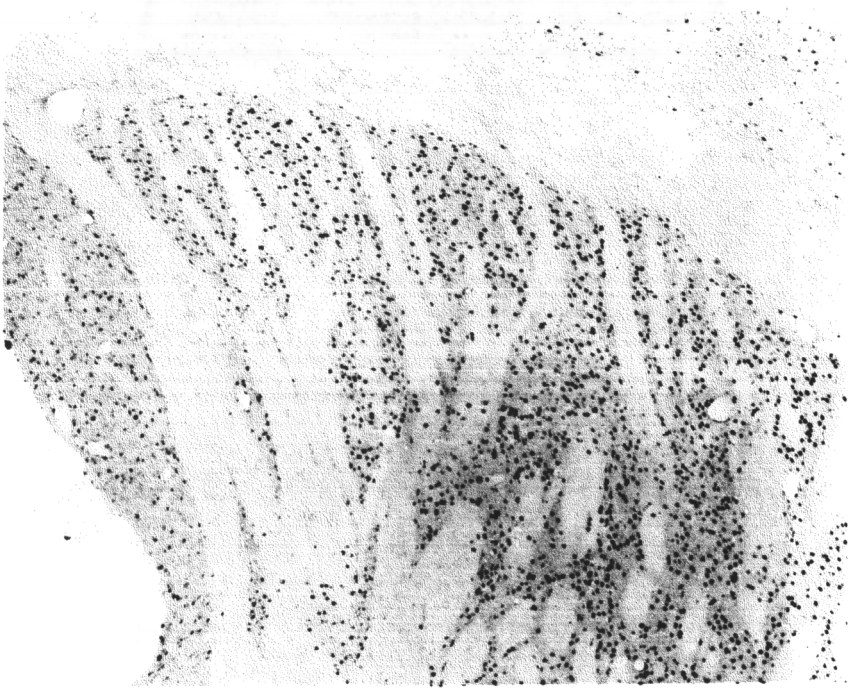

FIGURE 5. *Increase in Fos immunoreactivity 2 hours after IP injection of 20 mg/kg of GBR-12909. As formulated in this study, GBR-12909 produced essentially the same increases in c-fos mRNA and Fos-immunoreactive proteins that cocaine did. However, behaviorally, the time course of GBR's action was much longer than that of cocaine. This aspect was not systematically examined for its effect on immunocytochemical or molecular endpoints. GBR-12909 treatment caused marked staining throughout the rostral-caudal and dorso-ventral extent of the striatum as illustrated in the section through the posterior caudate. (64X magnification; antibody dilution, 1:10,000)*

and in ventral striatal areas (figures 5 and 6). We also observed a marked increase in *c-fos* mRNA with GBR-12909 in striatum at the 20 mg/kg IP dose when rats were sacrificed within 30 to 40 minutes of injection (not shown). GBR-12909 was also effective when given intravenously: Increases in *c-fos*

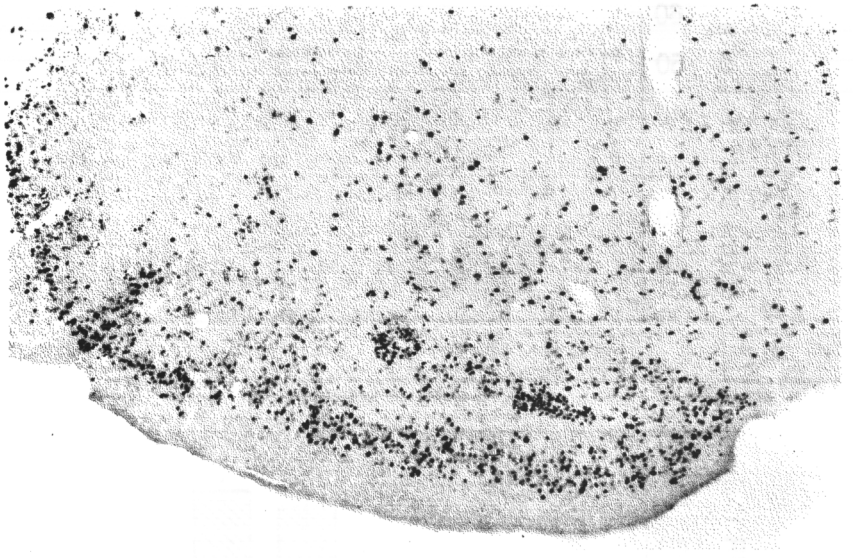

FIGURE 6. *GBR-12909 increases Fos immunoreactivity in the olfactory tubercle and islands of Calleja. Generally, control sections (see figure 4) are nearly blank except for the occasional darkly stained neuronal nucleus in the o/factory tubercle and adjacent pyriform cortex. After cocaine, large numbers of nuclei are densely stained in both the tubercle and the islands. (64X magnification; antibody dilution, 1:10,000)*

mRNA were seen as rapidly as 10 minutes and continued to accumulate at 20 and 60 minutes to reach a level (fivefold to eightfold increase) comparable to that obtained with the 30 mg/kg IP dose of cocaine (not shown). In general, our molecular observations of GBR-12909 were similar to those of cocaine.

The rapidity and selective regional distribution of the mRNA increase and subsequent increase in protein content suggest that treatment with a dopamine reuptake blocker rapidly induces *c-fos* gene expression. To test the involvement of dopamine receptors in mediating the effect of cocaine, we pretreated the rats with several doses of either the D1 selective antagonist SCH-23390 or the D2 antagonist sulpiride. Using immunocytochemistry and counts of Fos-immunoreactive cell nuclei, we observed that SCH-23390 pretreatment at a dose of 10 µg/kg partially attenuated the effect of 30 mg/kg of cocaine, and a dose of 50 µg/kg completely blocked the effect of cocaine (figures 8 and 9). Pretreatment with sulpiride partially attenuated the effect of

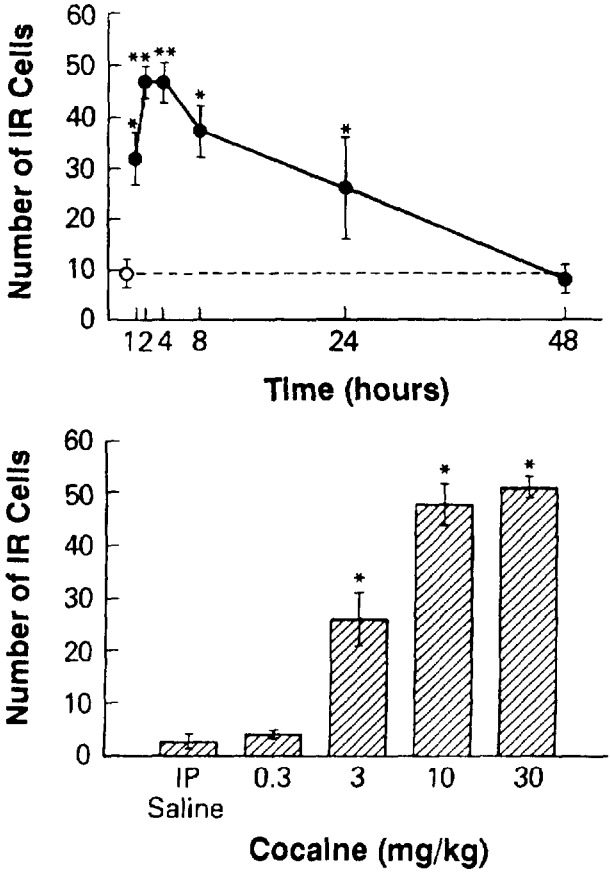

FIGURE 7. *Time course and dose response of the increase in Fos-immunoreactive (IR) cell nuclei following cocaine 30 mg/kg IP. Upper panel: The peak elevation of Fos-immunoreactive proteins is seen between 2 and 4 hours and returns to control over approximately the next 24 hours. This contrasts with the increase in c-fos mRNA, which peaked sharply at 30 minutes. Lower panel: The increase in Fos-immunoreactive protein staining occurred over the same dose increase in mRNA. Data were collected 2 hours after injection. Stained nuclei were counted regardless of intensity. However, the intensity increased progressively, especially between 10 and 30 mg/kg. At the latter dose many neurons were very dark brown, almost black, in color. *p<0.05, **p<0.01.*

SOURCE: Young et al. 1991

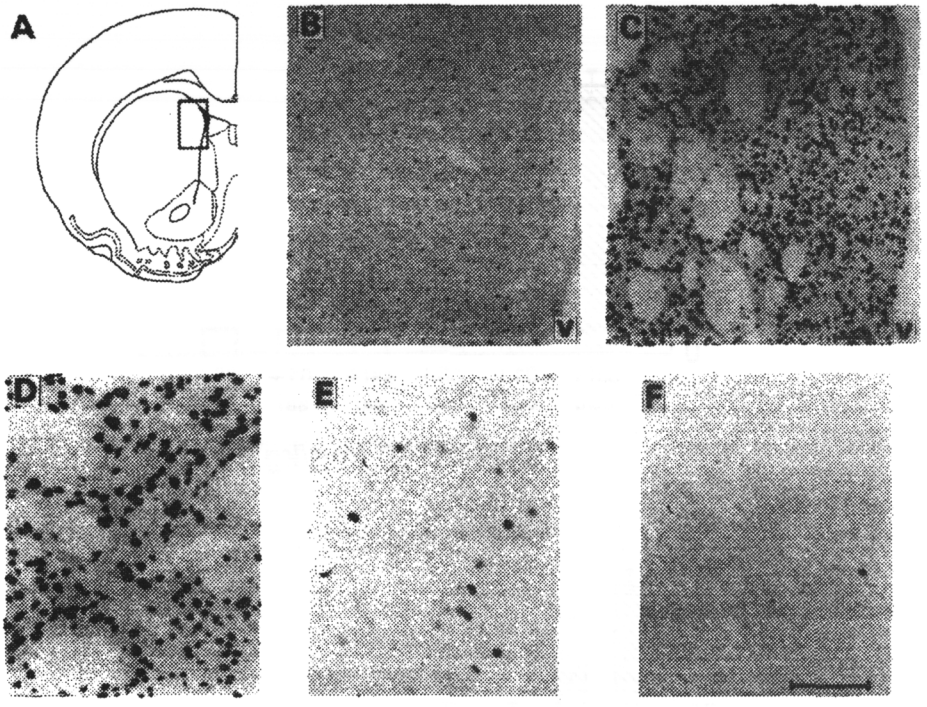

FIGURE 8. *Blockade of Fos induction by SCH-23390. (A) Diagram of forebrain section illustrating region of caudate shown in B through F. (B) Saline-treated control. (C) 2 hours after cocaine 30 mg/kg IP. (D through F) High-power micrographs of caudate from rats 2 hours after treatment with cocaine 30 mg/kg IP alone (D), or pretreated 30 minutes prior to cocaine with the D1 antagonist SCH-23390 at 10 µg/kg (E) and 50 µg/kg (F). The bar in all cases equals 100 µm; v=lateral ventricle. Preadsorption of the antibody with the antigenic peptide abolished all staining (not shown).*

SOURCE: Young et al. 1991

cocaine, but there was not a clear dose-related inhibition of the cocaine-induced elevation of Fos-immunoreactive nuclei (figure 9, lower panel). Administration of the antagonists alone produced no significant effect on the number of Fos-immunoreactive cell nuclei. These data suggest that the main receptor mediating the induction of *c-fos* in striatal neurons is the D1 receptor. Since the D1 receptor is positively linked to adenylate cyclase, it suggests the cyclic

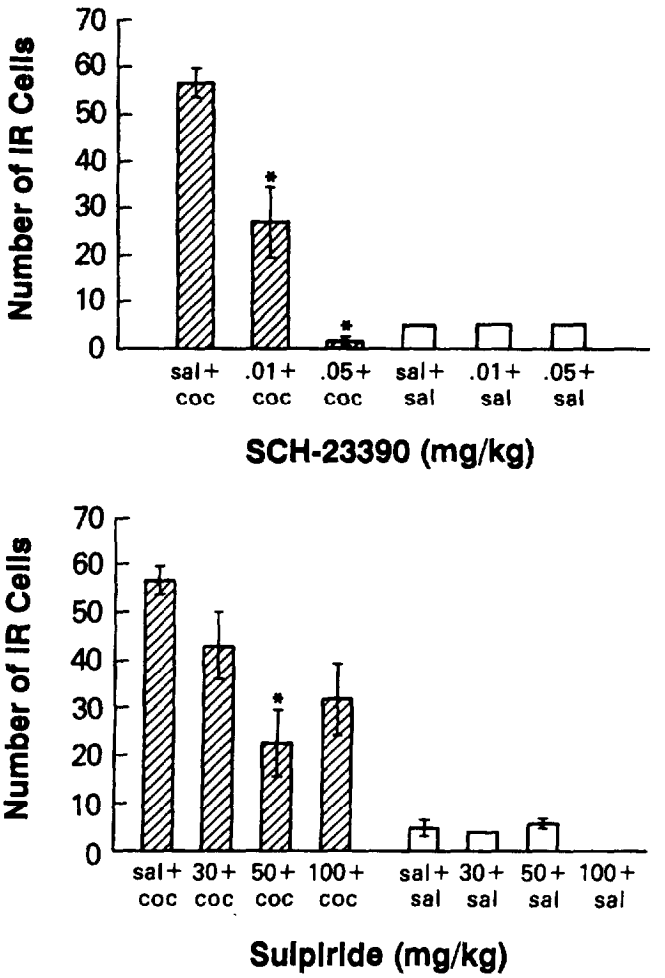

FIGURE 9. *Comparison of blockade of cocaine-induced Fos immunoreactivity produced by pretreatment with SCH-23390 or sulpiride. Neither antagonist alone had a marked effect on striatal c-fos staining. A dose-related decrease in the number of stained nuclei was seen with the D1 antagonist SCH-23390, whereas a clear dose-related decrease was not seen with the D2 antagonist sulpiride. (sal=saline, coc=cocaine) *p<0.05*

SOURCE: Young et al. 1991

AMP (cAMP) second-messenger system is an important element in the induction of c-fos gene expression in these cells.

The antibody used in the immunocytochemistry studies was directed against a c-*fos* sequence KVEQLSPEEEEKRRIRRERNKMAAA, which is highly conserved between species and among the four distinct members of the Fos/Fos-related antigen (Fra) family, which includes Fos-B (REETLTPEEEEKRRVRRERNKLAAA) (Zerial et al. 1989), Fra-1 (PCEQISPEEEERRRVRRERNKLAAA) (Cohen and Curran 1988), and Fra-2 (RDEQLSPEEEEKRRIRRERNKLAAA) (Nishina et al. 1990; Matsui et al. 1990). This suggested that more than one protein species may be elevated in the Fos-immunoreactive striatal nuclei as seen with immunocytochemistry. If multiple proteins were detected via Western blot methodology, they might correspond to one or more of the multiple Fos/Fra proteins or fragments thereof. To investigate this possibility, we performed Western blot analysis of striatal nuclear extracts (figure 10). After isolation of the nuclei by centrifugation through a 2.1-M sucrose solution, the nuclear pellet was homogenized directly in SDS sample buffer, assayed for protein content, and electrophoresed in a 4-percent stacking/10-percent separating polyacrylamide gel along with molecular weight markers. We observed a temporally distinct cascade of immunoreactive protein bands with the immunoblot technique (figure 10). The basal state contained a small but detectable amount of 55-kDa Fos protein (barely visible on the autoradiogram) as well as several, more distinct, lower molecular weight Fra proteins, especially one at 41 kDa. Within 2 hours an abundant amount of the 55-kDa Fos protein was seen and was followed thereafter by increases in the lower molecular weight Fra proteins (figures 10 and 11). We have confirmed the identity of the 55-kDa protein by probing with an antibody raised against an n-terminal peptide (DYEASSSRCSSASPAGDSLS) from the rat Fos protein. In these blots (not shown), an increase in the 55-kDa nuclear protein was detected as soon as 1 hour after a 30-mg/kg IP dose of cocaine. By 2 hours after cocaine treatment, and even more conspicuously after 4 hours, increases were seen in the lower molecular weight Fra proteins (figure 11). An analogous temporal pattern of Fos and Fra expression has been seen in hippocampus with convulsant stimulation (Sonnenberg et al. 1989).

One of the hallmarks of IEGs is that they do not require protein synthesis to undergo an increase in transcription, All the mechanisms and proteins needed to activate IEG transcription preexist in the cell's cytosol or nucleus. The activation of c-*fos* expression by cocaine fits this criterion, We observed that the potent protein synthesis inhibitor anisomycin, which crosses the blood-brain barrier, did not prevent the increase in c-*fos* mRNA (figure 12). In fact, we saw the phenomenon of superinduction, in which the stimulus produces an exaggerated elevation in mRNA levels in the presence of a protein synthesis

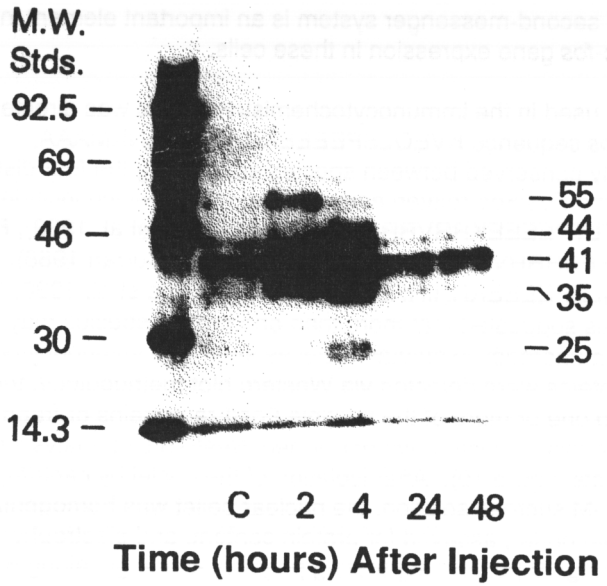

FIGURE 10. *Western blot analysis of Fos-immunoreactive proteins at different times following cocaine 30 mg/kg IP. The numbers at the left show the molecular weight markers (M. W. Stds in kDa); those on the right, the molecular weights for the Fos-immunoreactive proteins as recognized by the broadly specific anti-Fos antisera. The 55-kDa protein corresponds to the c-fos gene product; those below, to the various Fra's. An increase in Fos protein is seen as early as 1 hour in further repetitions of this experiment; the Fra proteins lag behind the increase in Fos protein.*

SOURCE: Young et al. 1991

inhibitor. Although this is interesting in itself, the main point of the anisomycin treatment was to determine whether it blocked or attenuated the increase in c-*fos* transcripts. We did not observe an attenuation, and this reinforces the idea that the increase in c-fos seen with cocaine is part of a broader spectrum of IEG activation in caudate-putamen and ventral striatal areas that involves not only fos but other IEGs as well (Hope et al. 1992).

We have shown that an acute injection of cocaine induces *c-fos* mRNA, Fos protein, and several other Fos-immunoreactive proteins (Fra's) and that these

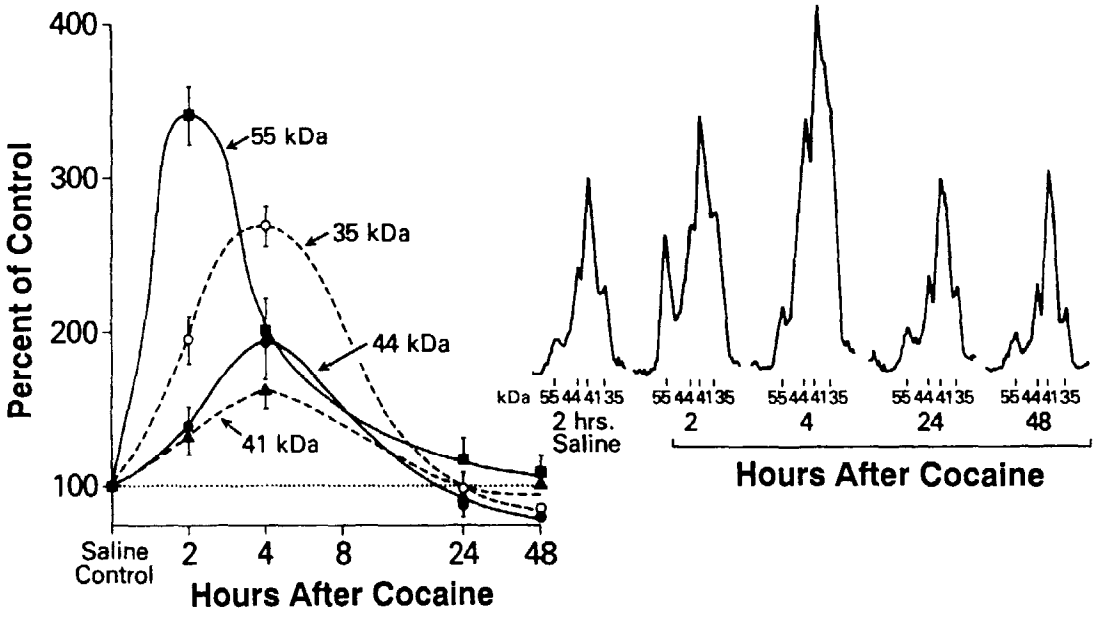

FIGURE 11. *Densitometric analysis of the increase in Fos and Fra proteins. The early increase in the 55-kDa Fos protein is clearly seen in the scans (first peak on the left in each scan record) and in the graph derived from the scan data. The Fra proteins, composed of the 44-, 41-, and 35-kDa components, reach their peak approximately 2 hours later than Fos.*

SOURCE: Young et al. 1991

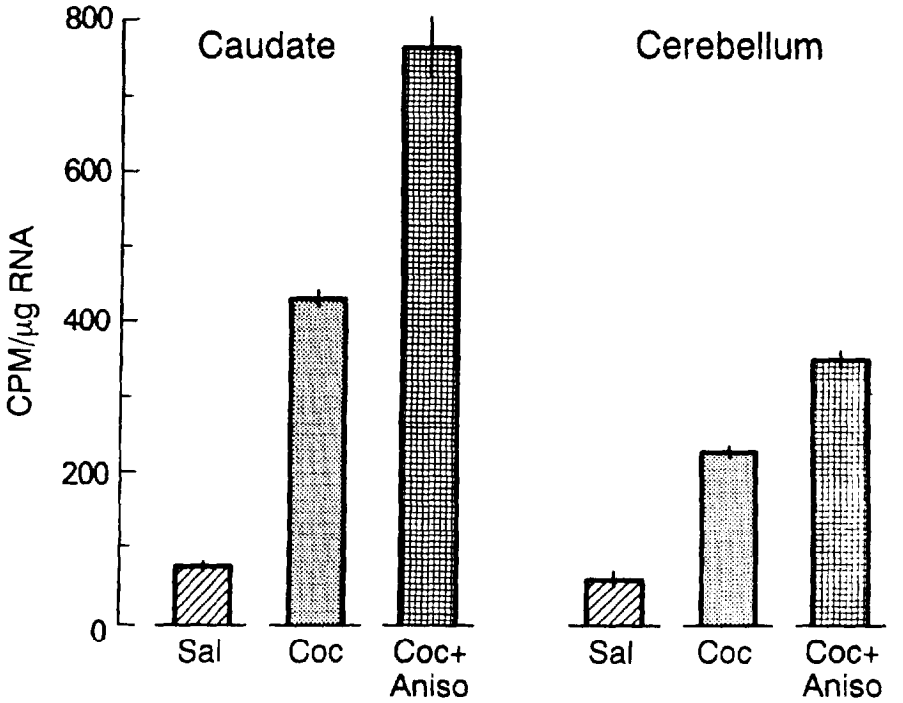

FIGURE 12. *The increase in c-fos mRNA is independent of protein synthesis, The bars represent results expressed as counts per minute (cpm) of protected c-fos mRNA from an RNase protection assay. Rats were treated with saline (sal); cocaine 30 mg/kg IP (coc); or anisomycin 100 mg/kg given 20 minutes prior to cocaine 30 mg/kg IP (coc+aniso). Anisomycin pretreatment did not prevent the increase in c-fos mRNA induced by cocaine. Anisomycin alone also stimulated an accumulation of c-fos mRNA (not shown). In fact, the increase produced by anisomycin alone was additive with that produced by cocaine. At the 30 and 40 mg/kg doses of cocaine, an increase in c-fos mRNA occurred in cerebellum. The cerebellar increase also was not attenuated by anisomycin.*

proteins are exclusively located in the cell nucleus. A further question is whether the induced proteins lead to an increase in the amount of AP-1 complex formation in the cell nucleus. Fos is a member of the leucine zipper family of transcription factors and is known to bind as a heterodimer with members of the Jun transcription factor family to the AP-1 enhancer element (but also with other proteins, see Hai and Curran 1991). The AP-1 consensus sequence is TGAGTCA. We used a small segment of the GALV enhancer that contains the AP-1 consensus in a gel shift reaction with extracts of nuclei isolated from normal and cocaine-induced striatum to determine whether the elevation in Fos protein content led to an increase in AP-1 complex formation. The gel mobility shift assay yielded an AP-1 shift in control striatum and an elevated amount of AP-1 complex within 1 hour following cocaine injection (figure 13). In both cases, the primary AP-1 shift contained Fos-immunoreactive proteins since the entire complex could be shifted further still by addition of the Fos antibody. The entire amount of complex was further retarded, which suggests that a Fos-immunoreactive protein was an element in all the complexes formed in both the control and cocaine-induced conditions. Thus, the elevated capacity for striatal extracts to form AP-1 complexes in the cocaine-treated condition is consistent with the Northern blot, Western blot, and immunocytochemical data demonstrating an increase in c-*fos* mRNA and Fos/Fra protein(s).

The most interesting effect of cocaine on IEG expression occurred when the effects of a multiple dosing regimen were examined (figure 14). With several doses given over the course of a single day (4 injections of 30 mg/kg IP, at 2-hour intervals), we observed a biphasic effect: The initial injection caused the familiar marked (fivefold to sevenfold) increase in *c-fos* mRNA, but the response to subsequent injections was nearly completely attenuated. Figure 15 shows that the c-fos induction was completely suppressed in response to the fourth injection of a series of 30 mg/kg injections given 2 hours apart over the course of a single day. We have mapped the time course of suppression following a single acute cocaine injection and have found that a short-lived period (approximately 1 to 2 hours) of suppression (or refractoriness to induction) occurs following a *single* injection of cocaine (not shown). However, the duration of suppression becomes much more prolonged with multiple injections. In the case illustrated in figure 15, where four successive injections were given, the duration of suppression lasted for 3 to 4 *days.* The long duration required for recovery of full sensitivity to cocaine was mapped out by giving challenge doses of cocaine at progressively greater intervals following the subchronic series of injections (figures 16 and 17). Our data indicate that the duration of suppression cumulates with multiple injections. Thus, multiple injections of cocaine produce a long-term uncoupling of dopaminergic neurotransmission from dopamine-regulated gene expression.

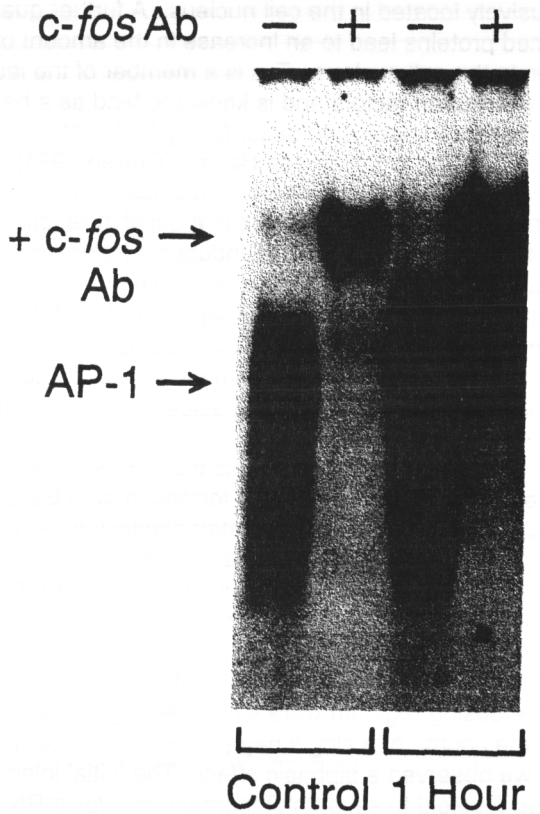

FIGURE 13. *Gel mobility shift analysis of AP-1 complex formation. In addition to stimulation of c-fos mRNA and Fos and Fra proteins, treatment with cocaine also increases the capacity for formation of a Fos-containing protein DNA complex. A double-stranded oligonucleotide from the GALV enhancer containing the AP-1 consensus was incubated with striatal nuclear extracts from control and cocaine-treated rats. The location of the basal AP- 1 complex is indicated. The entire complex can be shifted further still by concurrent incubation with the Fos antibody (Ab), which makes the complex larger and thus less mobile. The super-shift indicates that Fos-immunoreactive proteins are in the basal state AP-1 complex and are elevated after cocaine induction. Higher concentrations of antibody disrupt the complex completely via sequestration of Fos proteins; complex formation could also be competed by an excess of unlabeled AP-1 oligonucleotide but was unaffected by an excess of unrelated oligonucleotide (not shown).*

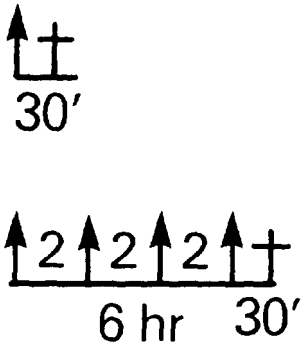

FIGURE 14. *Multiple dosage regimen used to obtain suppression. Rats were injected IP with a single dose of cocaine 30 mg/kg or with four successive doses at 2-hour intervals. In both cases animals were sacrificed 30 minutes after the last dose of cocaine. We refer to the four successive injections as subchronic treatment.*

The interplay between the induction and suppression mechanisms is shown diagramatically in figure 18. In the acute situation activation of the induction mechanism (shown by the upward deflection) is followed very shortly by actuation of the suppression mechanism. The temporal kinetics of the two are different: The induction is potent and short lasting, whereas the suppression process has a longer time course, somewhat similar conceptually to an action potential followed by a recurrent inhibitory postsynaptic potential. The cumulation of the inhibition with repeated injections is shown in figure 18. Here the induction process is eventually overwhelmed by the suppression, which becomes progressively greater with each injection. The effects of the suppression slowly reverse over the next several days, but complete recovery is not achieved until 4 days postinjection.

DISCUSSION

The data presented show that cocaine induces a dual abnormality in dopaminergic regulation of IEG expression. First, an acute injection induces a large increase in c-*fos* gene expression. Second, a suppression mechanism is induced, which renders the system refractory to induction by a subsequent system. This period of refractoriness or suppression is variable and depends on the frequency and number of multiple injections. Also, categorizing these effects as “abnormalities” may be conceptually misleading. Since cocaine blocks dopamine reuptake, it may be more accurate to think of cocaine as

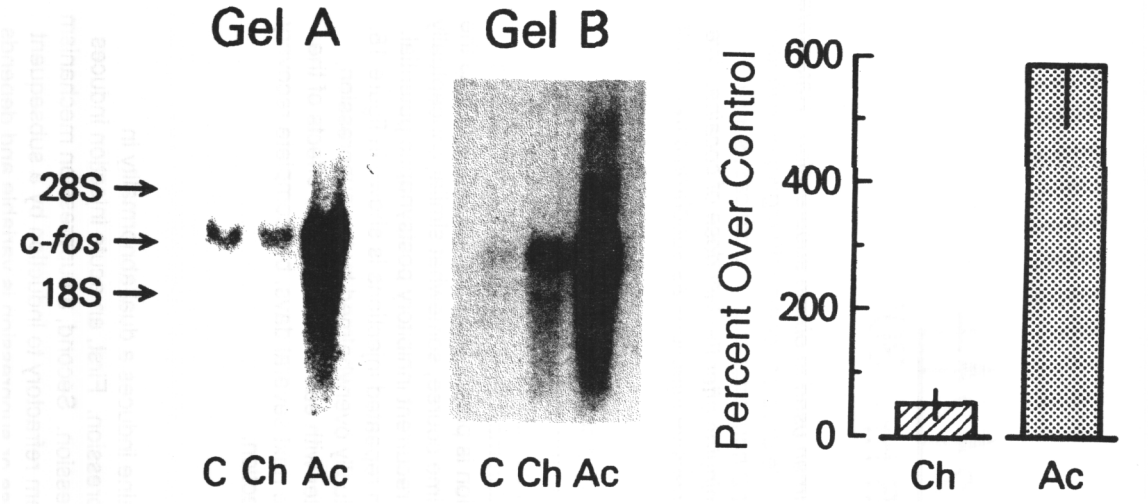

FIGURE 15. *Two representative RNA blots probed for c-fos (left) and a summary of the percent increase over control from three separate experiments (right). An acute injection (Ac) produces a marked (approximately sixfold) increase in c-fos mRNA in comparison to saline-injected controls (C). in contrast, 30 minutes after the last injection in the subchronic series (Ch) no substantial increase is seen (30 to 60 percent over control). The blots were reprobed for ß-actin, the c-fos mRNA to ß-actin mRNA ratio was calculated, and the data from three experiments expressed as the mean&EM percent of saline-injected controls are shown in the bar graph. The mutiple injections completely suppress the ability of cocaine to induce an increase in c-fos mRNA.*

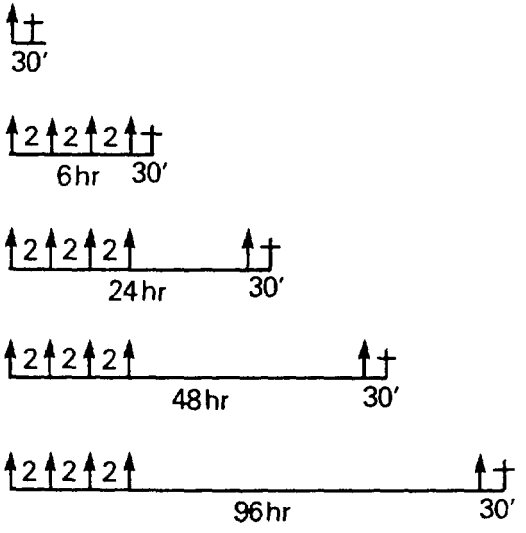

FIGURE 16. *Treatment schedule to determine time course of recovery from suppression following the multiple injection subchronic treatment. In all cases rats were sacrificed at 30 minutes after the last injection in a series. The dose of cocaine for all treatments was 30 mg/kg. Six groups of rats were treated: (1) a single injection of saline or (2) cocaine; (3) the subchronic series (i.e., four injections of cocaine at 2-hour intervals); (4) the subchronic series and 24 hours later a Challenge dose of cocaine; (5) the subchronic series with Challenge at 48 hours; and (6) the subchronic series with challenge at 96 hours.*

causing an exaggeration of ongoing dopaminergic activity, only potentiating processes that are already in place. Thus, to some extent, induction and suppression of IEG expression are likely to be normal components of the biochemical repertoire accompanying dopaminergic neurotransmission. This idea is supported by our Northern and Western blot data, which usually show a low, but detectable level of c-*fos* mRNA and Fos-immunoreactive proteins in basal state striatum. Modulation of expression either up (the usual case) or down is possible. A similar situation is seen for NGFI-A (not shown). However, the basal expression of NGFI-A mRNA is much higher than for *c-fos,* and large amounts of NGFI-A protein can be detected in control brain nuclear extracts. These data suggest that induction of NGFI-A, unlike c-fos, occurs on a fairly high level of constitutive expression.

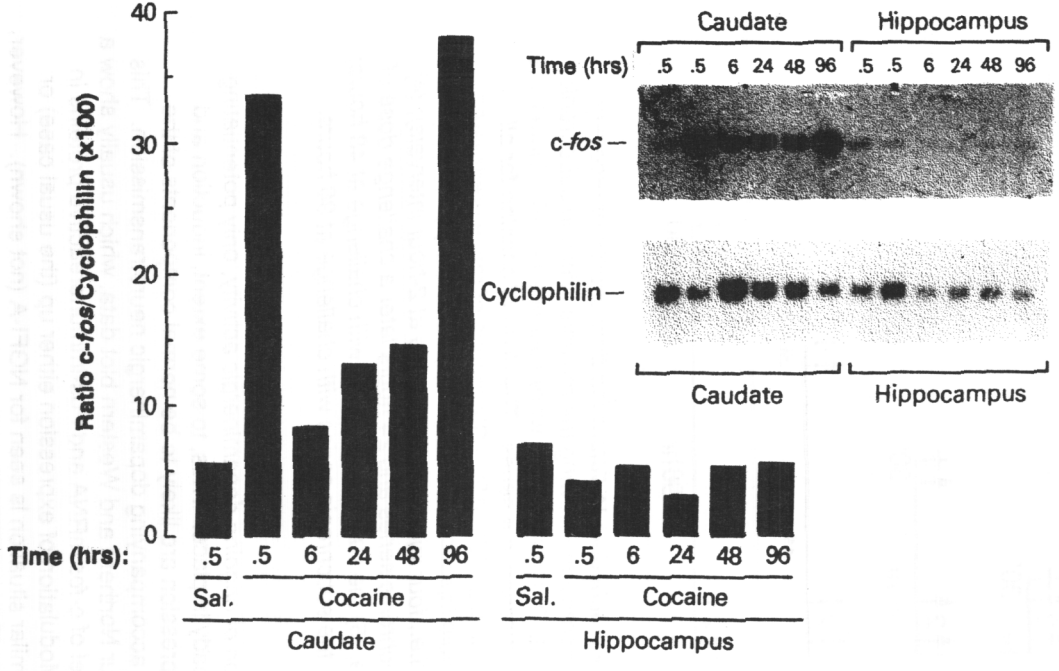

FIGURE 17. *Northern blot analysis of c-fos mRNA induction, suppression, and recovery from suppression. The bar graph shows the results from the six groups described in figure 16. RNA blots were prepared from* caudate-putamen *and hippocampus and probed for c-fos and cyclophilin mRNAs. The bars represent the ratio of c-fos mRNA to cyclophilin mRNA. Intensity of hybridization was determined with a phosphor storage screen system and lasar readout. This method increases the linear range of radiation detection several log units over x-ray film. The inset shows film autoradiograms for the two transcripts. Note the large (~sixfold) increase due to acute cocaine treatment and the nearly complete suppression at 6 hours following the subchronic series. The suppression is still evident at 1 and 2 days after the multiple injections. Full recovery is not seen until 4 days have elapsed after the subchronic series of injections.*

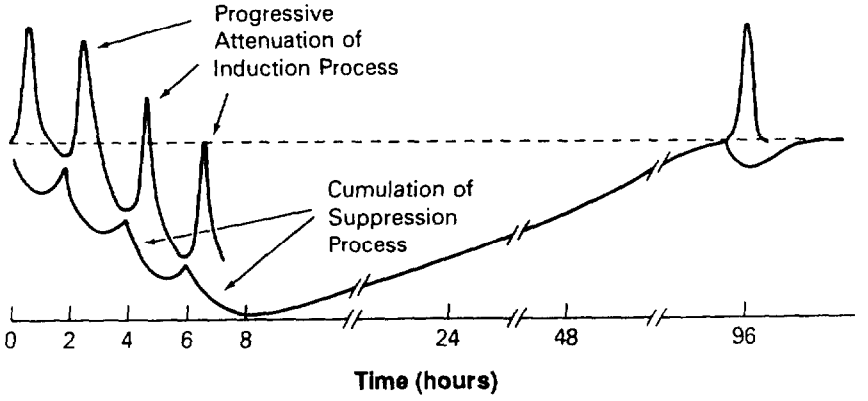

FIGURE 18. *Diagrammatic representation of the induction and suppression of c-fos expression by repeated cocaine injections. The induction process is triggered by excess dopamine at the synapse secondary to blockade of reuptake by cocaine. The effect is mediated by D1 receptors positively /inked to adenylate cyclase. This effect is transient but is accompanied by a concurrent activation of the suppression process. The mechanism(s) of suppression are not presently known but appear to taper off slowly. The suppression cumulates markedly with repeated cocaine administration, thereby resulting in a prolonged uncoupling of dopaminergic gene regulation from dopaminergic neurotransmission.*

Pretreatment with antagonists of the D1 and D2 subtypes of dopamine receptors demonstrated that the c-*fos* induction in response to cocaine was blocked by the D1 antagonist. The D1 receptor is linked in a positive fashion to adenylate cyclase, which suggests that c-*fos* gene expression is activated by a cAMP-dependent process. In support of this, many in vitro studies have shown that c-*fos* expression can be activated by stimulation of the cAMP second-messenger system (for review, see Montminy et al. 1990). Furthermore, one or more cAMP-responsive *cis*-acting elements are present in the promoter of the c-*fos* gene. In these studies, sequences at -60 in the c-*fos* promoter confer cAMP-dependent inducibility on a c-*fos* promoter-reporter construct in transient expression assays (Sassone-Corsi et al. 1988). Deletions or mutations in the cAMP response element (CRE) at -60 eliminate cAMP responsiveness in the transient expression assay. The CRE is a binding site for a transcription factor called CREB (Hoeffler et al. 1988), which is a member of the activating transcription factor (ATF) family of leucine zipper trans-acting factors (Hai et al.

1989). Thus, a likely scenario for c-*fos* induction, and possibly induction of other IEGs, by cocaine might be as follows: Cocaine enhances dopaminergic activity via blockade of reuptake, thereby activating dopamine receptors. Activation of the D1 receptor increases intracellular cAMP and the activity of protein kinase A, which then phosphorylates susceptible substrates, one of which may be CREB or another member of the ATF family. These in turn rapidly act as positive regulators of c-*fos* gene expression via the c-*fos* CRE sites. CREB is known to be phosphorylated by protein kinase A, and phosphorylation affects the binding of CREB to the CRE in in vitro binding assays (for review, see Montminy et al. 1990). Furthermore, CREB is constitutively expressed, suggesting that no new protein synthesis is required for the increase in c-*fos* gene expression. The latter conclusion is supported by the authors' data showing that the increase in c-*fos* mRNA still occurs in the presence of a high degree of protein synthesis inhibition produced by anisomycin.

GBR-12909, a cocaine analog without the susceptible ester bond found in cocaine, was also able to rapidly induce c-*fos* mRNA and Fos-immunoreactive proteins. Both compounds increased c-*fos* gene expression. Thus, by biochemical criteria they appear similar; however, the duration of action is longer for GBR-12909. This is exemplified by the duration of behavioral activation. lntraperitoneal injection of GBR-12909 at 30 mg/kg produced an intense stereotypy that lasted for at least 60 minutes. This is nearly four times longer than the increase in locomotor activity produced by an equal dose of cocaine. Although we did not formally analyze it, GBR produced more stereotypy, such as intense sniffing directed at the hole for the water spout in the cage, rather than locomotion. In comparison, cocaine produced stereotypic head movements, but at most doses they were superimposed on an increase in locomotion. Regardless of the behavioral differences, we observed an abundant elevation in *c-fos* immunoreactivity throughout the striatum, nucleus accumbens, olfactory tubercle, and islands of Calleja. The accumbens, in particular, has been implicated in the reinforcing properties of cocaine (see Kuhar et al. 1991 for review and Hope et al. 1992).

Several lines of evidence suggest cAMP-dependent mechanisms mediate the increase in c-*fos* expression seen with cocaine. Fewer data are available concerning the mechanisms underlying the suppression or refractoriness to induction seen with multiple cocaine injections. We speculate that suppression can be achieved at two levels of cellular organization. The first level is at the plasma membrane and involves receptor desensitization. In vitro studies with cells exposed to D1 agonists have shown acute and more prolonged desensitization depending on the duration of exposure to agonists of the D1 receptor in cells that express the D1 receptor naturally (Barton and Sibley

1990). The second level is at the cell nucleus and involves transcriptional mechanisms. Recently, a group of transacting factors that mediate down-regulation of c-*fos* transcription had been elucidated. One of these, called CREM (cAMP response element modulator), can act as an inhibitory modulator of cAMP-induced activation of c-*fos* gene expression (Foulkes et al. 1991a, 1991b). Thus, we propose that sequential activation of CREB followed by CREM forms the basis of the induction and suppression of c-*fos* expression following cocaine injection. Obviously, receptor desensitization and transcriptional modulation can act in combination to cause IEG suppression. Our data suggest that the suppression of IEG expression is a powerful regulatory mechanism governing the production of protein from this class of genes expression. The fact that, with repeated drug administration, the suppression mechanism(s) override the induction process reinforces the premium that the cell places on regulation of IEG expression. IEG expression is allowed to reach only a certain degree and duration before it is actively inhibited. Thus, the effective and prolonged suppression of IEG expression seen with repeated cocaine administration may be viewed as providing a protective or homeostatic function.

It is interesting to speculate on the meaning of some of these phenomena the authors have observed with cocaine administration. The data illuminate both the normal functioning of the dopaminergic system and its abnormal functioning subsequent to cocaine abuse. Normal dopaminergic functioning probably involves intermittent activation of c-*fos* and modulation of NGFI-A expression either up or down from the basal state of expression. In humans this may serve to couple positively rewarding or pleasurable stimuli to modifications in gene expression. Since cocaine is one of the most rewarding drugs in animal self-administration studies, it is tempting to suggest a relationship between psychological processes such as behavior modification with positive reinforcers and dopaminergic control of gene expression. C-*fos,* acting in concert with other transcription factors, constitutes a molecular interface between behavioral and environmental cues and gene regulation, whereby the IEGs integrate the various inputs received at the cell membrane into a coherent alteration in gene expression. The triggering stimulus may only briefly influence neurotransmission (e.g., cell depolarization, transmitter release) but may yield more permanent changes in neuronal biochemistry and function. A single intravenous injection of cocaine causing a brief “rush” fits this model very well. Subsequent regulation of target genes such as the dopamine D1 receptor or the preprotachykinin gene, both of which have AP-1 consensus elements in their promoter region, could lead to more permanent changes in neuronal function. Obviously, optimal changes in target gene expression depend on the rate of presentation and the intensity of the reinforcing cues to provide the optimal amount and duration of IEG expression, Too great an intensity at too frequent

a rate of presentation leads to a rapid uncoupling of the system as occurs with multiple injections of cocaine. Recent studies with once-daily injections suggest that even with this more mild treatment schedule full uncoupling can occur if cocaine is administered for a long enough duration (e.g., 14 days) (Hope et al. 1992).

The second area for speculation is the potential impact the induction and suppression processes may have on the human abuse situation. Because the uncoupling switches the cell from a transmitter-regulated mode of IEG expression to a purely constitutive mode, the normal response to hedonic cues at the molecular level is short circuited. Thus, certain gene products may accumulate to abnormal levels or fail to accumulate as necessary and thereby establish a new but abnormal complement of neuronal gene products. If the drug is abused *continually,* then maintenance of the new state (constitutive mode only) is relatively ensured, especially given the long duration of the suppression effect. In fact, the suppression could carry over from one session to another, even with a hiatus of 1 or more days as often occurs in human abuse (Spotts and Shontz 1980; Gawin and Ellinwood 1988; Gawin 1991). Such a scenario could yield a very prolonged state of dysregulation. Thus, it is possible that one or more of the problems related to addiction and craving may result, not from the prominent induction of IEGs seen with a single injection, but from the prolonged blockade of regulated expression engendered by the suppression process.

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