Molecular Neurobiology

ISSN0893-7648/1992/5/1:45–58/$2.80

The Molecular Biology of Addictive Drugs

Scott A. Mackler* and James H. Eberwine

Department of Pharmacology,
University of Pennsylvania School of Medicine,
Philadelphia, PA 19104

Contents

*Author to whom all correspondence and reprint requests should be addressed.

Abstract

The addictive drugs alcohol, morphine, cocaine, and amphetamine are each associated with the development of tolerance and physical dependence. Changes in gene expression occur in cell culture and in vivo with the administration of these centrally-acting drugs. This article reviews those experiments that have studied drug-induced alterations in gene transcription.

Ethanol has diverse effects on the amounts of messenger RNA molecules within the central nervous system. Ion channels, neuropeptides, membrane receptors, and immediate early genes represent several regulated mRNAs. The effects are selective, however, as many other specific products are not altered. Evidence for a genetic predisposition to ethanol use reinforces the importance of the genotype.

Opioids, cocaine, and amphetamine also affect gene transcription. Messenger RNAs studied have included many of those demonstrated to be altered by alcohol use. Interestingly, use of any of these drugs alters the expression of immediate early genes. These genes may represent an initial step in the pathway that leads to drug addiction.

The composite of drug-induced changes in gene expression results in the cellular responses of tolerance and dependence. The characterization of these changes should provide a better understanding of the molecular mechanisms of drug addiction.

Index Entries: Gene transcription; tolerance; physical dependence; drug addiction; ethanol; opiates; cocaine; amphetamine.

Introduction

The nonmedical use of alcohol and other addictive drugs has resulted in multiple problems for society (Kozel and Adams, 1986). Chronic self-administration of these centrally-acting drugs leads to the development of stereotypical behaviors that characterize drug addiction. Tolerance (defined by an increased dose of a drug necessary to achieve a desired effect) and physical dependence (manifested in an acute withdrawal syndrome) are principal features of addiction. The molecular mechanisms of tolerance and physical dependence are not well understood. The predictability of these behaviors, which occur in response to chronic and repeated drug use, suggests that changes in gene expression are necessary for their development (similar to studies of some other long-term behaviors (Goelet et al., 1986)). In addition, human addicts demonstrate conditioned responses, a form of long-term memory, in response to environmental stimuli. This contributes to withdrawal syndromes and drug craving (O'Brien, 1975). Alterations in the amounts of various messenger RNA (mRNA) molecules within the central nervous system (CNS) have been observed in laboratory animals with the repeated use of addictive drugs . This article will review experiments that have demonstrated changes in gene expression after the use of three types of addictive drugs: alcohol, opiates, and the psychostimulants cocaine and amphetamine.

Material describing changes in protein function and/or structure following addictive drug use exists and has been the subject of several previous reviews (Koob and Bloom, 1988; Simonds, 1988; Deitrich et al., 1989; Johnson and Fleming, 1989). This information, coupled with electrophysiological evidence (North, 1986; Deitrich et al., 1989), indicates that different types of addictive drugs affect similar neurochemical and neuroanatomical pathways. This raises the possibility that a common substrate exists within the dopaminergic pathways into and out of the limbic system of the CNS that contributes to the development of addiction (Koob and Bloom, 1988). Therefore, studies that detail the molecular and cellular mechanisms of drug addiction for one type of drug may be directly applicable to studies of other drugs.

The major hypothesis of this article is that changes in gene expression are associated with and result in the principal features of drug addiction. Recent advances in molecular biology now make it feasible to identify products of both known and novel genes whose expression are regulated by addictive drug use in cell culture, laboratory animals, and human subjects. Although many proteins have been demonstrated to be affected by addictive drug use, this review will discuss experiments that have directly attempted to detect changes within the CNS of mRNAs or genes involved in the development of drug addiction.

Ethanol

Background

Ethanol is a depressant that has multiple effects within the CNS (Deitrich et al., 1989). Ethanol partitions easily into lipid bilayers and a relatively high concentration is necessary to depress neuronal function (millimolar amounts). Therefore, ethanol affects many sites, and a single mode of action has not yet been identified. A previous theory that ethanol exerts its effects by perturbing all cell membranes based on its lipid solubility has gradually been replaced by evidence supporting specificity in the diverse effects of ethanol on the CNS.

Ethanol administration alters neuronal electrical activity in several areas of the brain. The majority of the effect is a depression in spontaneous or evoked action potential firing (locus ceruleus (Poherecky and Brick, 1977), raphe nuclei (Chu, 1984), and hippocampus (Sorensen et al., 1980)). Increases in electrical activity in other regions (cerebellar Purkinge cells (Sorensen et al., 1981), inferior olive nucleus (Rogers et al., 1986), ventral tegmentum (Brodie et al., 1988), and substantia nigra (Mereu et al., 1984)) are presumably mediated by disinhibition of inhibitory inputs mediated by augmentation of gamma amino butyric acid (GABA)-activated chloride ion channels. The dimunition in neuronal activity may also result from either a depression of ion flux through voltage-sensitive ion channels or reductions in synaptic transmission (most likely by interfering with intracellular Ca^{2+}) or a combination of these effects (Carlen and Wu, 1988).

Chloride ion channels, which respond to GABA as the primary ligand (GABA-A receptors), are typically composed of polypeptides from three separate subunits (alpha, beta, and gamma (Pritchett et al., 1989)). Multiple isoforms for each subunit exist and most likely contribute to the different responses exhibited in several regions of the mammalian CNS. Ethanol acts to enhance the flow of chloride ions through the GABA-A receptor in response to GABA stimulation. This has been demonstrated by measuring the flux of $^{36}Cl^-$ across cell membranes (Suzdak et al., 1986), but difficult to replicate in electrophysiological studies.

Ethanol also affects binding to several other membrane receptors and the synthesis and release of multiple neurotransmitters and neuropeptides (Deitrich et al., 1989). These findings further highlight the selective actions of ethanol, as not all molecules studied are altered by ethanol administration.

Effects on RNA

General Studies of RNA Synthesis

Early studies in both whole brain and cell-free systems demostrated that chronic ethanol exposure reduces both RNA and protein synthesis (Tewari et al., 1975). These experiments utilized pulse-chase labeling with radioactive precursors of RNA and protein synthesis, illustrating the diffuse influence ethanol may have. A 10-d treatment of 100 m*M* ethanol in cultured astrocytoma cells resulted both in a decrease in RNA synthesis and a reduction in the total number of polysomes (Fleming et al., 1981). These experiments suggested a decrease in the stability of the majority of mRNA species as a partial explanation for the diminished protein synthesis. Fourteen days of ethanol vapor inhalation in adult rats

(resulting in a similar blood alcohol concentration) did not lead to a decrease in total ribosomal and total polyadenylated RNA within the cerebral cortex, as studied with dot blots (Montpied et al., 1991). Furthermore, expression of the mRNA for the constitutively expressed beta-actin also did not change in this study. A separate study, also employing 14 d of ethanol inhalation, found no reduction in total RNA amounts in the anterior and intermediate lobes of the pituitary gland (Dave et al., 1986). Therefore, the effects of ethanol on RNA synthesis or stability most likely are a function of both the individual RNA molecule and its location within the CNS (Table 1).

Ion Channels

Exposure to ethanol inhibits the flow of calcium and the generation of calcium-dependent potentials through voltage-sensitive Ca^{2+} channels in several types of cultured neurons (Deitrich et al., 1989). Chronic exposure to ethanol in adult rats and in cell culture has been associated with an increase in the binding of ligands for the dihydropyridine-sensitive Ca^{2+} channel (Dolin et al., 1987). Interestingly, treatment with an appropriate Ca^{2+} channel antagonist may inhibit the alcohol withdrawal syndrome in adult rats (Little et al., 1986), suggesting a possible role for Ca^{2+} channel regulation in alcohol addiction. The increase in number of dihydropyridine-sensitive Ca^{2+} channels is prevented in cultured adrenal cells simultaneously treated with anisomycin (an inhibitor of RNA synthesis) (Harper et al., 1989). Thus, transcription is necessary for the observed upregulation of one class of Ca^{2+} channels that occurs in alcohol-tolerant rats.

Inhalation of ethanol in adult rats over two weeks (resulting in tolerance) leads to a dimunition in GABA-A receptor $^{36}Cl^-$ flux (Morrow et al., 1988). These studies, performed in cerebral cortical synaptoneurosomes, suggest a role for GABA-A-mediated currents in the development of tolerance and withdrawal. A reduction in the amount of alpha1 and alpha2 subunit transcripts (two of the three GABA-A alpha subunits studied) occurs in cerebral cortical RNA after identical treatment (Montpied et al., 1991). This effect was selective, as no decrease was noted for the GABA synthetic enzyme glutamic acid decarboxylase mRNA or other RNAs. Similar findings for a decrease in GABA-A alpha subunit transcription products has been demonstrated in cultured chick neurons, an effect blocked by application of the GABA-A antagonist SR 95531 (Montpied et al., 1991).

Membrane Proteins

Neurotransmitter and neuromodulator receptors occupy a central role in the transduction of an extracellular signal onto individual neurons. Although many experiments have demonstrated changes in receptor number and affinity to their selective ligands in response to ethanol treatment (Valverius et al., 1989), similar changes in mRNAs encoding many of these receptors have not yet been investigated.

Exposure of neuroblastoma-glioma cells (NG108-15) to 100 m*M* ethanol for 48 h resulted in a 30% reduction in the mRNA for the alpha subunit of the stimulatory guanine nucleotide binding (G) protein (Mochly-Rosen et al., 1988). Similar magnitudes of decrease were also noted for the corresponding alpha subunit protein and cyclic AMP stimulation in response to isoprenaline treatment. The central position G proteins play in signal transduction may help to explain some of the diverse effects of alcohol without directly altering multiple neurotransmitter systems.

Ethanol treatment of NG108-15 cultured cells increases delta opiate receptor binding (Charness et al., 1986). However, this upregulation of the opiate receptor occurs in the presence of actinomycin D (a RNA synthesis inhibitor). Ethanol-induced opiate receptor upregulation in cell culture is apparently independent of transcription. The development of alcohol tolerance in rats was associated with changes in *in situ* hybridization signals for the muscarinic type three receptor (in a preliminary study that used ogligonucleotides as probes designed for several muscarinic subtypes (Vincent et al., 1990)).

Table 1
Studies of Ethanol Administration

Regulated mRNAs	Nonregulated mRNAs
GABA-A receptor (cortex)	Ribosomal (18S and 28S) RNA (cortex)
Dihydropyridine-sensitive calcium channel	Total polyadenylated RNA (cortex)
Muscarinic receptor type III (cortex)	Beta-actin (cortex)
Proopiomelanocorticin (pituitary)	Glutamic acid decarboxylase (cortex)
Vasopressin (hypothalamus)	Prolactin (pituitary)
c-*myc* (cell culture)	Opiate receptor, delta subtype[b] (cell culture)
c-*myb* (cell culture)	
Tumor necrosis factor (cell culture)	
c-fos[a]	

[a]Studied in alcohol withdrawal.
[b]Indirect evidence only.

Neuropeptides

Alcohol is associated with changes in the release and synthesis of several neuropeptides in vivo (Deitrich et al., 1989). In addition, some of the behavioral effects of acute ethanol exposure are similar to (neurotensin) or reversed (thyrotropin releasing hormone) by various neuropeptides. Acute alcohol administration (less than 24 h) results in increases in the opioid peptides and their precursors. However, chronic adminitration in vivo and in cell culture is accompanied by a reduction in proopiomelanocorticin (POMC) mRNA levels. POMC mRNA decreases in both the anterior and intermediate lobes of the rat pituitary after 14 d of alcohol inhalation (Dave et al., 1986). This effect may be mediated by alterations in the corticotrophin releasing factor (CRF) membrane receptor in the pituitary, because there is a comparable reduction in CRF binding activity and adenylyl cyclase activity. The same ethanol treatment did not affect prolactin mRNA levels in the pituitary gland.

Acute alcohol use results in a reduced release of vasopressin from the hypothalamus in human subjects and rodents (Brinton et al., 1986), resulting in a vigorous diuresis. This effect persists with chronic ethanol use. Seven days of ip ethanol administration leads to an approximate 50% reduction of vasopressin mRNA autoradiographic signals in both the paraventricular and supraoptic nuclei of the hypothalamus, as assessed by *in situ* hybridization (Ishizawa et al., 1989).

Immediate Early Genes

Immediate early genes are transcriptional activators that respond to multiple types of stimuli in the CNS by a rapid increase in their mRNA and protein levels (Sheng and Greenberg, 1990). The increase in mRNA levels does not require new protein synthesis and several oncogenes act as immediate early genes. Administration of acute ethanol does not alter total brain *c-fos* expression (Le et al., 1990). Pentylenetetrazole is a convulsant that induces *c-fos* expression in the CNS. However, concomitant administration of ethanol with pentylenetetrazole reduced the expected expression of the *c-fos* gene (Le et al., 1990). Ethanol-induced withdrawal seizures also produced an increase in *c-fos* expression in the mouse brain (Dave et al., 1990). This effect occurred throughout the CNS and was greatest in the hippocampus (a similar finding to other seizure-induced increases in *c-fos* transcription).

Ethanol (at a concentration equal to or less than 200 m*M*) has an effect on oncogenes in cultured leukemia cells (Datta et al., 1990). Both *c-myc* and *myb* transcripts were decreased in a concentra-

tion-dependent manner. Expression of the cytokine tumor necrosis factor was increased in these same cells. The protein products of these RNAs may also play an important role within the CNS (Sheng and Greenberg, 1990).

Genetic Predisposition to Alcohol Use

Animal Studies

Several strains of rats and mice have been bred for successive generations, which demonstrate preferences for or differences in the behavioral response and metabolism of alcohol (Belknap et al., 1983). These inbred strains help indicate the role that genetic factors play in the development of alcohol addiction. Selection for these rodent lines has included differences in the righting reflex in response to ethanol use (a measure of alcohol's acute hypnotic effect), preference of ethanol vs other forms of caloric intake, predeliction to ethanol withdrawal-induced seizures, in addition to other characteristics. Reports have attempted to identify individual chromosomes and their gene products that are associated with a genetic susceptibility to alcohol use (Goldman et al., 1987). However, a rigorous documentation of a gene and its identified product contributing to alcohol addiction has not yet been demonstrated.

Detailed experiments in mice strains that differ in the righting reflex following acute ethanol administration have implicated a contribution by the GABA-A receptor (Wafford et al., 1990). GABA-A receptor expression studies in *Xenopus* oocytes revealed differences between total mRNA isolated from whole brains of these two strains (SS for short sleep and LS for long sleep). Oocytes with mRNA injected from the ethanol-"sensitive" LS mice demonstrated a facilitation of ethanol's effects on GABA-mediated currents. The "insensitive" SS mouse RNA was associated with an inhibition of GABA-mediated currents. Barbiturate and benzodiazipene stimulation did not differ in GABA current activation between mRNA from the two mouse lines. Furthermore, no differences were seen in the expected inhibition of NMDA receptor-mediated responses in the oocytes. This selective effect of ethanol studied in an in vivo transcription system suggests a molecular mechanism for the genetic difference in the hypnotic effects of ethanol. Future studies should identify the differences between the mRNA molecules.

Human Studies

A large number of epidemiological studies have implicated a genetic susceptibility for the development of alcohol addiction (Schuckit, 1985). Children with a family history for alcoholism demonstrate a higher prevalence of developing alcohol-related problems later in life. Concordance rates in monozygotic twins are roughly twice the rates for those seen in dizygotic twins. The most compelling investigations have employed cross-rearing studies. Adoption of a son with a biologic alcoholic parent into a nonalcoholic family is still associated with an increased rate of alcohol-related problems.

A recent analysis of cadaver brain tissue was performed in alcoholics and nonalcoholics for an allelic association of the dopamine D_2 receptor by restriction fragment-linked polymorphism (Blum et al., 1990). The choice of the D_2 receptor resulted from the suspected central role of dop-aminergic pathways in the limbic-nucleus accumbens in drug addiction (Koob and Bloom, 1988) and the identification of the human chromosome containing this gene. An allelic association of 70% was described by Southern blot analysis for one allelle (A_1) and alcoholism. A combination of a limited-sample size, questions about the means of ascertaining the diagnosis of alcoholism, and a low allelic association, each have suggested that additional work must be performed. Further work (including a large and well-defined kindred analysis) is necessary in identifying which molecules contribute to a genetic predisposition for alcohol use.

Opiates and Opioid Peptides

Background

Endogenous opioid peptides are widely distributed throughout the mammalian CNS. The genes for the precursors of the three major classes of opioid peptides (POMC, prodynorphin, and proenkepahalin) have been previously cloned (Evans et al., 1988). This work has allowed for detailed neuroanatomical studies into the regulation and function of the endogenous opiates. Selective ligand analysis, utilizing both agonists and antagonists, has resulted in a classification of opiate receptor subtypes. Activation of opiate receptors directly inhibits the receptor expressing cell (North, 1986), which in some cases leads to disinhibition of other neurons. Inhibition is in part mediated by signal transduction via the G protein system (Brandt et al., 1976). The location of these receptor subtypes within the CNS has been described by autoradiographic studies (Kuhar et al., 1973), resulting in attempts to define opiate receptor subtype function. The detailed information about the endogenous opiates and their receptors has not, however, been sufficient to explain the addictive properties of opiate use.

Early studies demonstrated that treatment of adult rats with RNA synthesis inhibitors prevented the development of morphine-induced analgesia (monitored by behavioral assays (Cox and Osman, 1970). Infusion of morphine to pregnant rats in amounts sufficient to result in tolerance did not significantly alter total RNA amounts in the brains of the maternal or fetal rats (Johannesson et al., 1972). Therefore, selective synthesis of certain mRNA molecules may be necessary for the development of tolerance in response to opiate stimulation. Recent experiments are beginning to demonstrate changes in the amounts of several identified mRNAs (Table 2 and below).

Stimulation of opiate receptors with morphine in neonatal rats also inhibits incorporation of radioactive thymidine into newly synthesized DNA (Kornblum et al., 1987). This inhibition of DNA synthesis in neonatal rat brain was prevented by concurrent use of naloxone and also did not occur in the spleen. This regional and selective effect of morphine on DNA synthesis, in addition to altering cellular division, may also directly interfere with gene transcription.

Studies of mRNA Levels

Studies in Cell Culture

The NG108-15 cell line has been extensively studied for changes in opiate receptor binding. The delta receptor subtype is expressed on these cells (Klee and Nirenberg, 1974) (and also on another glioma cell line (Chang et al., 1978)), as determined by selective ligand-binding studies. Changes in receptor number and binding affinity result from agonist treatment (Law et al., 1983). These actions occur within less than 24 h and proceed in the absence of RNA synthesis (Law et al., 1985). However, upregulation of receptor numbers is inhibited by prevention of RNA synthesis (Law et al., 1985). This suggests that formation of new opiate receptors is dependent on transcription. The cloning of a complementary (c)DNA clone encoding an opiate receptor subtype could aid further studies into the transcriptional effects of opiate use. The NG108-15 cells also contain proenkephalin mRNA and proenkephalin-derived peptides (Schwartz, 1988). Treatment with a delta opiate receptor agonist or morphine for more than 4 d resulted in a threefold increase in proenkephalin mRNA. These increases were prevented by concurrent administration of the opiate antagonist naloxone, directly implicating mediation by opiate receptor stimulation.

Another study of G proteins showed that the mRNA encoding the alpha subunit of the stimulatory G protein increased approx 30–50% in response to morphine (Von Zastrow et al., 1991). This change was naloxone-reversible, showing the opiate specificity of this response.

Table 2
Study of Opiate Receptor

Regulated mRNAs	Nonregulated mRNAs
Opiate receptor, delta subtype[a] (cell culture)	Somatostatin (striatum)
Enkephalin (cell culture, striatum, pituitary)	Enkephalin (hypothalamus)
Dynorphin (striatum, hippocampus)	Corticotrophin releasing factor (hypothalamus)
Dynorphin[b] (hypothalamus)	Vasopressin (hypothalamus)
Tyrosine hydroxylase (locus ceruleus)	Oxytocin (hypothalamus)
Ornithine decarboxylase[a] (hippocampus)	Tyrosine hydroxylase (substantia nigra)
c-fos (striatum, locus ceruleus)	*c-myc*[b] (locus ceruleus)
c-fos[b] (multiple CNS regions, cell culture)	GABA-A receptor (cell culture)
c *jun* (cell culture)	
c-*jun*[b] (multiple CNS regions)	
Corticotropin releasing factor[b] (hypothalamus)	
Vasopressin[b] (hypothalamus)	
Stimulatory guanine binding protein, alpha subunit (cell culture)	
Voltage-sensitive potassium channel (cell culture)	
Voltage-sensitive calcium "A" channel[b] (cell culture)	
Voltage-sensitive sodium brain channel, type II[b] (cell culture)	

[a]Indirect evidence only.
[b]Studied in opioid withdrawal.

This alteration occurred while the alpha subunit of the inhibitory G protein (which interacts with the delta opiate receptor) remained unchanged. These data suggest that changes in the stimulatory G protein to inhibitory G protein ratio may be important in cellular responses to opioid use.

Studies of Tolerance in Adult Rats

Rats develop tolerance to morphine, and tolerance may occur either from self-administration or treatment in which the experimenter applies the opiate drug (Blasig et al., 1973). Daily subcutaneous placement of a delayed release pellet of morphine is a standard method used to achieve tolerance, as monitored by various behavioral assays. Physical dependence, as manifested by a withdrawal syndrome, may be precipitated by a single injection of an opiate receptor antagonist. Several investigators have employed these techniques over a period of 5–12 d in order to study the expression of selected mRNA molecules.

Neuropeptides. Preproenkephalin levels decline in the striatum to 66% of control values in morphine-tolerant rats (Uhl et al., 1988). This decrease is selective, however, because the neuropeptide somatostatin does not demonstrate any changes in mRNA amounts in the same animals (mRNA levels for dynorphin nay also be reduced by morphine treatment (Romualdi et al., 1990)). The striatum is a CNS region containing a large number of opiate receptors (as determined by receptor autoradiography studies). Use of an opiate antagonist to interfere with these effects would further identify a specific role of opiate receptors in this regulation of gene epression. Prodynorphin mRNA amounts are also decreased in the hippocampus (Romualdi et al., 1990).

The use of *in situ* hybridization helps to define the regional and cellular localization of probes designed to hybridize to specific mRNA molecules. Changes in the number of silver grains after emulsion autoradiography may be quantitated for statistical analysis. Morphine treatment resulted in an increased number of grains for dynorphin in the hypothalamus but had no effect on signals for enkephalin, CRF, vasopressin, and oxytocin (Lightman and Young, 1988).

Metabolic Enzymes. The phosphorylation state of several proteins extracted from the locus ceruleus (another opiate receptor-rich area) of morphine-tolerant rats is altered. These detailed studies, including in vitro experiments of known

proteins (e.g., cAMP-dependent protein kinase (Nestler and Tallman, 1988) and components of the G protein pathway (Nestler et al., 1989)) have identified the mRNA encoding the synthetic enzyme tyrosine hydroxylase as one transcript regulated by opiate use (Guitart et al., 1990). An increase in the amount of mRNA for tyrosine hydroxylase occurs within the locus ceruleus but not the substantia nigra of morphine-tolerant rats. Presumably, there are changes in transcription for the many other proteins that are altered by opiate use (or in the proteins that regulate the synthesis or phosphorylation of these molecules). Indirect evidence (by use of RNA synthesis inhibitors) suggests that opioid peptides potentiate a transcription-dependent increase in ornithine decarboxylase activity studied in hippocampal slices (Baudry et al., 1988).

Immediate Early Genes. A single iv injection of morphine (not enough for the development of tolerance) results in an increase in striatal amounts of the immediate early gene *c-fos* (Chang et al., 1988). This increase in *c-fos* transcription (documented with dot blot analysis) is blocked by administration of the opiate antagonist naloxone. Immunohistochemical staining for the Fos protein displays a similar increase in protein amounts in the same experiments. Interestingly, the opioid peptide precursor preproenkephalin may itself be regulated by immediate early gene expression (Sonnenberg et al., 1989). Thus, induction of the mRNA for one or more immediate early genes may directly lead to the transcriptional regulation of mRNAs directly involved in neural pathways that mediate the responses to opioid use. The effects of chronic opiate stimulation on *c-fos* expression in the striatum have not yet been described, although the initial increase in mRNA amounts is rapidly reversed. Acute and chronic morphine treatment is associated with a reduction in *c-fos* mRNA amounts in the locus ceruleus.

Studies of Withdrawal in Adult Rats

Withdrawal in morphine-tolerant rats also is associated with changes in mRNA amounts. Although not as many studies have been performed as in experiments of opiate-induced tolerance, the results nonetheless are informative.

Neuropeptides. Preproenkephalin levels in the striatum increase from the reduced levels resulting from morphine stimulation (Uhl et al., 1988). This increase, however, does not reach the levels observed in control animals. *In situ* hybridization studies revealed several changes in grain amounts for probes of various neuropeptides in the hypothalamus and pituitary (Lightman and Young 1987,1988). Naloxone-precipitated withdrawal increased grains for CRF and enkephalin in paraventricular cells and grains for POMC in the anterior pituitary. No changes were noted for vasopressin, dynorphin, and oxytocin. Withdrawal of morphine without addition of naloxone resulted in different alterations in grain numbers. Signals in the hypothalamus increased for vasopressin and dynorphin but did not change for CRF. The discrepancy in the results between naloxone-precipitated and simple morphine withdrawal remains unexplained.

Immediate Early Genes. Use of the mixed opiate agonist/antagonist naltrexone to precipitate withdrawal in morphine-tolerant rats led to increases in *c-fos* expression in several regions of the adult rat CNS (Hayward et al., 1990). These included the locus ceruleus, ventral tegmentum, amygdala, nucleus accumbens, striatum, and cortex, areas rich in monoaminergic pathways felt to be integral in the development of opiate addiction. The effects were selective, because similar changes were not observed in the hippocampus, dorsal raphe, and periaqueductal grey region. Levels of *c-jun* mRNA also increased in a region-specific manner, involving the locus ceruleus and amygdala. The protooncogene *c-myc* did not demonstrate changes within the locus ceruleus during withdrawal in the same study.

Expression Profiling

The above experiments demonstrate that several mRNA molecules are affected by opiate use. Tolerance and physical dependence most likely are a function of changes in many of these mRNAs and do not result from a single mRNA and its protein. Although experiments can be

performed to illustrate alterations in individual cDNA clones for specific mRNAs, this approach can be time-consuming. Furthermore, the functional importance of changes in mRNA expression may be reflected in the relative amounts of altered gene expression. For example, documenting that the mRNA for a neurotransmitter decreases may not be as significant as also knowing if changes also occur for another neurotransmitter or neuromodulator involved in the same neuronal pathway.

A method of in vitro transcription that amplifies the polyadenylated RNA fraction of small amounts of tissue has recently been described (Van Gelder et al., 1990). This method utilizes specific bacteriophage RNA polymerase promoter sequences in the first strand cDNA synthesis. Positioning this promoter sequence 5' to an oligothymidine primer leads to the eventual amplification of the polyadenylated RNA population in a distribution proportionate to the starting material. These antisense RNA transcripts may be directly used as probes with the incorporation of radioisotopic nucleotides. This antisense RNA has been hybridized to large numbers of previously isolated cDNA clones immobilized on a single blot in equimolar amounts, providing profiles of mRNA expression patterns from the original tissue (Mackler and Eberwine, 1990). Results have demonstrated changes within several groups of neuronal cDNA clones in cell culture and from discrete regions of the rat CNS.

The time-course of changes of mRNA patterns of expression in NG108-15 cells treated with an opioid peptide or precipitated withdrawal of this peptide has been determined (Mackler and Eberwine, 1991). Voltage-sensitive potassium channels show decreased amounts of mRNAs following opiate stimulation associated with receptor down-regulation. In contrast, mRNAs for the voltage-sensitive calcium and sodium channels are unchanged with stimulation but increased in precipitated withdrawal. The oncogenes *c-fos* and *c-jun* also respond with increases in precipitated withdrawal and *c-jun* mRNA alone increases with opioid stimulation. The induction of *c-jun* expression lasts a longer time-period than that seen in *c-fos* expression. The alpha subunit of a stimulatory G protein also demonstrates an increase in its mRNA levels.

The combination of antisense RNA amplification has been performed after *in situ* transcription (Tecott et al., 1989) in rat brain sections (Mackler and Eberwine, 1991). This approach can be performed on specific regions of interest, and dramatically limits the amounts of tissue necessary to perform the experiments. Probes obtained from the striatum of morphine-tolerant rats have demonstrated similar results as those in cell culture experiments. Identifying the relative changes of mRNA expression profiles should permit the determination of whether the coordinate changes in mRNA levels are important in eliciting opiate-mediated behaviors.

Approaches to Cloning Novel cDNAs

The development of tolerance and physical dependence may result from changes in transcription of both previously isolated and novel cDNA clones. Experiments designed to identify novel, drug-regulated cDNA clones will require knowledge about the protein product (either partial peptide sequence information or antibodies selective for the protein) or a method relying on the nucleotide sequence.

Proteins that are altered by opiate use may be directly visualized via polyacrylamide gel electrophoresis. The physical characteristics of those proteins that are phosphorylated can also be identified with autoradiography of an acrylamide gel. One approach in identifying novel protein products induced during opiate addiction would be to generate polyclonal antibodies directed toward these regulated proteins. The antibodies can next be used to screen a cDNA library that expresses peptides. Another approach, if the protein that is regulated exists in relatively large amounts, would be to purify this protein and perform partial peptide sequence. The peptide sequence is used in designing oligonucleotide probes in the screening of a cDNA library of interest. In either case, a cDNA clone isolated

from a library would next be fully characterized with respect to its DNA sequence, tissue distribution, and function. Studies of protein phosphorylation for the locus ceruleus in response to opiate use have identified several interesting candidates. One such protein was already previously identified as tyrosine hydroxylase and the result was verified (Guitart et al., 1990). No novel cDNA clones for opiate-regulated mRNAs have been published for either of the above methods to date.

Hybridization strategies that depend on differences between cDNAs generated from either control or drug-treated tissues may be attempted. This may involve either subtractive hybridizations (in order to enrich for low abundance, unique mRNAs) or differential hybridization. Clones that demonstrate differences in hybridization signals may be isolated and further studied. Probes synthesized from rat striatal tissue with the combined *in situ* transcription–antisense RNA technique have been used to screen a NG108-15 library in the bacteriophage vector lambda ZAP (Von Zastrow et al., 1991; Mackler and Eberwine, 1990). Differences in the intensity of the hybridization signal between probes from control and morphine-tolerant rats were used to select individual clones. These clones are now being further characterized to assess their presumptive role in this paradigm of opioid tolerance.

Cocaine and Amphetamine

Background

Cocaine and amphetamine are both CNS stimulants that mediate their effects (at least partially) via indirect catecholaminergic receptor activation (Koob and Bloom, 1988). Cocaine interferes with the monoamine reuptake proteins located on the presynaptic surface, which normally remove monoamines present within the synaptic cleft. Amphetamine causes the release of dopamine from presynaptic terminals. The dopaminergic pathways located in the nucleus accumbens appear to play a critical role in repeated use of these drugs (Chiara and Imperato, 1988). Only recently have investigators begun to demonstrate changes in the amounts of mRNAs altered by use of either cocaine or amphetamine (Table 3).

Studies of mRNA Levels

Metabolic Enzymes

Use of amphetamine in tissue culture leads to an induction of mRNA for tyrosine hydroxylase (Fontenot et al., 1987), as seen with many other transsynaptic stimulators of dopamine receptors (Zigmond et al., 1989). Chronic cocaine treatment of adult rats resulted in an increase in immunoreactivity for tyrosine hydroxylase in the ventral tegmentum (Beitner-Johnson and Nestler, 1991). Further work is needed to show if this increase in immunoreactivity is associated with higher levels of tyrosine hydroxylase mRNA (as observed with morphine treatment (Guitart et al., 1990)). Selective stimulation of dopamine receptors in the rat striatum decreases mRNA for the enzyme glutamic acid decarboxylase (Caboche et al., 1991). It seems likely that either cocaine or amphetamine would have similar effects, but this remains to be proven.

Immediate Early Genes

Single ip injections of either cocaine or amphetamine in adult rats resulted in induction of *c-fos* expression in the striatum, determined by *in situ* hybridization, combined with immunohistochemical staining for the Fos product (Graybiel et al., 1990). Surprisingly, the patterns of *c-fos* activation differed in the striatum between the two drugs. One hypothesis for how cocaine regulates activity appears to be via activation of D1 receptors (Robertson et al., 1989), and possibly other monoamine receptors. Northern blot analysis confirmed the cocaine-induced activation of *c-fos* in the striatum and also revealed a smaller magnitude of activation in the cerebellum (Iadarola et al., 1990). Single iv injections of cocaine showed a time-dependent increase in *c-fos* mRNA in sev-

Table 3
Studies of Cocaine or Amphetamine Administration

Cocaine-regulated mRNAs	Amphetamine-regulated mRNAs
c-fos (striatum, cerebellum, hippocampus, amygdala, cortex)	Tyrosine hydroxylase (tissue culture)
Zif/268 (striatum)	*c-fos* (striatum)

eral CNS regions (including the striatum, cerebellum, amygdala, hippocampus, and cortex (Cohen et al., 1990)). Another immediate early gene, *Zif/268,* was also activated in this study in similar regions. The use of the alkaloidal form of cocaine ("crack") should result in similar effects on gene transcription.

Summary

Drug addiction associated with the use of many commonly abused substances (including alcohol, opiates, and psychostimulants) occurs only after repeated and chronic drug use. The stereotypical behaviors of addiction and evidence for a genetic predisposition toward developing drug dependence both strongly suggest a causative role mediated by the regulation of gene expression. Acute and chronic use of addictive drugs may alter the neuropharmacology and synaptic transmission within several regions of the CNS. However, changes in the levels of mRNA molecules are hypothesized to play a critical role in the development and maintenance of drug adiction (Fig. 1). The alterations in mRNA amounts may result from increases in transcription and/or changes in metabolism. Studies of RNA synthesis (nuclear run-on assays) and stability will be needed to address this issue.

Experiments are being performed at an increasing rate that demonstrate the effects of addictive drug use on neuronal gene expression. Because of the diverse effects of drug use within the CNS, it is prudent to expect that multiple gene products contribute to the development of addiction. These gene products may encode structural proteins or involve the regulation of further transcription and translation. The role of immediate early gene activation resulting from acute drug use may serve as an initial trigger in activating gene expression of other mRNA molecules. It seems likely that immediate early gene activation (as hypothesized in other systems (Sheng and Greenberg, 1990)) in drug-responsive neurons would activate the expression of a number of genes, resulting in manifestations of addiction.

Identification of previously characterized mRNAs that are regulated by addictive drug use may not be sufficient in completely defining the molecular basis of addiction. Strategies designed to isolate novel cDNAs that are involved in drug addiction need to be employed. Analysis of their protein products will help in resolving the pathophysiology of addiction. Furthermore, experiments limited to cell culture need to be complemented by in vivo studies, in order to ensure that the effects of transsynaptic regulation of gene expression in these behaviors is not ignored.

A large number of experiments have demonstrated individual neurochemical and neurophysiological changes resulting from addictive drug use. However, no effective treatment strategies presently exist for the addicted patient. A more detailed description of the molecular basis of drug addiction should help in the development of new treatment strategies directed toward the underlying pathophysiology (e.g., Little et al., 1986; Trujillo and Akil, 1991). Common mechanisms of action between alcohol, opiates, and psychostimulants may mean that results obtained from studies of one type of addictive drug may be directly applicable to other forms of addiction.

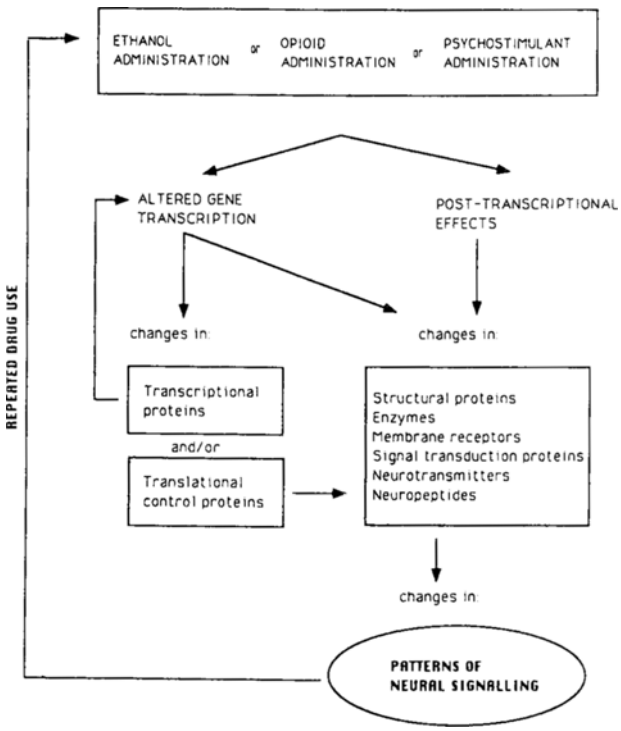

Fig. 1. Administration of ethanol, opioids, cocaine, or amphetamine has been shown to affect mRNA amounts (altered gene transcription) and alterations in several different proteins (posttranscriptional effects). Regulated gene transcripts may include those that activate or inhibit further gene transcription (e.g., immediate early genes), or others that directly affect protein translation. Both of these pathways will lead to changes in the synthesis of several types of proteins. Alterations in these proteins involved in the neural pathways that mediate drug addiction behavior will cause repeated drug use.

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