National Institute on Drug Abuse

# RESEARCH

MONOGRAPH SERIES

# Molecular Approaches to Drug Abuse Research Volume I

111

U.S. Department of Health and Human Services • Public Health Service • National Institutes of Health

# Molecular Approaches to Drug Abuse Research Volume I: Receptor Cloning, Neurotransmitter Expression, and Molecular Genetics

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Research Monograph 111
1991

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Alcohol, Drug Abuse, and Mental Health Administration

National Institute on Drug Abuse
5600 Fishers Lane
Rockville, MD 20857

For sale by the Superintendent of Documents, U.S. Government Printing Office
Washington, D.C. 20402

**ACKNOWLEDGMENT**

This monograph is based on the papers and discussion from a technical review on "Molecular Approaches to Drug Abuse Research" held on August 24-25, 1989, in Bethesda, MD. The review meeting was sponsored by the National Institute on Drug Abuse.



NIDA Research Monographs are indexed in the *Index Medicus.* They are selectively included in the coverage *of American Statistics Index, BioSciences Information Service, Chemical Abstracts, Current Contents, Psychological Abstracts,* and *Psychopharmacology Abstracts.*

DHHS publication number (ADM)91-1798
Printed 1991

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# Introduction

***Theresa N.H. Lee***

The extraordinary advances in molecular biology since the early 1970s have created unprecedented opportunities for progress in every field of biological research. The sensitivity and specificity of these techniques have enabled scientists to acquire an enormous amount of knowledge about the genetic control of the physiological and pathological processes and to resolve these processes at the molecular level.

The opportunity for progress presented by molecular biology in understanding the fundamental nature of the addictive process is particularly timely in light of the national consensus to develop new approaches to the problem of drug addiction.

Despite advances in many fields, molecular biological approaches remain underutilized in research related to understanding the actions and consequences of abused substances. In recent years, extensive progress has been made on the structure, biosynthesis, modification, degradation, and regulation of opioid peptides and their precursors. However, similar studies on the receptors, transporters, and ion channels relevant to many abused substances are still lacking.

To stimulate more research in these and other areas, the first technical review by the National Institute on Drug Abuse (NIDA) on "Molecular Aproaches to Drug Abuse Research" was held on August 24-25, 1989, in Bethesda, MD. The proceedings of this conference are presented in the following chapters of this monograph.

All the speakers either have successfully cloned a gene relevant to drug abuse or have been employing molecular approaches to drug abuse research. However, due to limited research carried out in some areas, the specific abused substances studied by the speakers do not necessarily represent NIDA's current programmatic preference. For example, because the muscle nicotinic acetylcholine receptor genes have been cloned and studied for a number of years, results of experiments manipulating the expression of these subunit genes instead of the neuronal genes were presented at this meeting.

This monograph provides many elegant examples of what researchers can do with the molecular approaches to drug abuse research. For drug abuse researchers interested in molecular approaches, the decade to come undoubtedly will prove to be both challenging and fruitful, with ground-breaking results contributing to understanding the underlying basis of addiction and the consequences of long-term drug abuse to generate better strategies for effective diagnosis, treatment, education, and prevention.

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# Brain Nicotinic Receptor Genes

***Stephen F. Heinemann, Jim Boulter, John Connolly, Evan Deneris, Robert Duvoisin, Melissa Hartley, Irm Hermans-Borgmeyer, Michael Hollmann, Anne O'Shea-Greenfield, Roger Papke, Scoff Rogers, and Jim Patrick***

Nicotine is one of the most widely taken addictive drugs, and the history of the cultivation of tobacco suggests that early man appreciated the behavioral effects of nicotine. About one-third of the adult population of the United States is addicted to nicotine, which is mostly delivered to the body through the smoking of cigarettes. Nicotine addiction has led to an epidemic of lung cancer and heart disease in the United States and in many other industrialized countries. The less developed countries of the world increasingly face a similar fate. As is the case for chemical addiction in general, the mechanism of nicotine addiction is not known.

To develop a rational theory of nicotine addiction, it is necessary to understand how nicotine exerts its effects on the nervous system. With this knowledge, it might be possible to develop drugs that would help patients overcome their nicotine addiction. Many drugs that alter brain function or have proven useful for the treatment of mental disorders are known to affect synaptic transmission. Thus, the synapse is a plausible place to look for the site of nicotine action in the brain. During the past decade, the availability of radiolabeled nicotine with high specific activity has led to the discovery and mapping of nicotine binding sites in the mammalian brain (Clarke et al. 1985, 1986; for review, see Martin 1986; Clarke 1988). These data suggested that the mammalian brain contains an important nicotinic receptor system.

To investigate this possibility that the mammalian brain contains a major nicotinic receptor system, the authors decided to use a molecular genetic approach to identify genes that code for nicotinic receptors expressed in the brain. We reasoned that the brain nicotinic receptors might be evolutionarily related to the *Torpedo* electric organ and skeletal muscle nicotinic receptors that we had cloned previously (Ballivet et al. 1982; Claudio et al. 1983; Patrick et al. 1983; Boulter et al. 1985, 1986a). Based on this assumption, genomic

and brain cDNA libraries were screened at low stringency using the muscle nicotinic receptor cDNAs as probes. The subject of this chapter Is the family of brain nicotinic acetylcholine receptors that the authors have discovered in the past few years through the use of this molecular genetic approach (Boulter et al. 1985, 1986a, 1986b, 1987; Goldman et al. 1986, 1987; Deneris et al. 1988, 1989; Wada et al. 1988; Duvoisin et al. 1989).

The authors have identified seven genes in the rat or mouse genome that code for proteins with homology to the nicotinic acetylcholine receptor. These genes are expressed in the mammalian brain and in some peripheral neurons. We have named these genes α2, α3, α4, α5, β2, β3, and β4. In our terminology, the genes coding for the muscle nicotinic acetylcholine receptors are called al, β1, γ, δ, and ε. Thus, the nicotinic acetylcholine receptor family is encoded by at least 12 genes in the mammalian genome. A similar set of genes has been identified in chicken (Ballivet et al. 1988; Nef et al. 1988). Recently, the GABA and glycine receptors also have been cloned and shown to be structurally related to the nicotinic receptor (Grenningloh et al. 1987; Schofield et al. 1987; Levitan et al. 1988). Thus, the major inhibitory receptors in the nervous system are similar In structure to the nicotinic receptor, an excitatory receptor. This was an unexpected finding and raises the exciting possibility that all ligand-gated channels are members of one superfamily of related proteins. Thus, it is reasonable to expect that knowledge about one receptor will be useful in understanding the role and function of other ligand-gated channel receptors.

The primary structures of the brain nicotinic receptor subunits expressed in the brain have been deduced from the sequences of the cDNA clones (figures 1 and 2). Analysis of the hydrophobicity profiles of the brain nicotinic receptor subunits suggests that they fold through the membrane in an identical manner to the *Torpedo* fish electric organ nicotinic receptor. Each subunit contains four stretches of about 20 to 24 amino acids that are hydrophobic. We have suggested that these hydrophobic stretches are membrane-spanning regions. We therefore proposed that each subunit spans the membrane four times, placing the N-terminal and C-terminal outside the cell (Claudio et al. 1983). This four transmembrane model places about half the protein bulk outside the cell. This model is also consistent with structural studies of the *Torpedo* receptor (Stroud and Finer-Moore 1985; Toyoshima and Unwin 1988). In addition, this model predicts that the C-terminal is outside the cell, and this prediction has been confirmed by McCrea and coworkers (1987).

The proposed model (Claudio et al. 1983) also predicts that several potential phosphorylation sites are cytoplasmic. In one case, phosphorylation at one of these sites is known to regulate receptor function (Huganir et al. 1986; Safran et

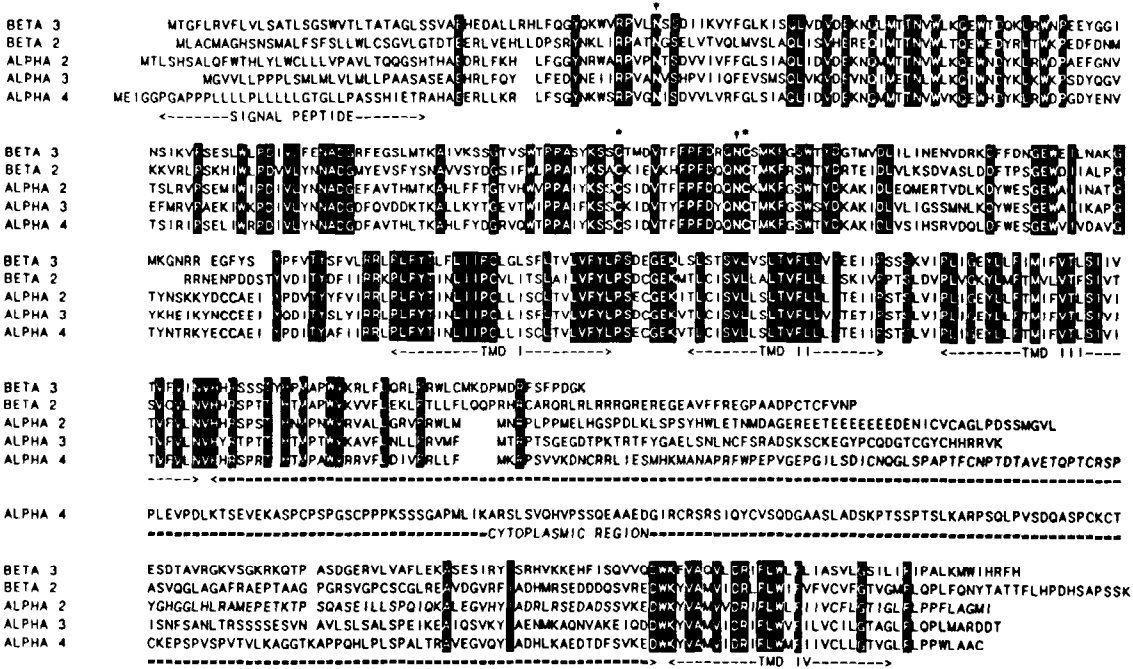


**FIGURE 1.** *Amino acid sequence alignment of the* ***β3*** *subunit with neuronal nAChR subunits. Aligned with the* ***β3*** *subunit are the rat* ***β2, α2, α3, and α4-1 subunits.*** *Indicated in the figure are the positions of the predicted leader peptide, potential N-linked glycosylation sites* ***(†)*** *cysteine residues conserved in each member of the neurotransmitter-gated ion-channel subunit superfamily (*), putative transmembrane domains (TMD I-IV), and cytoplasmic domain.*

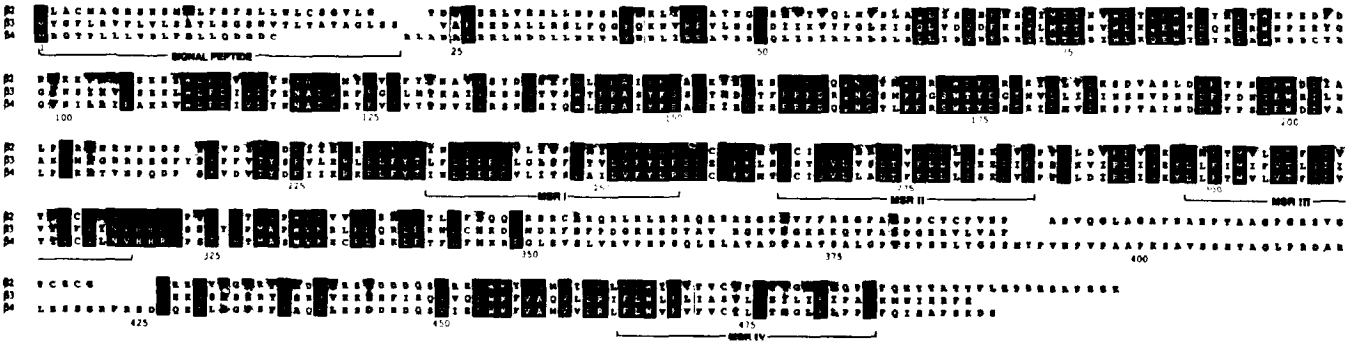


**FIGURE 2.** *Amino acid alignment of the rat neuronal β-type subunits. Aligned with the ß4 subunit are the ß2 (Deneris et al. 1988) and ß3 (Deneris et al. 1989) sequences. Identical residues in all three subunits are shown on a black background. Conservative changes are indicated by a gray background. Putative signal peptides and membrane-spanning regions (MSR) are identified below the sequences. The region referred to as the extracellular domain is located between the amino terminus and MSR I, and the putative cytoplasmic domain is located between MSR Ill and MSR IV. The numbering is that of the precursor ß4 subunit.*

SOURCE: Duvoisin et al. 1989, copyright 1989, Cell Press

al. 1986). The brain α-subunits contain adjacent cysteines at homologous positions to the *Torpedo* α-subunit cysteines 192 and 193, which are known to be near the acetylcholine binding site (for review, see Heinemann et al. 1986).

At present, it is not known how many copies of each subunit form a brain nicotinic receptor. However, because of the observed structural homology between the ligand-gated channels, we have proposed that the brain receptors are a pentameric structure, as has been shown to be the case for the *Torpedo* fish nicotinic acetylcholine receptor and, recently, the glycine receptor (Langosch et al. 1988; for review, see Popot and Changeux 1984). The results of the physiological experiments described below indicate that the brain nicotinic receptors are composed of two different gene products, an α- and a ß-subunit. This conclusion is also compatible with the recent biochemical analysis of a nicotine binding site isolated from rat brain (Whiting and Lindstrom 1987).

The availability of cDNA clones coding for the brain nicotinic receptors has made it possible to study their function. Expression studies in *Xenopus oocytes* have shown that, in general, two nicotinic receptor gene products—an α- and a ß-subunit—are necessary for the formation of a functional nicotinic receptor. Although the α4-subunit alone produces a weak response to nicotine in oocytes, α2 and α3 are inactive (figures 3 and 4) (Boulter et al. 1987). However, when α2, α3, or α4 is combined with ß2, a strong reproducible response is observed (figures 3 and 4) (Boulter et al. 1987; Wada et al. 1988). Thus, ß2 is a promiscuous subunit that can combine with three different α-subunits to form a functional receptor. This is consistent with its wide distribution of expression in the brain (figure 5) (Wada et al. 1989; and see below). A similar result is seen when the ß4-subunit is expressed in the presence of each of the three α-subunits (i.e., α2ß4, α3ß4, or α4ß4) (figure 6) (Duvoisin et al. 1989). At present, we have not been able to demonstrate any function for the α5 or ß3 subunits. Thus, their designation as nicotinic receptor subunits remains unproven.

These results demonstrate that, in general, two different subunits—an α and a ß—are required to form a functional brain nicotinic receptor. The brain a-subunits α2, α3, and α4 contain adjacent cysteines found in all muscle a-subunits (see above for discussion), while the brain ß-subunits ß2, ß3, and ß4 are missing these cysteines. We have called the non-α-subunits ß2 and ß4 because they can functionally substitute for the muscle ß1 subunit to form muscle-type nicotinic receptors (i.e., $\alpha 1\beta 2\gamma\delta$ and $\alpha 1\beta 4\gamma\delta$) (Boulter et al. 1987; Duvoisin et al. 1989). On the basis of these results and by analogy with the

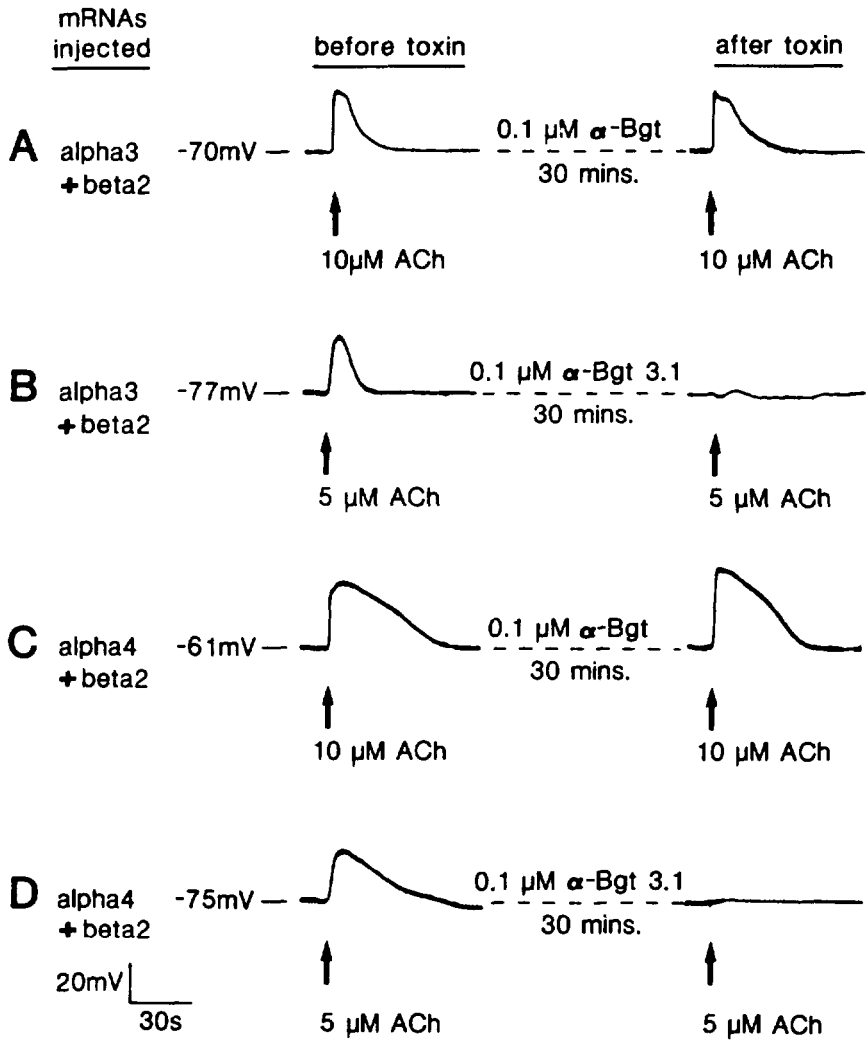


**FIGURE 3.** *This figure shows the effect of two different neurotoxins on the activation by acetylcholine of two neuronal nicotinic acetylcholine receptor subtypes, α3ß2 and α4ß2. The voltage tracing on the left shows the response before application of the toxin, and the voltage tracing on the right shows the response following a brief washing and a 30-minute incubation in the indicated concentrations of the two toxins.*

SOURCE: Boulter et al. 1987, copyright 1987, National Academy of Sciences

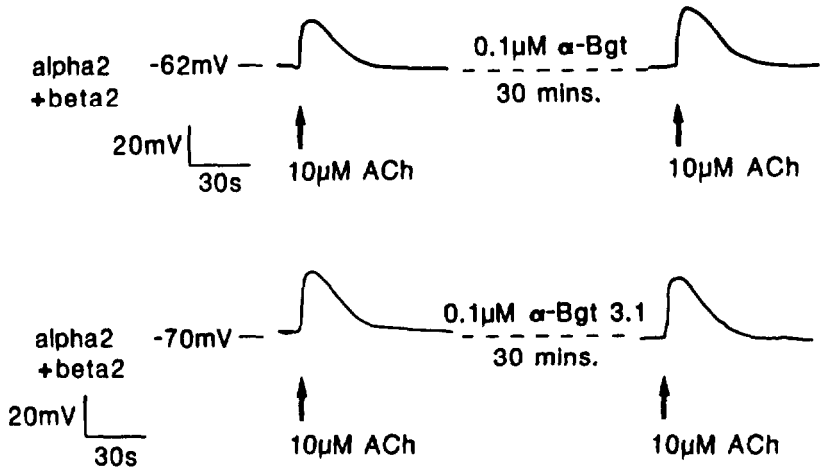


**FIGURE 4.** *This figure shows the effect of two different neurotoxins on the activation by acetylcholine of the neuronal nicotinic acetylcholine receptor subtype α2ß2. The voltage tracing on the left shows the response before application of the toxin, and the voltage tracing on the right shows the response following a brief washing and a 30-minute incubation in the indicated concentrations of the two toxins.*

SOURCE: Wada et al. 1988, copyright 1988, American Association for the Advancement of Science

we propose that the brain nicotinic receptors are a pentameric structure made from α- and ß-subunits in some as yet unknown stochiometry. That we have shown that the brain ß-subunits can function as part of the muscle nicotinic receptor, which is known to be pentameric, supports our proposal.

We have used the patch-clamp technique to record from single receptor molecules to characterize the biophysical properties of individual nicotinic receptor subtypes. The single unit conductances and channel open times of four subtypes have been analyzed thus far: α2ß2, α3ß2, α4ß2, and α4ß4. These data demonstrate that each of the subtypes has unique biophysical properties that can be used to identify the subtype (Papke et al. 1989). Each of the six functional combinations of subunits forms a pharmacologically distinct subtype that is activated by acetylcholine and nicotine and is resistant to α-bungarotoxin (figures 3, 4, 6, and 7). The α3ß2 and α4ß2 subtypes are

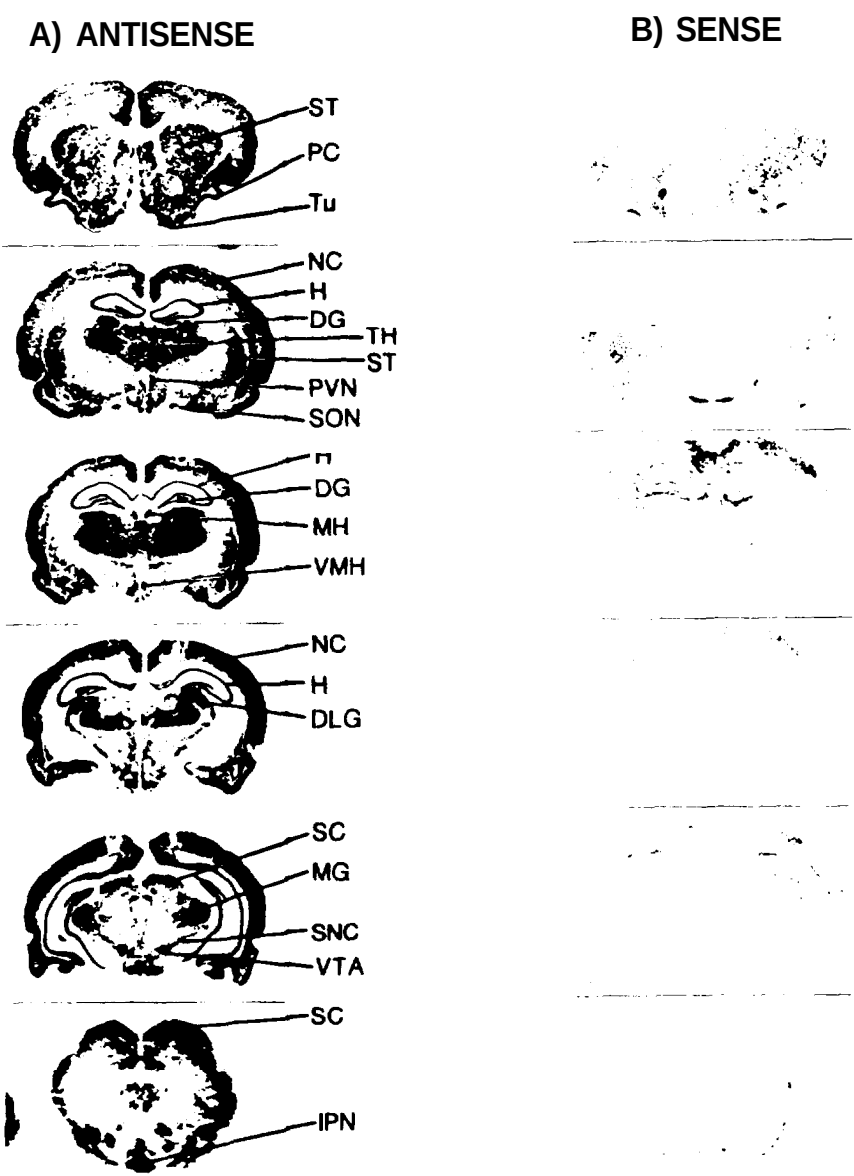


**FIGURE 5.** *In situ hybridization analysis. Rat forebrain and midbrain sections were probed with PSI-radiolabeled antisense (A) or sense (B) ß2 RNA transcribed in vitro using a plasmid into which a 571bp Pst1/ EcoR1 fragment of PCX49 was subcloned.*

Key: DLG=lateral geniculate nucleus (dorsal part); DG-dentate gyrus; H=Ammon's horn (hippocampus); IPN=interpeduncular nucleus; MG=medial geniculate nucleus; MH=medial habenular nucleus; NC=neocortex; PC=piriform cortex; PVN=paraventricular hypothalamic nucleus; SON=supraoptic hypothalamic nucleus; SNC=substantia nigra, pars compacta; SC=superior colliculus; ST=striatum; TH=thalamus; Tu=olfactory tubercle; VTA=ventral tegmental area; VMH=ventromedial hypothalamic nucleus

SOURCE: Deneris et al. 1988, copyright 1988, Cell Press

blocked by a toxin isolated from the venom of *Bungarus multicinctus,* called 3.1 toxin. This result is consistent with a ganglionic nicotinic-type pharmacology (figure 3). However, the α2ß2 and α3ß4 receptor subtypes are resistant to the 3.1 toxin and, therefore, represent new receptor subtypes with a pharmacology that has not been observed previously (figures 4 and 7) (Boulter et al. 1987; Wada et al. 1988; Duvoisin et al. 1989).

One important and unexpected result is that, while the α3ß2 subtype is blocked by 3.1 toxin, the α3ß4 subtype is resistant to this snake toxin (figure 7). This was unexpected because it generally has been accepted that the α-subunit contains the ligand- and toxin-binding site. This result shows that the ß-subunit, at least in this case, determines the sensitivity to 3.1 toxin (Duvoisin et al. 1989).

To determine where the individual nicotinic receptors are expressed in the brain, we have utilized the method of *in situ* hybridization to visualize the distribution of expression of the mRNA coding for each nicotinic receptor subtype. These experiments demonstrated that each of the three α-subunit mRNA's has a unique distribution of expression, consistent with the proposal that they are part of three independent nicotinic receptor systems (figure 8). The ß2 transcript is distributed throughout the brain, consistent with the hypothesis that it is a common subunit used to form at least three different receptor subtypes (figure 5). On the other hand, the ß4 transcript shows a much more localized distribution of expression (figure 9). In general, the distribution of mRNA coding for the nicotinic receptor family parallels the map of nicotine binding (Boulter et al. 1986b, 1987; Goldman et al. 1986, 1987; Deneris et al. 1988, 1989; Wada et al. 1988, 1989; Duvoisin et al. 1989).

The finding that these nicotinic receptor genes are expressed widely in the brain indicates that the nicotinic receptor is a major excitatory system. In the past few

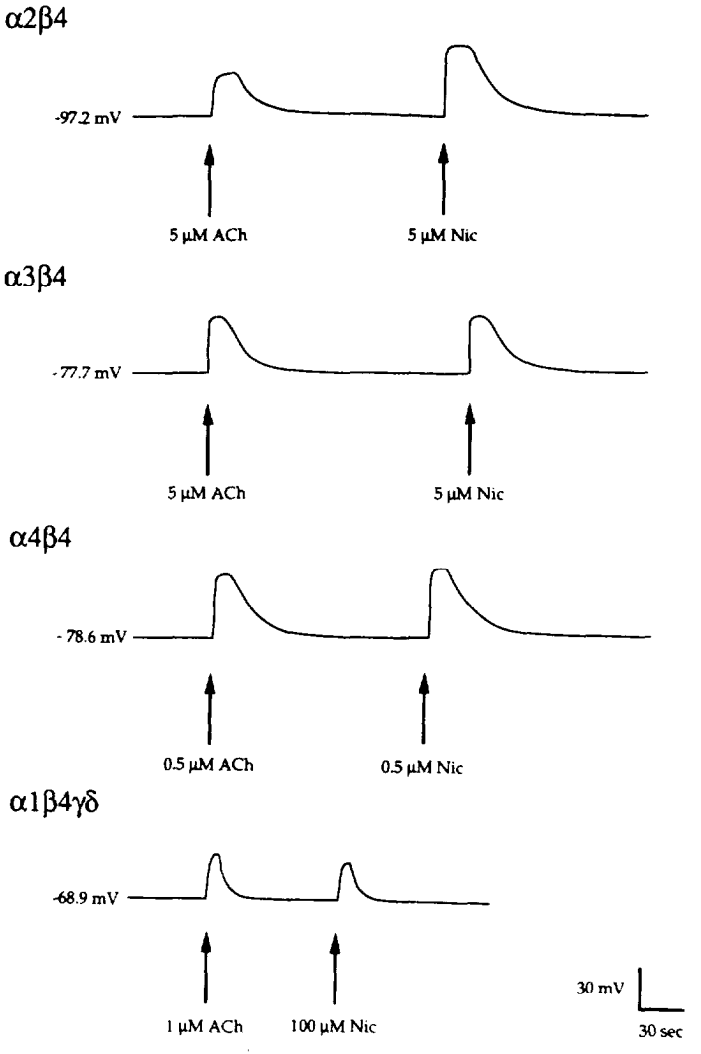


**FIGURE 6.** *Electrophysiological recordings of Xenopus oocytes injected with in vitro synthesized RNA encoding the nAChR's subunits in the indicated combinations. Representative responses induced by acetylcholine and nicotine stimulations at the given concentrations are shown. Potential measurements were monitored on a digital voltmeter and recorded on a pen recorder (Gould). Voltage traces were scanned and prepared for publication using a personal computer.*

SOURCE: Duvoisin et al. 1989, copyright 1989, Cell Press

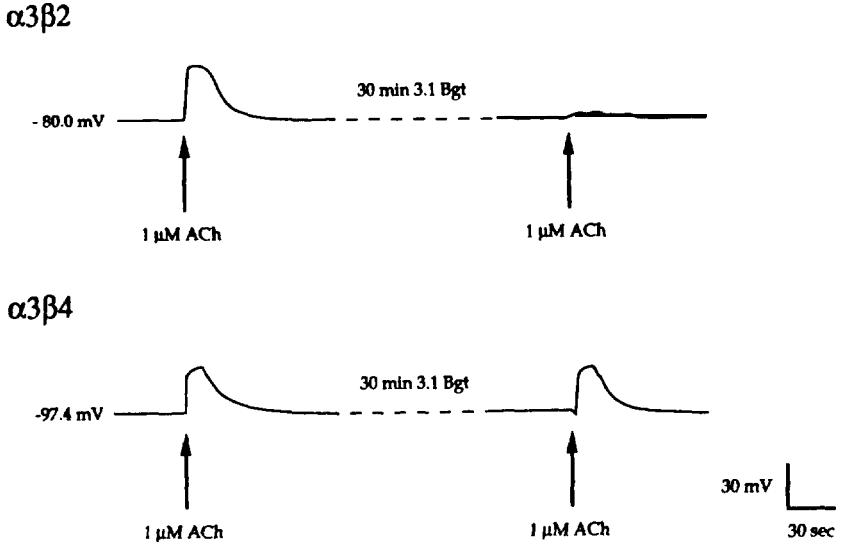


**FIGURE 7.** *Voltage recordings of Xenopus oocytes injected with α3 and either ß2 or ß4 before and after exposure to an estimated concentration of 0.1 mM 3.1 toxin. Experiments were performed as described in figure 6.*

SOURCE: Duvoisin et al. 1989, copyright 1989, Cell Press

years, physiologists using recently developed sophisticated techniques, such as the slice preparation and patch-clamp recording methods, have found extensive evidence for nicotinic receptor function in the brain, The *in situ* hybridization results demonstrated that the α3, α4, ß2, ß3, and ß4 genes are expressed at high levels in the medial habenula (figures 5, 8, and 9). The presence of functional nicotinic receptors has been confirmed in the medial habenula by intracellular recording techniques (McCormick and Prince 1987a). Nicotine also has been shown to increase glucose utilization in the medial habenula (London et al. 1988). There is now good evidence for functional nicotinic receptors in many other regions of the brain that have high levels of nicotinic receptor gene expression, These areas include the interpeduncular nucleus, the retina, the lateral and medial geniculate, and the neocortex (Brown et al. 1984; Lipton et al. 1987; Lipton 1988; McCormick and Prince 1987b; Vidal and Changeux 1989).

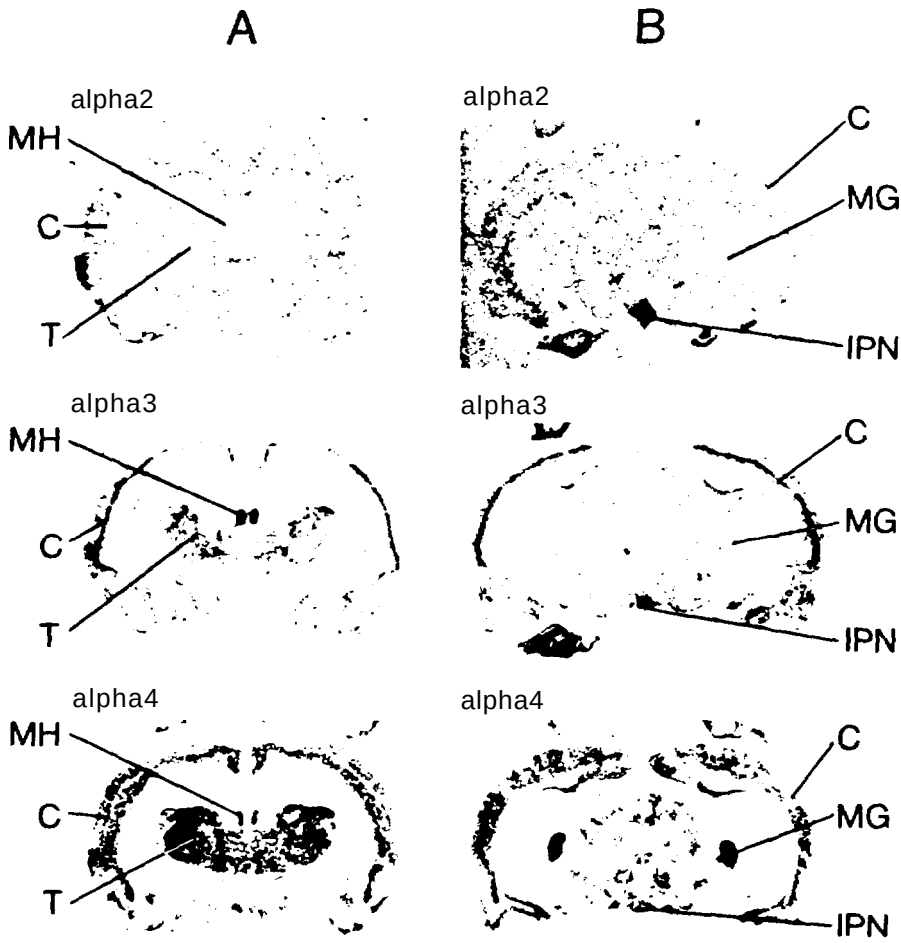


**FIGURE 8.** *Comparison of the distribution of α2, α3, and α4 transcripts by in situ hybridization histochemistry. Serial coronal sections through the medial habenula (A) and the interpeduncular nucleus (B) were hybridized with the probes for α2, α3, and α4. In (B) slides contain sections of the trigeminal ganglion. Tissue preparation and hybridization were performed with minor modifications. Briefly, paraformaldehyde-fixed rat brain sections (25mm) were mounted on poly-L-lysine-coated slides, digested with proteinase K (10 mg/mL, 37°C, 30 min), acetylated, and dehydrated, Hybridization with $^{35}$S-radiolabeled RNA probe (5-10x10$^{6}$cpm/mL) was performed at 55°C for 12 to 18 hours in a solution containing*

*50 percent formamide, 0.3M NaCI, 10mM Tris (pH 8.0), 1mM EDTA, 0.05 percent tRNA, 10mM DTT, 1xDenhardt's solution, and 10 percent dextran sulfate. Because of the high sequence similarities in the protein coding regions of the cDNA's, 3' untranslated sequences were used to make probes. The EcoRI/ 3' end, Ball/3' end, and Bgll/3' end fragments derived from Cl 83, PCA48, and α4-2 cDNA clones, respectively, were subcloned into the plasmid, pSP65 and used to synthesize antisense RNA probes in vitro. After hybridization, sections were treated with RNaseA (20mg/mL, 37°C, 30 min) and washed in 0.1xSSC at 55°C. Dehydrated slides were exposed to x-ray films for 3 to 16 days at 4°C. An RNA probe coding the sense strand of C183 clone was used as a control.*

Key: C=cortex; IPN=iterpeduncular nucleus; MH=medial habenula; MG=medial geniculate nucleus; T=Thalamus

SOURCE: Deneris et al. 1988, copyright 1988, Cell Press

The discovery of a family of genes coding for nicotinic acetylcholine receptors expressed in the brain brings for the first time the full power of molecular biology to the study of this important receptor system. The existence of multiple subtypes and the fact that they are expressed throughout the brain suggest that the nicotinic receptor is a major excitatory system. The anatomical distribution of these receptors now can be studied using specific antibodies to localize the receptor subtypes in the brain. *In situ* hybridization methods can be used to localize the cell bodies that synthesize the receptor protein. The regulation of receptor gene expression can be studied under a variety of conditions. Understanding the promoters of these receptor genes will provide insight into how the brain builds, regulates, and maintains specific neural networks. At present, there is little reliable pharmacological data on the properties of the nicotinic acetylcholine receptor in the brain. The receptors now can be expressed in the oocyte system where rigorous pharmacology can be performed without the complications that are inherent in pharmacological studies in the brain. By engineering mammalian cell lines, it will be possible to study these receptors in several convenient cell systems to ask specific questions about their function. The availability of the primary structure of the brain and muscle receptors makes it possible to build models that relate structure to pharmacology and function. These models can be tested by site-specific mutagenesis. At present, the only ligand-gated ion channel protein that can be isolated in milligram amounts is the *Torpedo* nicotinic receptor. Thus, it

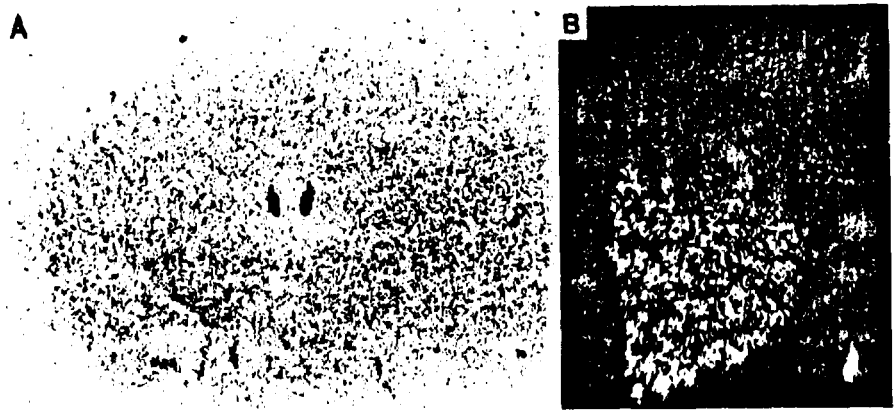


**FIGURE 9.** *Analysis by in situ hybridization histochemistry of the distribution of ß4-transcripts. Adult rat brain coronal sections (30mm thick) were hybridized with $^{35}$S-radiolabeled sense and antisense probes derived from KpnI- and BamHI-linearized pGDD15, respectively. A. Only sections across the thalamus as the one shown here gave above background signals by x-ray film autoradiography. In parallel experiments, a sense probe was hybridized to adjacent sections and gave background levels of hybridization (data not shown). B. Dark-field microphotograph of a medial habenula from an identical section to that shown in A after emulsion dipping.*

SOURCE: Duvoisin et al. 1989, copyright 1989, Cell Press

is the only candidate for structural studies leading to a complete, high-resolution, three-dimensional structure. Because of the structural homologies between the ligand-gated channels, it should be possible to apply the results of the structural studies of the *Torpedo* nicotinic receptor to the muscle and brain nicotinic receptors. It is very likely that these studies can be extended to the glycine and GABA receptors and even to the glutamate receptor system, which also may be a member of this ligand-gated channel superfamily.

Interesting mutations can be introduced into the receptors and expressed in transgenic mice. The mutant mice then can be studied to gain insight into the function of the nicotinic receptors. The diversity of structure and function in this receptor system now can be studied with the long-range goal of understanding the functional roles that the nicotinic receptors play in the nervous system.

At present, little is known about the roles of nicotinic receptor systems in the brain. However, nicotine is one of the most widely consumed and addictive drugs. Many behavioral effects have been observed, including mood changes, effects on learning and memory, and weight loss (Wellman et al. 1986; for review, see Clarke 1988). Under some conditions, nicotine is a relaxant; under other conditions, nicotine is a stimulant. Many of these effects may be beneficial, and this raises the possibility of designing better and more specific nicotinic drugs (Luyten and Heinemann 1987).

Recently, another nicotinic receptor subtype has been identified in the rat dorsalateral septal nucleus. Electrophysiological experiments indicate that activation of this receptor leads to a hyperpolarization of the membrane mediated by an increase in potassium conductance (Wong and Gallagher 1989). It will be interesting to see whether this receptor is a member of the same family of nicotinic receptors that we have described. One can predict that, if it is a member of the family, it will have an altered channel region. The mammalian brain also contains another nicotinic receptor that binds α-bungarotoxin (Patrick and Stallcup 1977). The receptors described above are not blocked by this toxin, and the structure and function of the brain α-bungarotoxin sites remain to be elucidated.

The nicotinic receptor system has been implicated in several serious health problems. Nicotine is a widely consumed and addictive drug and is a major factor in the present smoking epidemic. Behavioral studies have linked the nicotinic receptor system to learning and memory, which is intriguing given the finding that Alzheimer's patients have a deficiency in memory function and cortical nicotinic receptors (Perry et al. 1987). The possibility that one or more of the nicotinic receptor subtypes is depressed in patients with Alzheimer's disease now can be explored, That in Alzheimer's disease the number of nicotine binding sites is depressed and the fraction of high- and low-affinity sites is altered suggests that one nicotinic receptor subtype may be specifically affected by the disease (Nordberg et al. 1988; Whitehouse et al. 1986).

The existence of receptor subtypes and the availability of cDNA clones may make it possible to design new drugs that are subtype-specific that will prove useful in the battle against smoking-related illnesses and Alzheimer's disease. The known ability of nicotine to affect mood suggests that new nicotinic drugs also may prove useful in the treatment of various mood disorders.

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## ACKNOWLEDGMENTS

The work reviewed in this chapter was supported by National Institute of Neurological and Communicative Disorders and Stroke grant 5 R01 NS-11549-18 (SH and JP) and a grant from the Muscular Dystrophy Association of America (SH and JP).

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# Regulation of Acetylcholine Receptor Gene Expression

*Alex M. Simon, Emma K. Dutton, and Steven J. Burden*

## INTRODUCTION

The skeletal muscle acetylcholine receptor (AChR) is a ligand-gated channel composed of four structurally related subunits that assemble into a pentamer ($\alpha_2$, $\beta$, $\gamma$, and $\delta$) (Karlin 1980; Anderson 1987). The genes encoding the subunits are activated coordinately during embryonic development as myoblasts withdraw from the cell cycle and fuse to form multinucleated myotubes (Baldwin et al. 1988). Following activation of AChR genes during myogenesis, expression of these genes is regulated subsequently by physiological signals that include the pattern and intensity of muscle cell electrical activity (Merlie and Kornhauser 1989; Tsay and Schmidt 1989). To understand how AChR genes are regulated by physiological signals in mature myofibers, knowledge of how these genes are activated initially during development is required. Thus, the authors' initial studies have focused on the mechanisms involved in activation of the AChR delta subunit gene during myogenesis.

Analysis of the regulation of skeletal muscle genes provides a particularly attractive system to understand mechanisms that are responsible for cell-type specific gene expression. In particular, identification of a small family of myogenic basic-helix-loop-helix proteins (e.g., *myoD1)* that is capable of altering the fate of mesodermal cells and activating the myogenic phenotype provides one of the clearest examples in vertebrates of genes that are critical for early decisions in cell determination and differentiation (Davis et al. 1987; Wright et al. 1989; Edmondson and Olson 1989; Weintraub et al. 1991). An understanding of how these myogenic basic-helix-loop-helix proteins activate the myogenic program is likely to provide an important framework for understanding the mechanisms that control cell-type specific gene expression and cell differentiation.

## RESULTS AND DISCUSSION

We have characterized the muscle-specific regulatory region of the AChR delta subunit gene by transfecting gene fusions between the delta subunit gene and the human growth hormone (hGH) gene into cell lines and assaying expression of hGH. These studies have shown that ~500-bp of 5' flanking DNA from the delta subunit gene (nucleotides -501 through +24) is necessary and sufficient for muscle-specific gene expression.

Mutational analysis reveals that this *cis*-acting regulatory region contains three elements that collectively limit activation of the delta subunit gene to myotubes. One element, an enhancer, is necessary for maximal gene expression in muscle cells, but does not confer muscle-specific expression; rather, this enhancer is similarly active in muscle and nonmuscle cells. The other two regulatory elements limit enhancer activity to myotubes and are responsible for muscle-specific gene expression. One of these muscle-specificity elements is necessary for repressing the delta subunit gene in nonmuscle cells and is not required for activating the gene in myotubes. The other muscle-specificity element, a binding site for myogenic basic-helix-loop-helix proteins, is required both for activating the delta subunit gene in myotubes and for repressing the gene in other cell types.

Currently, the authors are identifying regulatory elements that control gene expression by electrical activity, producing transgenic mice harboring a gene fusion between ~1.8 kbp of 5' flanking DNA from the delta subunit gene and the hGH gene, and showing that hGH RNA levels are low in innervated skeletal muscle and high in denervated muscle. Thus, 1.8 kbp of 5' flanking DNA from the delta subunit gene contains the control elements that are necessary to confer innervation-dependent gene regulation.

We have also established a cell culture system to study electrical activity-dependent regulation. Primary rat myotubes, which form in cell culture, are electrically active, and this spontaneous activity can be abolished by tetrodotoxin, which blocks action potentials. We have transfected primary myotubes with delta subunit-hGH gene fusions and have shown that ~1.8 kbp of 5' flanking DNA from the delta subunit gene is sufficient to confer electrical activity-dependent gene expression in this cell culture system. We are exploiting these two systems further to delineate more precisely the critical *cis*-acting regulatory elements and to characterize the regulatory pathway that couples changes in the pattern of electrical activity to alterations in gene expression.

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## ACKNOWLEDGMENT


This work was supported by National Institutes of Health grant NS-27963.


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# Mutations Affecting Local Anesthetic Block of the Nicotinic Acetylcholine Receptor Ion Channel

***Reid J. Leonard, Pierre Charnet, Cesar Labarca, Nancy J. Vogelaar, Linda Czyzyk, Annie Gouln, Norman Davidson, and Henry A. Lester***

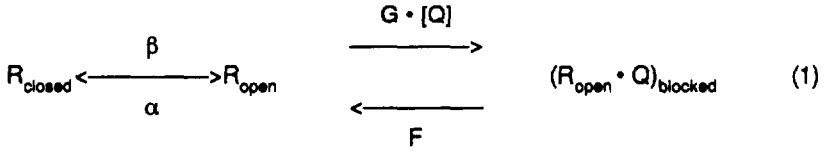
A common goal in molecular pharmacology is to understand how the structure of a complex transmembrane protein determines its behavior. One such protein is the nicotinic acetylcholine receptor (AChR), which transduces a chemical signal (binding of ACh) into an electrical signal (change in the postsynaptic membrane potential) by acting as a ligand-gated pore for the conduction of ions across the cell membrane (for review, see Popot and Changeux 1984; Stroud and Finer-Moore 1985). The functional AChR is an oligomer composed of four homologous subunits that form a pentamer with stoichiometry $\alpha_2\beta\gamma\delta$ (Raftery et al. 1980). Functional AChR's are expressed in *Xenopus* oocytes by injecting them with a mixture of mRNA transcripts from cDNA's encoding each of the subunits. Site-directed mutagenesis (Kunkel et al. 1987) is used to alter the coding sequence of the subunit cDNA's, ultimately expressing "mutated" AChR's in the plasma membrane of the oocyte. By comparing the electrophysiological characteristics of the mutated AChR's with those of the unaltered channels, inferences can be drawn about the roles that various portions of the primary sequences play in shaping the behavior of the normal receptor-channel molecule.

We focused on a region of the primary sequence that had been implicated by the elegant experiments of Hucho and colleagues (1986) and Giraudat and coworkers (1986, 1987) as forming part of the ion conduction pathway of the *Torpedo* AChR. Those experiments used channel-blocking compounds as photoaffinity reagents to map the binding site for noncompetitive antagonists. The drugs were attached covalently to the receptors under conditions that favored the open channel conformation, and the covalently modified receptors were subjected to peptide cleavage and microsequencing analysis. The labeled residues were determined to lie within a proposed membrane-spanning helix (M2) of the $\alpha$, ß, and $\delta$ subunits. The stoichiometry of binding of the photoaffinity reagents to the AChR was 1:1; however, the label was

incorporated into residues occupying homologous positions in at least three of the four subunit polypeptides. These results supported a hypothesized structure (Noda et al. 1983a; Claudio et al. 1983; Devillers-Thiery et al. 1983) for the ion channel of the AChR in which homologous domains (in this case, the M2 helix) from each of the subunit polypeptides contributed equally to form the walls of the pore, similar to staves of a barrel.

To test this hypothesis, we altered the amino acid side chains at the homologous positions in the mouse muscle AChR subunits (figure 1) and looked for changes in the properties of the channel, measured electrophysiologically. In particular, we wanted to examine the effects of such mutations on the interaction between the channel and a local anesthetic analog, with the hope that the drug could act as a probe for the lumen of the channel. (For details on experimental methods, see Leonard et al. 1988 and Charnet et al. 1990.) One advantage of working with AChR's expressed from mouse cDNA's, rather than Torpedo, Is that the effects of local anesthetics on the native mammalian AChR's have been studied extensively (Neher and Steinbach 1978; Neher 1983).

Local anesthetics interact with nicotinic acetylcholine receptors in a noncompetitive fashion (with respect to agonist binding) to interfere with ion flux through the channel. A simple model for this interaction is given below:




(1)

where R=AChR; Q=blocker; ß is the channel opening rate; a is the channel closing rate; and G and F are the respective rates for blocking and unblocking. This model proposes that local anesthetics block current through the channel by entering the pore and plugging it, much like a stopper in a bottle. Two important features of this model are that the blocker cannot enter the channel until it opens and that the channel is not free to close while the blocking molecule resides in the pore. In the case of the permanently charged quaternary amine derivative of lidocaine, QX-222, these conditions appear to hold true (Neher 1983). Many other noncompetitive antagonists of the AChR, however, have actions that are not limited to those proposed for a simple "open channel blocker" (Changeux et al. 1986).

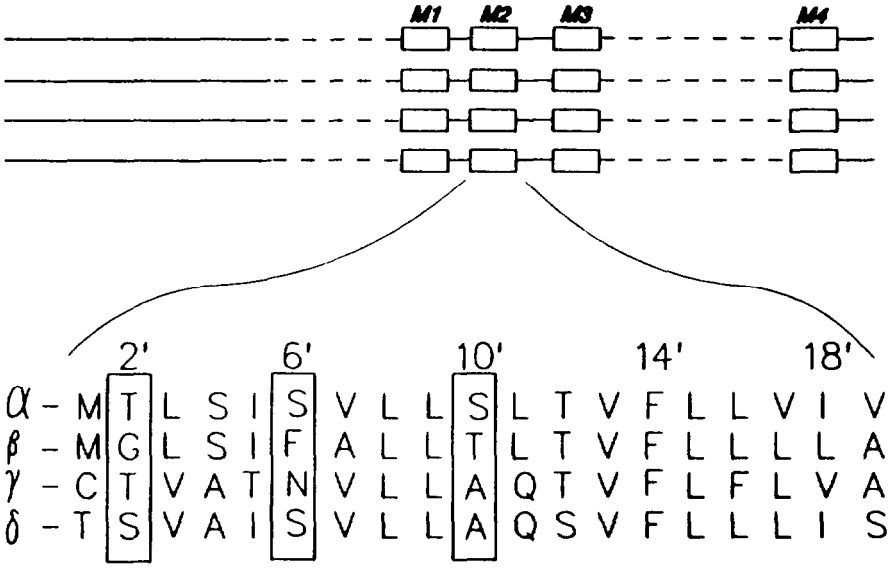



**FIGURE 1.** *Aligned amino acid sequences of the M2 hydrophobic domains of the mouse skeletal muscle nicotinic acetylcholine receptor subunits, Residues are numbered 1' through 19' with 1' at the presumed cytoplasmic end of the transmembrane helix and 19' at the extracellular end. The boxed residues are hypothesized to face the lining of the ion channel pore.*

SOURCE: Leonard et al. 1991, copyright 1991, *Annals of the New York Academy of Sciences*

The most compelling evidence for the action of QX-222 on the pore of the AChR comes from direct observations of the current through individual channel proteins using the giga-seal "patch clamp" technique. Such recordings (figure 2) show that addition of QX-222 to the extracellular face of the pore transforms the normal open state of the channel into a burst of rapid transitions between the conducting and blocked states. Kinetic analysis of channel activity before and during exposure to QX-222 yields an estimate of the affinity of QX-222 for the open channel. The forward rate of block (G in equation 1) can be determined from measurements of mean open time at various concentrations of blocker, according to the formula

$$(1/t_{open}) = \alpha + G \cdot [Q] \qquad (2)$$

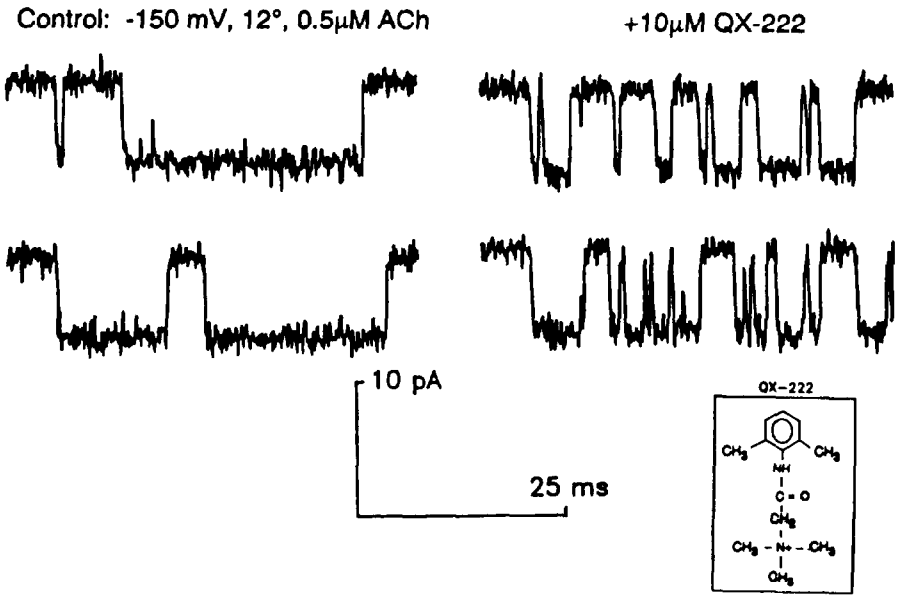



**FIGURE 2.** *Effect of QX-222 on ACh-induced single channel currents. Recordings made from a patch of membrane excised ("outside-out") from a Xenopus oocyte that had been injected 48 hours earlier with mRNA transcripts of cDNA's encoding the mouse skeletal muscle AChR subunits. Channel openings are downward deflections in the traces. Left: Channel openings in the presence of 0.5 μm ACh. Right: Same patch after addition of 10 4M QX-222 to the bath. Inset: Structure of QX-222.*

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which simply states that the reciprocal of the open time is the sum of the rates for leaving the open state. As stated in equation 1, $\alpha$ is the channel closing rate measured in the absence of QX-222. The unblocking rate can be determined from the mean blocked time within a burst

$$(1/t_{blocked})=F \quad (3)$$

and is independent of blocker concentration. The apparent equilibrium dissociation constant of QX-222 for the *open* channel, therefore, may be calculated

$$K_Q = F/G. \qquad (4)$$

The effect of QX-222 also can be measured macroscopically, on a cell's entire population of AChR channels. At low ACh concentrations, voltage-jump relaxations (Adams 1977) provide an independent measure of the total lifetime of the agonist-activated state, including brief nonconducting periods (such as those induced by QX-222). As mentioned above, the channel is not free to close while the QX-222 is in the pore. The burst duration is therefore *prolonged* in the presence of blocker by an amount equal to the sum of the individual blocked times. This property of block of AChR's by QX-222 also was observed directly from single channel records: Neher and Steinbach (1978) and Neher (1983) noted that the integral open time during a burst is unaffected by the presence of the blocker. That is, the sum of the conducting periods within a burst induced by the rapid blocking and unblocking by QX-222 is the same as the normal duration of the uninterrupted burst in the absence of blocker. The "clock" (the conditional probability that the channel will enter the closed state) only runs when the channel is unblocked. This prolongation of the burst duration increases the time constant of an exponential voltage-jump relaxation as follows:

$$\tau_Q = \tau(1 + [QX\text{-}222]/K_Q) \qquad (5)$$

where $\tau_Q$ and $\tau$ are the decay time constants of voltage jumps in the presence and absence of QX-222, respectively. $K_Q$, the apparent dissociation constant for QX-222, therefore, is obtained easily by comparing the decay constants before and after addition of blocker. An example of the effect of QX-222 on AChR voltage-jump relaxations is shown in figure 3.

Using the two complementary approaches outlined above, we examined the effect of amino acid substitutions in the M2 domain upon the interaction between the channel and QX-222. For each new mutant construct, we first used single channel recording to measure the mean blocked times and to verify the open channel block model of QX-222 as given in equation 1. Following that, it was much more efficient to use macroscopic recording to measure $K_Q$

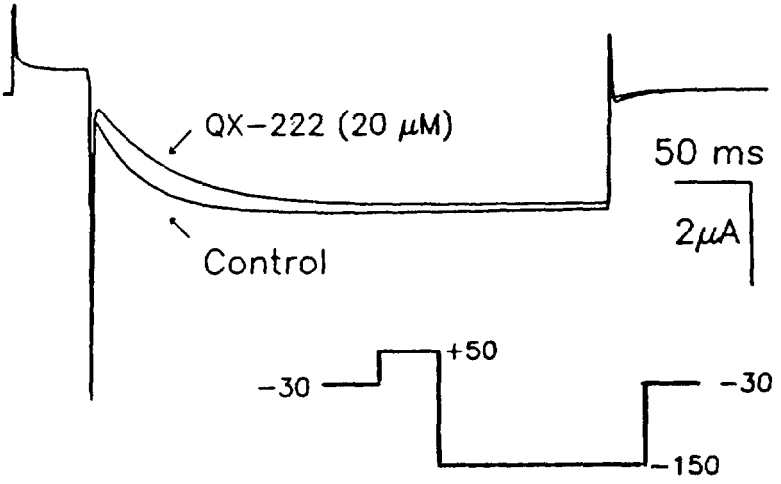


**FIGURE 3.** *Prolongation of voltage-jump relaxation of ACh-induced currents by QX-222. Whole-cell recording from a Xenopus oocyte that had been injected 48 hours earlier with mRNA transcripts of cDNA's encoding the mouse skeletal muscle AChR subunits. Upper: Currents elicited in the presence of 1 μM ACh before and during addition of QX-222 to the bath. QX-222 reduces the initial amplitude of the current elicited by the jump to -150 mV and prolongs the decay phase. Lower: Voltage-clamp protocol used to elicit the currents.*

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from the various mutants. In all cases, the values obtained from single channel and macroscopic recording were in good agreement.

The first series of mutations were carried out at the position where photoaffinity reagents bound in the *Torpedo* sequence. This position (6' in figure 1) contains serines in every subunit of *Torpedo* (Sumikawa et al. 1982; Noda et al. 1982, 1983b; Ballivet et al. 1982; Claudio et al. 1983), but only in the α and ß subunits of the mouse AChR (Boulter et al. 1985, 1986; LaPolla et al. 1984). Our first mutation was to change the δ--$Ser_6$, to Ala, α substitution from a polar to a nonpolar residue. The result of this mutation is shown in figure 4.

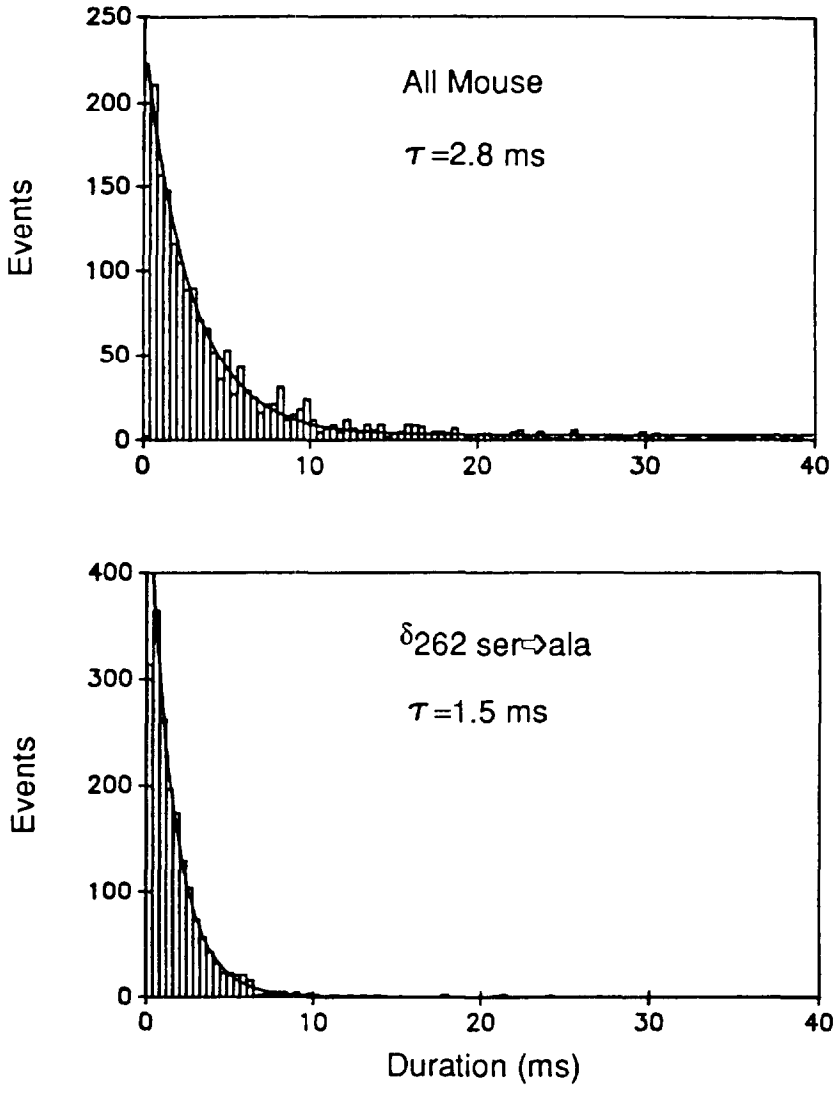


**FIGURE 4.** *Comparison of average residence time of QX-222 in normal vs. mutated AChR's expressed in oocytes. Residence time of the blocker equals the time constant of an exponential fit to the histogram of nonconducting periods (blocked times) caused by QX-222.*

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The residence time of QX-222 for the AChR's containing the $\delta_{S6'A}$ mutation was decreased by -40 percent relative to nonmutated AChR's. The apparent dissociation constant for QX-222 ($K_Q$) derived from voltage jumps to -130 mV increased from 27 µM to 45 µM. The second mutation, $\alpha_{S6'A}$, produced an increase in QX-222 residence time and a decrease in $K_Q$, but the magnitude of the change was twice as large as that seen for the $\delta_{S6'A}$ mutation ($K_Q$=62 µM). This was interesting, since a mutation in a produces two serine to alanine substitutions per pentamer, compared with the single substitution achieved with the δ mutant. For receptors containing both mutated α and mutated δ together, the effects were additive ($K_Q$=134 µM for $\alpha_{S6'A}\beta\gamma\delta_{S6'A}$). The final mutation constructed at site 6' replaced the phenylalanine of the ß subunit with a serine ($\alpha\beta_{F6'S}\gamma\delta$ to produce AChR's with a total of four serines at position 6'. This mutant, in contrast to the ones in which the number of serines was reduced, exhibited an increased residence time for QX-222 and a decrease in the $K_Q$ to 23 µM.

The effects of the site 6' mutations on affinity for QX-222 can be summarized (figure 5) as follows: Polar to nonpolar substitutions decreased the affinity of the open channel for QX-222 in a graded fashion, with each successive serine to alanine substitution producing a lower affinity, while the nonpolar to polar substitution (Phe to Ser) had the opposite effect. These results indicate that the serines normally present at site 6' exert a stabilizing influence on the binding of QX-222 to the pore, presumably via favorable interactions between the quaternary ammonium group of the blocker and the polar (and net electronegative) -OH side chains. Replacement of the hydroxyl side chains by hydrogen (in the Ser to Ala mutation) therefore renders the channel less attractive to the blocker. It is important to note that while the change in free energy of the QX-222 binding produced by the mutations amounts to only 0.2 kcal/mol/serine (much less than even a single hydrogen bond), the aggregate stabilizing influence of all the residues forming the annulus of the channel at site 6' is comparable to that exerted by a 50 mV hyperpolarization of the membrane.

Additional evidence that the residues at position 6' lie within the ion conduction pathway comes from measurements of the single channel conductance. None of the single-subunit mutations affected the conductance of the channel, but the conductance of the $\alpha_{S6'A}\beta\gamma\delta_{S6'A}$ channels, in which *no* polar residues remained at site 6', exhibited a dramatic change: The conductance for ions flowing in the inward direction (extracellular to cytoplasmic) was unaffected, but the conductance for current in the outward direction was reduced by half (figure 6). A change such as that observed would be expected from an increased barrier to permeation (Lauger 1976; Hille 1984) toward the intracellular end of the pore. Hydroxyl side chains have been shown to facilitate conduction through synthetic pore-forming peptides (Lear et al. 1988), perhaps because they can interact

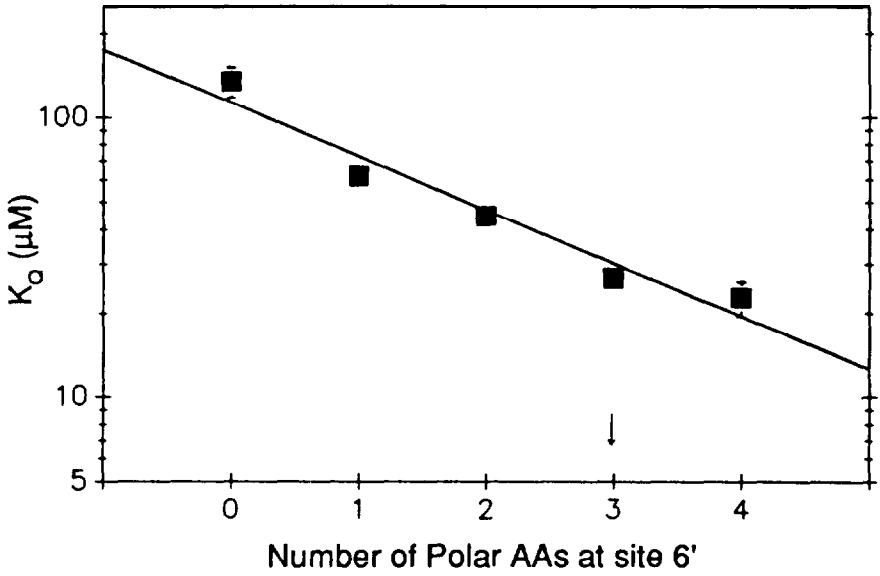


**FIGURE 5.** *Effect of mutations (Ser->Ala or Phe->Ser) at site 6' on the apparent equilibrium binding constant for the interaction between QX-222 and the open AChR channel. $K_Q$ was determined from macroscopic voltage jumps and single channel recording. The arrow on the abscissa represents the number of serines normally present at site 6'. Removal of serines lowers the affinity for QX-222.*

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with the hydration shells of permeant ions. Removal of the -OH groups at site 6 could then restrict the ability of ions to enter the channel at the intracellular end, thus producing the observed rectification in channel conductance.

Charged molecules, such as ions or local anesthetics, within ion channel sense the transmembrane potential. The degree of voltage dependence of block by QX-222 in muscle AChR's (Neher and Steinbach 1978) suggests that the blocker travels approximately two-thirds of the distance across the membrane field to reach its binding site. The same voltage dependence was observed in the AChR's expressed in oocytes, and this value was unaffected by our mutations. Although it is incorrect to equate electrical distance across the field with *physical* distance across the membrane, the lack of change in voltage

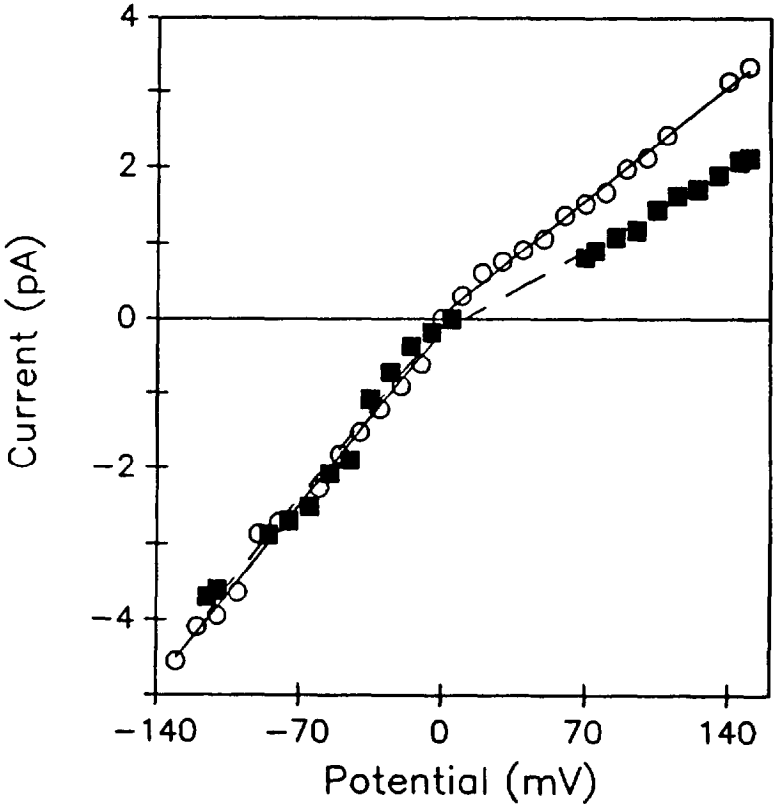


**FIGURE 6.** *Change in single channel conductance of AChR's caused by removal of all serines at position 6'. The amplitude of the current through ACh-gated channels is plotted against the voltage across the membrane. Open Circles: Normal mouse AChR's expressed in Xenopus oocytes. Closed Squares: Mutated receptors* ($\alpha_{S6'A}\beta\gamma\delta_{S6'A}$). *The selective decrease in the conductance for outward current (positive limb) implies a change in an energy barrier at the cytoplasmic end of the pore.*

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dependence implies that the mutations affected only the affinity, and not the site, of the QX-222 binding.

Several lines of evidence therefore converge to support the hypothesis that the residues of the proposed M2 helix form part of the lining of the ion channel: Site

6' is predicted from hydropathy (Kyte and Doolittle 1982) analysis to lie at the cytoplasmic end of a transmembrane α-helix. The voltage dependence of QX-222 block places the binding site for the drug toward the cytoplasmic end of the pore. Photoaffinity labeling of the *Torpedo* AChR by channel-blocking drugs occurs at site 6'. Mutations of the mouse AChR subunits at site 6' change the apparent affinity of QX-222 for the open channel, suggesting that those residues form part of the drug's binding site. Replacement of all the polar serines at site 6' by nonpolar alanines reduces the channel conductance for cations entering the cytoplasmic end of the pore.

To test this hypothesis and extend our studies of the interaction between the channel and QX-222, we performed a series of mutations at site 10' (figure 1), which in an α-helix should be approximately one full turn away from site 6' and therefore would be expected to contribute to the lining of the pore. When we examined the effects of polar to nonpolar substitutions at site 10', we observed changes in QX-222 block that were of the same magnitude as those for the site 6' mutations, but in the opposite direction. For example, the mutant channel $\alpha_{S10'A}\beta\gamma\delta$ exhibited a *longer* residence time for QX-222 block and a *lower* $K_Q$ value compared with controls. Figure 7 shows the effect of the entire series of site 10' mutations on the affinity of the open channel for QX-222. Polar to nonpolar substitutions *increased* the affinity of the open channel for QX-222 in a graded fashion, with each successive serine or threonine to alanine substitution producing a *higher* affinity, while the nonpolar to polar substitution (Ala to Ser) *decreased* the affinity. Once again, there was no change in the voltage dependence of the block by QX-222. The single channel conductance was not altered by any combination of site 10' mutations tested.

An explanation for the opposite influence of polar to nonpolar substitutions at positions 6' vs. 10' is suggested by the structure of QX-222. As shown in the inset to figure 2, QX-222 consists of a nonpolar aromatic moiety whose center, in CPK modeling, is separated by 5-6 Å from the positively charged quaternary amine. This spacing (or its integral multiple) between aromatic and amine moieties is a common structural feature of many anesthetics (for reviews, see Ritchie and Greengard 1966; Courtney and Strichartz 1987). Since the 5-6 Å spacing coincides with the repeat distance of one face of an α-helix, we propose that QX-222 "partitions" itself within the lumen of the channel as shown in figure 8, with the quaternary amine interacting with the site 6' residues and the aromatic ring in the vicinity of site 10'.

The clinical targets of local anesthetics are the voltage-gated sodium and potassium channels of heart and nerve. If our model of QX-222 binding is correct, then at least some of the structural features proposed for the lining of the AChR pore, such as rings of amino acids with polar side chains, also should

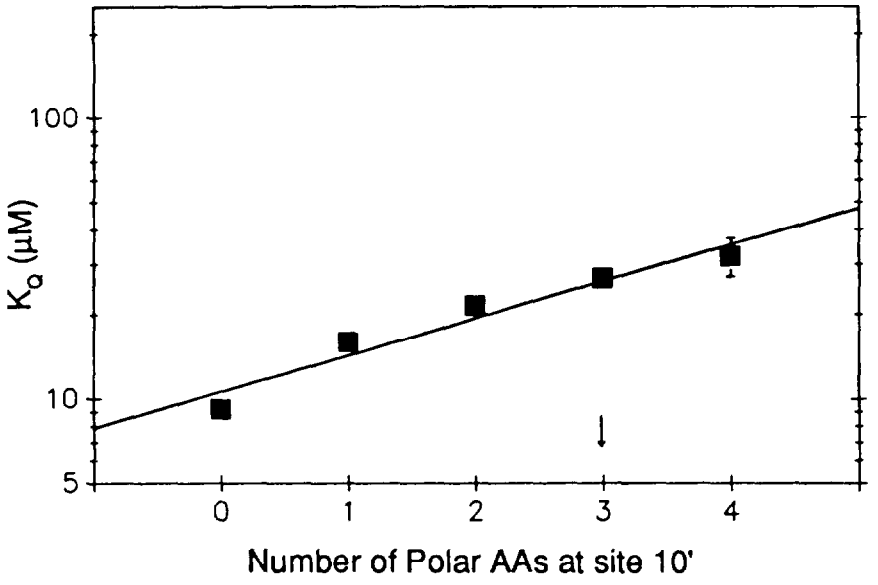


**FIGURE 7.** *Effect of mutations (Ser/Thr->Ala or Ala->Ser) at site 10' on the apparent equilibrium binding constant for the interaction between QX-222 and the open AChR channel. $K_Q$ was determined from macroscopic voltage jumps and single channel recording. The arrow on the abscissa represents the number of serines and threonines normally present at site 6'. Removal of serines raises the affinity for QX-222.*

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be found in the lumen of $Na^+$ and $K^+$ channels. With the availability of cDNA clones for several subtypes of these channels becoming available, it will be possible to study the effects of structural changes on the action of local anesthetics on these channels as well. Experiments similar to those conducted on the nicotinic AChR may help to identify structural elements comprising the lumen of other ion channels. Furthermore, it will be possible to test the generality of the mechanism of local anesthetic binding proposed here.

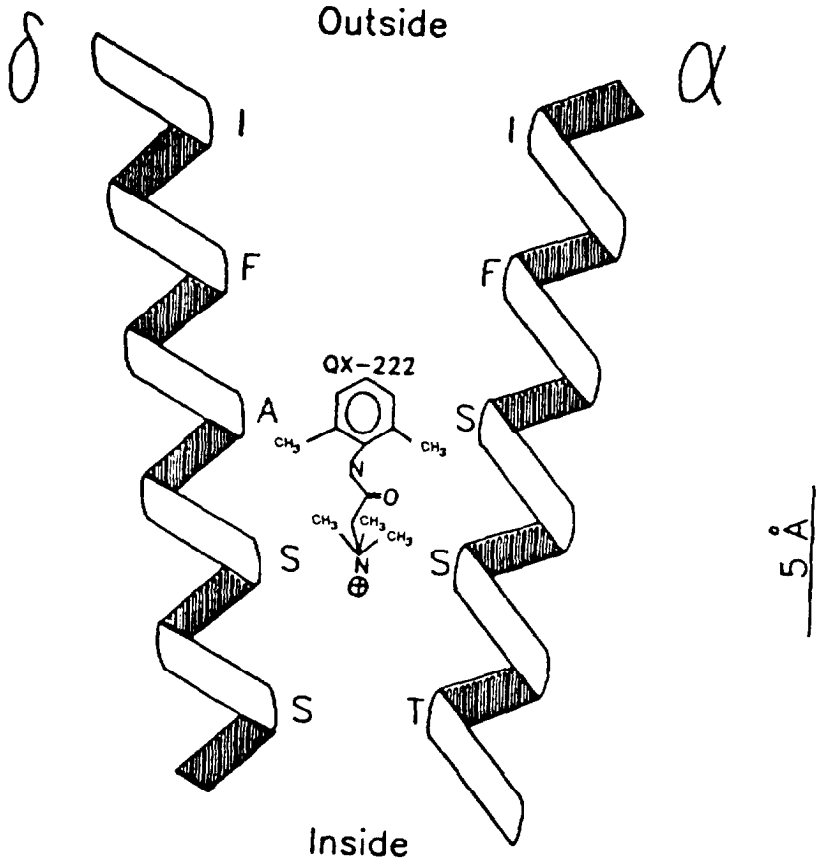


**FIGURE 8.** *Schematic model of the interaction between QX-222 and adjacent turns of α-helical M2 domains of the AChR subunits. The M2 domains from all five subunits ($\alpha_2\beta\gamma\delta$) of the channel are presumed to contribute equally to the lining of the pore. Only the α and δ helices are shown in the drawing. In this model, the blocker can enter the channel only from the extracellular side and can go no deeper than the level of the 6' residues,*

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# Molecular Biology of the Dopamine $D_2$ Receptor

***Olivier Civelli, James Bunzow, Paul Albert, Hubert Van Tol, and David Grandy***

## INTRODUCTION

Dopamine is the dominant catecholamine neurotransmitter in the mammalian brain. It is found throughout the central nervous system but is predominant in the nigrostriatal, mesocorticolimbic, and tuberoinfundibular tracts (Creese et al. 1983). Dopamine exerts its effects through binding to two types of receptor, the $D_1$ and $D_2$ receptors (Kebabian and Caine 1979). Binding of dopamine to its receptors induces several second messenger systems, most importantly affecting cAMP levels (Vallar and Meldolesi 1989). Activation of the $D_1$ receptor stimulates adenylyl cyclase activity, which results in an increase in intracellular cAMP levels, whereas binding of dopamine to the $D_2$ receptor inhibits the cyclase activity (Caron et al. 1978).

The importance of the dopamine $D_2$ receptor is evidenced by the number of physiological activities it modulates, including control of movement, maintenance of emotional stability, and regulation of prolactin secretion (Hess and Creese 1987). The $D_2$ receptor has been implicated specifically in the pathophysiology of most of the dopamine-associated disorders. It has been proposed that the $D_2$ dopamine receptor is involved in the etiology of schizophrenia (Seeman 1987), Parkinson's disease, and Tourette's syndrome. In this respect, it is noteworthy that many drugs used in treating mental disorders have high affinity for the $D_2$ receptor site, making this receptor the focus of numerous studies (Seeman and Lee 1975). Though progress has been made in understanding the pharmacology and physiology of the $D_2$ receptor, its biochemical characterization has been difficult. Only recently has the $D_2$ dopamine receptor been purified to homogeneity (Senogles et al. 1988). We discuss here our studies on the molecular cloning and expression of the rat brain dopamine $D_2$ receptor.

## CLONING OF THE RAT DOPAMINE $D_2$ RECEPTOR

With the cloning of the $ß_2$-adrenergic receptor in 1986 (Dixon et al. 1986), it was observed that its sequence was very similar to rhodopsin, the retinal receptor. Since both receptors are transduced by G proteins, a new concept evolved that proposed that G-protein-coupled receptors might share certain sequence similarities with each other and thus be part of a large gene family.

Indeed, with the recent cloning of several other G-protein-coupled receptors (Stevens 1987; Hall 1987; Dohlman et al. 1987; Masu et al. 1987; Julius et al. 1988), it is clear that all these receptors are evolutionarily related. In particular, it was shown that G-protein-coupled receptors share three structural characteristics: seven hydrophobic domains, about two dozen conserved amino acid residues (some of which might be targets of posttranslational modifications), and a significant degree of sequence similarity at both the peptide and the nucleotide levels. It should be noted that not all the G-protein-coupled receptors necessarily will share these characteristics; but to date, all the proteins having these structural characteristics are G-protein-coupled receptors.

Bunzow and colleagues (1988) embarked on the cloning of G-protein-coupled receptors by taking advantage of their sequence homology. They used the $ß_2$-adrenergic receptor coding sequence as a hybridization probe to screen a rat genomic library under nonstringency hybridization conditions and were able to isolate numerous clones. One, RGB-2, was studied in detail.

The RGB-2 genomic fragment was found to hybridize to a 2.8 kb rat brain mRNA and was used subsequently to screen a rat brain cDNA library. One clone containing a 2.5 kb insert was sequenced and its corresponding peptide sequence was determined. This clone encodes a 415 amino acid protein with all the expected characteristics of a G-protein-coupled receptor: It has seven hydrophobic domains, the 21 amino acid residues conserved among all cloned G-protein-coupled receptors and potential glycosylation and phosphorylation sites, and a significant degree of sequence similarity with the other receptors in this gene family. Therefore, it appeared that RGB-2 could be a G-protein-coupled receptor.

## PHARMACOLOGY OF THE CLONED DOPAMINE $D_2$ RECEPTOR

To determine the ligand specificity of RGB-2, we first analyzed the tissue distribution of its mRNA to relate RGB-2's expression to the distribution of a known receptor. Northern blot analysis showed that RGB-2 mRNA sequences are expressed throughout the rat brain, with the highest levels found in the

striatum. We also determined that RGB-2 mRNA is present in high levels in the intermediate lobe of the pituitary and at much reduced levels In the anterior lobe. These data suggested that RGB-2 could encode a dopamine $D_2$ receptor.

The full-length RGB-2 cDNA then was cloned into a plasmid containing the metallothionein promoter, and this construct was cotransfected with pRSVneo (a selectable marker conferring resistance to the antibiotic neomycin) into mouse Ltk- cells. This fibroblast cell line does not express endogenous mouse RGB-2 mRNA sequences. Stable transfectants expressing RGB-2 were isolated, and membranes from one of these clones, L-RGB2Zem-1 (also called LZR-1, Neve et al. 1989) were prepared and analyzed for their ability to bind dopamine ligands (Bunzow et al. 1988).

L-RBG2Zem-1 membranes bound dopamine $D_2$ agonists and antagonists with the same pharmacological profile as do rat striatal membranes. These studies used the antagonist [$^3$H]-spiperone whose binding was shown to be saturable (950 fmol/mg protein) and of high affinity (48 pM). [$^3$H]-Spiperone binding to L-RGB2Zem-1 membranes was displaced by several antagonists with the stereospecificity expected of a $D_2$ receptor and with the same $K_1$'s as determined in rat striatal membranes. Finally, as determined using compounds that detect more than one receptor type, the transformed Ltk- cells expressed only one type of receptor. Therefore, we had demonstrated that RGB-2 encodes a protein that possesses the $D_2$ receptor binding characteristics. The next step was to show that this $D_2$ receptor was functional (i.e., that it couples to a second messenger system).

**G-PROTEIN COUPLING AND SECOND MESSENGER INDUCTIONS**

$D_2$ receptors are present on lactotroph cells of the anterior pituitary where they regulate prolactin secretion. The somatomammotroph cell line $GH_4C_1$ is derived from a rat pituitary tumor and is known to secrete prolactin. This cell line, however, does not bind dopamine and therefore represents an excellent cell system in which to study exogenously expressed $D_2$ receptor activity.

$GH_4C_1$ cells were transfected with the RGB-2 cDNA metallothionein construction, and several stably transformed cells were cloned and raised. One, $GH_4ZR_7$, was found to express high levels of RGB-2 mRNA. Membranes prepared from $GH_4ZR_7$ cells bound PHI-spiperone with saturable kinetics, a $K_d$ of 96 pM, and a $B_{max}$ of 2.3 pu/mg protein (Albert et al. 1989).

To analyze second messenger coupling, we first showed that dopamine inhibits PHI-spiperone binding with an $IC_{50}$ of 49 µM and a Hill coefficient of 0.69. A Hill coefficient less than unity is suggestive of high- and low-affinity binding sites.

Addition of GTP and NaCl increased dopamine $IC_{50}$ value twofold (109 µM) with a Hill coefficient approaching unity (0.93). Thus, the presence of GTP and NaCl converted the dopamine receptor from a population of high- and low-affinity receptors to the low-affinity state. These results indicated that the cloned dopamine receptor interacts with G proteins.

We next analyzed the effects of dopamine binding on the levels of intracellular and extracellular cAMP. Since the $D_2$ receptor is expected to inhibit cAMP levels, vasointestinal peptide (VIP) was used first to stimulate endogenous cAMP production. Dopamine inhibited both basal and VIP-stimulated cAMP levels in media from $GH_4ZR_7$ cells. Furthermore, intracellular cAMP levels were inhibited, albeit at a less pronounced level, probably due to the lower recovery of intracellular cAMP. The stereospecificity of these inhibitions was demonstrated using isomers of sulpiride: The active enantiomer (-)sulpiride blocked the inhibition while (+) sulpiride had no effect.

To demonstrate that the changes in cAMP levels were the result of an inhibition of adenylyl cyclase, dopamine was added to membranes of VIP- or forskolin-stimulated $GH_4ZR_7$ cells, and adenylyl cyclase activity was measured. Dopamine inhibited adenylyl cyclase activity by 45 percent. This inhibition was stereoselective, since the agonist quinpirole was active while its enantiomer LY181990 did not have any significant effect. Moreover, since receptors that couple to inhibitory G proteins are known to be sensitive to pertussis toxin, adenylyl cyclase activity was measured in membranes prepared from $GH_4ZR_7$ cells pretreated with pertussis toxin and stimulated with forskolin or VIP. Pertussis toxin was able to uncouple dopamine-mediated inhibition of adenylyl cyclase and dopamine-stimulated cAMP accumulation. Therefore, the $D_2$ receptor expressed in $GH_4ZR_7$ cells is capable of inhibiting adenylyl cyclase activity through a pertussis-toxin-sensitive mechanism.

Finally, the inhibition of prolactin (PRL) secretion by dopamine was assayed in $GH_4ZR_7$ cells. VIP and thyrotropin-releasing hormone (TRH) are known to enhance PRL release by a CAMP-dependent and a CAMP-independent mechanism, respectively. Dopamine was able to inhibit PRL secretion stimulated by both hormones. These inhibitions were reversed by the active antagonist (-)sulpiride but not (+)sulpiride. Therefore, we have demonstrated that the RGB-2 cDNA encodes a dopamine $D_2$ receptor that is functional, since it can couple to inhibitory G protein and since this coupling results in an inhibition of adenylyl cyclase activity as measured by the drop in cAMP levels and in an inhibition of PRL secretion.

## GENE LOCALIZATION OF THE HUMAN $D_2$ RECEPTOR

As mentioned above, several human neurological diseases have been associated with imbalances in the dopaminergic system, and in some cases, $D_2$ receptor involvement has been proposed. For example, several lines of evidence have linked the $D_2$ receptor to schizophrenia. First, a linear relationship exists between the affinity of different neuroleptic drugs for the $D_2$ receptor and their therapeutic dosage (Seeman et al. 1976). Also, the brains of some schizophrenics have been reported to possess an unusual density of $D_2$ receptors in their striatum (Seeman 1987). In addition, the dopamine $D_2$ receptor has been proposed to be involved in Parkinson's disease and Tourette's syndrome. To study the relationship between human diseases and the $D_2$ receptor, we have cloned the human gene.

A human genomic library was screened with the rat $D_2$ cDNA. Several clones were detected and one, λHD2G1, contained a 1.6 kb BamHl fragment, which was sequenced and found to code for the last 64 amino acids of the $D_2$ receptor. It should be stressed that the $D_2$ receptor gene has introns and that its entire sequence spans more than 30 kb.

The λHD2Gl DNA was hybridized to human metaphase chromosomes and to DNA prepared from a panel of rodent-human hybrids that have selectively lost human chromosomes (Grandy et al. 1989a). All the data were consistent with localizing the dopamine $D_2$ receptor to the long arm/of chromosome 11 in the region q22-q23. Moreover, when the 1.6 kb BamHl fragment from λHD2Gl was used to probe human genomic DNA digested with different restriction enzymes, only single fragments were detected. This result, plus the fact that the total size of hybridizing bands in the hybrid panel analysis was consistent with the size of the λHD2Gl phage DNA, indicates the human genome contains only one $D_2$ receptor gene. It should be mentioned that, although our studies were done under stringent hybridization conditions, these conditions would have detected genes whose sequences are closely related (75-percent identity).

The presence of one dopamine $D_2$ receptor gene is of pharmacological importance. The dopamine receptors have been classified into at least two subtypes, $D_1$ and $D_2$. The $D_2$ receptors themselves have been further subdivided into autoreceptors and postsynaptic receptors (Carlsson 1975). The autoreceptors have a $D_2$-like pharmacological profile but are found on neurons that synthesize dopamine. In these neurons, upon binding of dopamine, the autoreceptors regulate dopamine production through a feedback mechanism.

Our data, therefore, indicate that either the diverse $D_1$ and $D_2$ receptors are encoded by different genes whose sequences are divergent, at least more than

the sequences of the muscarinic receptors, or that these dopamine receptors are encoded by the same unique gene and that the pharmacological differences are the results of either alternative posttranscriptional events or diverse posttranslational modifications. In view of the current knowledge about the differences between the $D_1$ and $D_2$ receptors, it seems that they may be coded for by different genes. On the other hand, some recent experiments indicate that the $D_2$ autoreceptors are similar in sequence to the postsynaptic $D_2$ receptor and that these two receptors are products of the same gene (Meador-Woodruff et al. 1989).

The human genomic clone, λHD2G1, has been used in several laboratories to investigate the linkage of the $D_2$ receptor gene and several human genetic disorders. Thus far, the studies have focused on families afflicted with schizophrenia (Moises et al. 1989; Byerley et al. 1989), on other kindreds afflicted with Tourette's syndrome (Gelernter et al. 1989; lsenberg et al. 1989), and on families with a high incidence of manic depression (Byerley et al. 1989). The $D_2$ receptor was not linked directly to disease in any of these cases. It should be mentioned, however, that more families currently are being studied.

## DOPAMINE $D_2$ RECEPTOR SUBTYPES

By using the rat cDNA as a probe, a human dopamine $D_2$ receptor was cloned and sequenced from a pituitary cDNA library (Grandy et al. 1989b). The deduced sequence of the human clone revealed a surprising result. The human pituitary $D_2$ receptor is highly similar to its rat brain counterpart since they share 96-percent identity in their amino acid sequence. However, we found that the human pituitary receptor contains a stretch of 29 amino acid residues that are unaccounted for in the rat receptor. These additional residues are found in the putative third cytoplasmic loop of the receptor, suggesting that they might be important for differentiating G-protein couplings. When the human pituitary receptor is expressed into mouse Ltk- cells, it binds dopamine ligands with the same pharmacological profile as does the rat brain receptor, indicating that the 29 amino acid extension does not affect ligand binding significantly. Furthermore, we have since shown that a rat equivalent of the human pituitary receptor exists and that it is also possible to find a human $D_2$ receptor that does not contain the 29 amino acid residues. Therefore, presence of this peptidic extension is not species-specific.

Finally, the human dopamine $D_2$ receptor gene was isolated and sequenced. This analysis revealed that the dopamine receptor gene is split into exons and that the coding sequence of the receptor is carried by seven different exons. Moreover, we demonstrated that the 29 amino acid extension is encoded by a specific exon that does not carry sequences of any other part of the molecule,

thus strongly indicating that the existence of the two forms of $D_2$ receptors is the result of an alternative splicing event occurring during pre-mRNA maturation.

## CONCLUSIONS

We have demonstrated that the G-protein-coupled receptor that we have cloned has the pharmacological profile and the biological function expected of the dopamine $D_2$ receptor. Furthermore, we have localized the dopamine $D_2$ receptor gene to the long arm of chromosome 11 in humans and have shown it to be represented as a single copy in the genome. Finally, we have shown that there are two subtypes of $D_2$ receptor that differ by a stretch of 29 amino acid residues found in the putative third cytoplasmic loop. These additional residues do not affect ligand binding and are the result of an alternative splicing event occurring during the pre-mRNA maturation.

This work has established the foundations on which further studies of the $D_2$ receptor can be based. For example, the $D_2$ receptor has been shown to induce not only inhibition of adenylyl cyclase, but also other second messenger systems. Cell lines transfected with the cloned receptor will be of great interest when analyzing the coupling of the $D_2$ receptor to other second messenger pathways. In addition, the human $D_2$ gene is the focus of many attempts to relate this gene to specific genetic diseases.

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# Characterization of Mammalian Potassium Channel Genes

***James O. Douglass, Macdonald Christie, John P. Adelman, and R. Alan North***

## INTRODUCTION

Potassium channels are transmembrane proteins that form a pore for the passage of potassium ions through the membrane lipid bilayer. Of the membrane ion channels characterized to date, potassium channels are the most widespread and diverse. In endocrine and exocrine cell types, they play an important role in the regulation of secretion of hormonal factors. In the heart, they are responsible for repoiarization of myocardial ceils, and they determine the frequency of firing in Purkinje fibers. In lymphocytes, they are involved with the development of mature T cells from immature thymocytes and are necessary for T-lymphocyte activation.

Potassium channels are most prevalent in neuronal cell types, where they play a central role in regulating the level of neuronal excitability. Some types of potassium channels are opened by depolarization to limit the duration of single action potentials (delayed rectifier) or to set the pattern of bursts of action potentials (transient, or "A," currents). Other potassium channels are opened or closed by second messengers, such as intracellular calcium, to mediate the actions of synaptic transmitters (Ca-activated potassium currents). Additional types of potassium channels are involved in mediating entry of potassium ions into neurons (inward rectifiers) and in controlling excitation following activation of neurotransmitter receptors (neurotransmitter- and second messenger-regulated channels). These various types of potassium channels are present in unique regions and cell types of the mammalian central and peripheral nervous systems where they serve to imprint on each neuron its particular properties of plasticity and excitability (for review, see Rudy 1988; Latorre et al. 1984; Hille 1984).

The potassium channel responsible for the generation of the transient outward current (Ia current) is present on the majority of neuronal cell types and is a characteristic of excitable cells. Ia channels in excitable membranes are

essential for a variety of cellular functions, including neuron bursting, the determination of resting potentials, the duration of action potentials, and the modulation of cardiac pacemaking functions. They also have been implicated in some model systems of learning.

The molecular cloning of the Shaker locus of *Drosophila melanogaster* has provided the starting point for the molecular analysis of the potassium A-channel. *Shaker* mutations phenotypically show a loss of A currents without affecting other potassium currents. Therefore, the *Shaker* locus was perceived to encode the structural component of the A-channel. Genomic DNA rearrangements in five distinct *Shaker* mutants were genetically mapped to a 60 kb segment of the *Drosophila* genome (Papazian et al. 1987). Genomic DNA from this region was isolated and then subcloned, and specific restriction DNA fragments were used as hybridization probes to screen adult fly head cDNA libraries (Papazian et al. 1987; Tempel et al. 1987; Schwarz et al. 1988; Pongs et al. 1988). Isolation and sequence analysis of cDNA clones, followed by comparison with genomic DNA sequences, revealed that transcription from the wild-type *Shaker* genomic locus results in the production of multiple species of mRNAs, each encoding a potassium channel protein, Distinct *Shaker* mRNAs result from alternate splicing of precursor mRNA, and the resulting potassium channels share a common *core* sequence with different amino and carboxyl termini.

Various Shaker cDNA clones have been expressed in *Xenopus* oocytes and characterized electrophysiologically (Timpe et al. 1988a, 1988b; Iverson et al. 1988). RNA synthesized *in vitro* from each cDNA clone directs the synthesis of a voltage-dependent potassium channel. Unique kinetics of inactivation are seen for each channel type, indicating that the *Shaker* products may contribute to kinetic diversity in A-channels of the fly and that sequences in the amino terminal region may be important for inactivation.

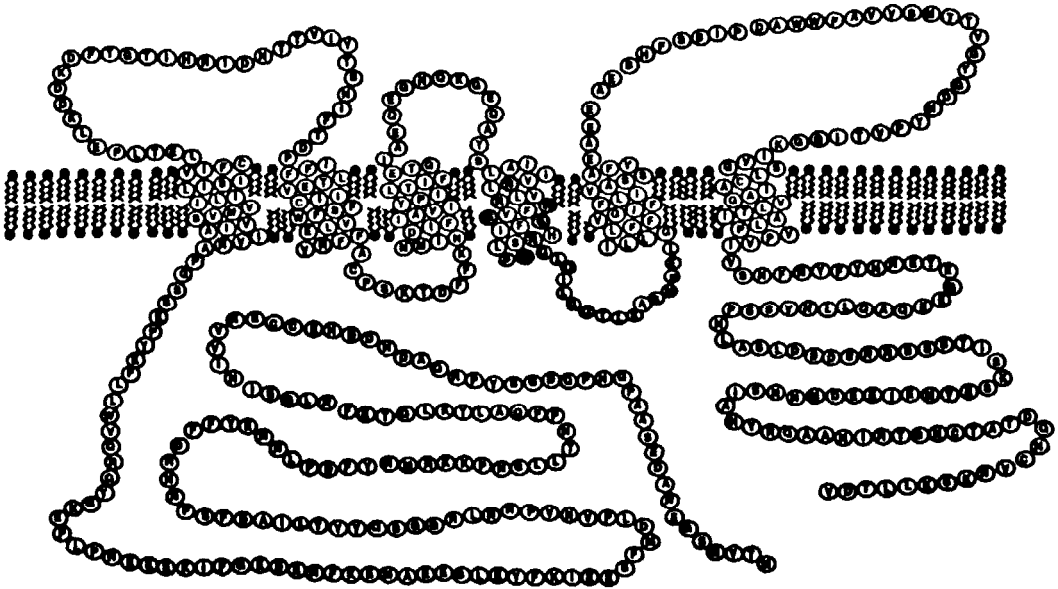
*Shaker* sequences have been used as hybridization probes to isolate potassium channel cDNA clones from mouse brain (Tempel et al. 1988) and rat brain (Baumann et al. 1988; Christie et al. 1989; McKinnon 1989) cDNA libraries, This chapter presents a short summary of efforts to characterize mammalian potassium channel genes and their products, A brief discussion of the possible structure of a typical, mammalian, voltage-gated potassium channel is presented. Genomic Southern blot analysis suggests that rat and human voltage-gated potassium channels have been highly conserved during evolution, and they represent a large gene family. Finally, some electrophysiological properties of cloned rat potassium channels are described, using the *Xenopus* oocyte system to define specific channel properties,

**AMINO ACID SEQUENCE AND PREDICTED STRUCTURE OF THE RAT VOLTAGE-GATED POTASSIUM CHANNEL, RBK1**

A cDNA library constructed from rat hippocampal polyadenylated mRNA was screened with two radiolabeled oligonucleotide probes whose sequences were based on conserved amino acid residues between the *Shaker* A potassium channel of *Drosophila* (Tempel et al. 1987) and the mouse potassium channel equivalent, MBK1 (Tempel et al. 1988). One of the rat hippocampai cDNA clones (designated as RBK1), which was hybridization-positive with both oligonucleotide probes, was purified, and the nucleotide sequence of the 1.7 kb cDNA insert was determined (Christie et al. 1989). The sequence contains a single, long open reading frame that encodes a protein of 495 amino acids. The predicted amino acid sequence is 69 percent homologous to the 453 residues representing the conserved core region of the *Shaker* A potassium channel and is 99 percent homologous to the MBK1 channel.

The amino acid sequence and location of predicted transmembrane regions of the RBK1 potassium channel are diagramed in figure 1. Both amino and carboxyl terminal domains are located on the cytoplasmic side of the cell membrane. Six regions of clusters of hydrophobic residues represent putative transmembrane domains of the channel (designated as S1 to S6). Residues between Sl/S2, S3/S4, and S5/S6 are presumably extracellular, with residues between S2/S3 and S4/S5 located on the cytoplasmic side of the membrane. Conservation of amino acid sequence motifs between *Shaker* A, MBK1 , and RBK1 potassium channels reveal specific moieties that are of functional significance. For example, seven positively charged amino acids (arginine-$R_1$ and lysine-K) that repeat at every third position are in the S4 transmembrane domain. It is probably this region of the channel that responds to changes in membrane potential, regulating potassium ion gating. Five possible sites for N-linked glycosylation are present in the predicted sequence, as well as one possible A kinase phosphorylation site in the carboxyl terminal intracellular region.

In contrast to the predicted structure of mammalian voltage-dependent potassium channels, mammalian voltage-dependent calcium and sodium channels contain four repeating units, with six putative transmembrane domains located in each repeat unit (Noda et al. 1984; Trimmer et al. 1989; Tanabe et al. 1987). Thus, by analogy with the latter two types of ion channels, functional potassium channels *in vivo* may represent a complex of four individual subunits (figure 2). This hypothesis is of note in that there exists a variety of functionally specialized voltage-dependent potassium channels, and some of this diversity may be due to the formation of potassium channels by assembly of different subunits (i.e., heteropolymerization).



**RBK1 Rat brain potassium channel**

**FIGURE 1.** *Amino acid sequence and predicted transmembrane domains of the rat voltage-gated potassium channel, RBK1*

NOTE: Amino acid sequence is predicted from the nucleotide sequence of the RBK1 cDNA clone (Christie et al. 1989). Predicted transmembrane domains represent clusters of hydrophobic amino acids. In the fourth transmembrane domain (S4), positively charged R and K residues are noted as darkened circles.

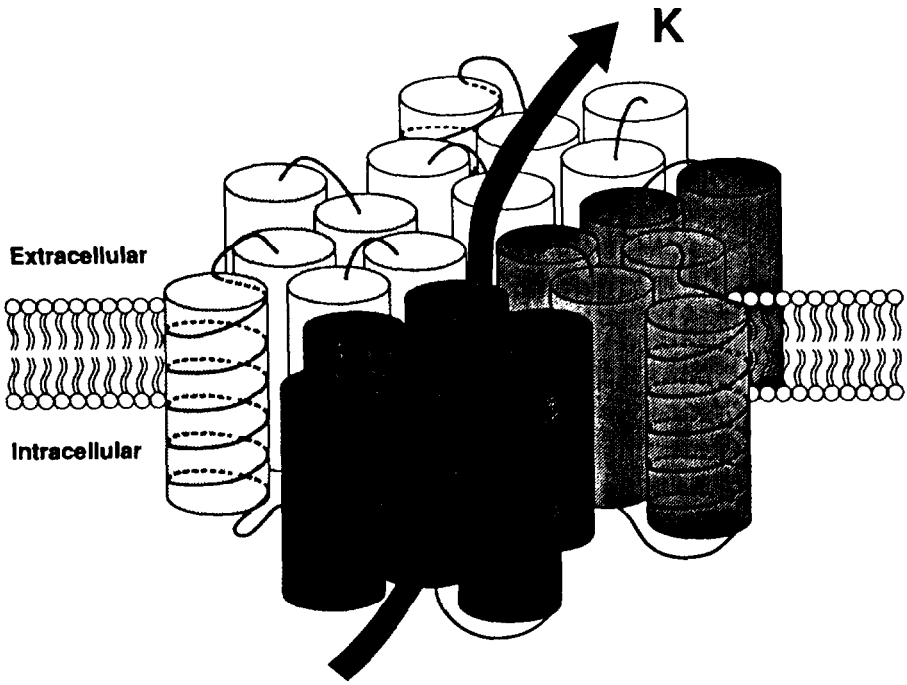



**FIGURE 2.** *Schematic representation of the possible general structure of a functional mammalian voltage-gated potassium channel*

NOTE: Transmembrane domains are represented as cylinders through the cell membrane (pseudocylindrical, α-helical in nature). tines represent regions separating the transmembrane domains, both extracellular and intracellular. Individual subunits contain six transmembrane domains. Different patterns of shading represent the notion that four individual subunits assemble as a tetramer to form a functional potassium channel with a central conducting pore (by analogy with the voltage-dependent calcium and sodium channel).

## MULTIPLE GENES ENCODE MAMMALIAN VOLTAGE-GATED POTASSIUM CHANNELS

In *Drosophila,* transcription at the *Shaker* locus results in the production of multiple species of mRNAs (via alternate splicing mechanisms) encoding unique potassium channel proteins. The coding region of the *Shaker* transcript(s) encompasses approximately 70,000 bases of genomic DNA, with

11 to 12 introns separating exonic sequences (Schwarz et al. 1988). The 10 or 11 exons encoding specific portions of the *Shaker* potassium channels are from 100 to 450 bases in length.

To determine if a similar situation exists at the genomic locus encoding the RBK1 potassium channel, a rat genomic DNA library was screened under high stringency conditions (50 percent formamide, 42°C hybridization) with $^{32}$P-labeled RBK1 cDNA. The hybridization positive λ genomic DNA clone, RGK1 , was isolated and then purified via successive rounds of low-density screening; phage DNA was subjected to Southern blot analysis using the 1.7kb RBK1 cDNA as a hybridization probe. A 1.9kb, RGK1 Hind III restriction fragment was observed to be hybridization-positive following autoradiography. This observation suggested that the majority of the RBK1 coding region was present on a single exonic domain in the corresponding (RGK1) gene. To determine if this was indeed the case, the 1.9kb Hind III fragment was isolated from the RGK1 rat genomic DNA clone, restriction mapped, and subcloned into M13 for dideoxynucleotide sequence analysis. This analysis revealed that the 1.9kb Hind III fragment contained an *uninterrupted* sequence representing 250 bases of RBK1 mRNA 5' untranslated region, the entire 1,485 bases of RBK1 mRNA coding region, and 165 bases of RBK1 mRNA 3' untranslated region. Thus, the entire series of nucleotides encoding the RBK1 voltage-gated potassium channel is contained on a single exonic domain-a situation dramatically different from that seen at the Shaker locus. It follows, then, that alternate splicing at the RGK1 locus cannot result in the production of multiple species of mRNAs encoding unique potassium channel products.

At our present level of understanding, it appears that diversity of mammalian voltage-gated potassium channels is due to the presence of multiple, structurally related genes in the genome, with each member of the gene family encoding a distinct potassium channel product. In *an* effort to begin characterizing the multitude of mammalian potassium channel genes, Southern blot analysis of rat and human genomic DNA was performed under different hybridization stringencies (figures 3 and 4).

High molecular weight rat and human genomic DNA was incubated with several restriction enzymes (Eco RI, Bgl II, Pst I, and Hind Ill), electrophoresed on a 1 -percent agarose gel, and transferred to nylon membranes. The Southern blots were hybridized overnight under stringent conditions (50 percent formamide/42°C hybridization; figure 3) with $^{32}$P-labeled RBK1 cDNA. In all lanes, a single hybridization signal was observed following autoradiography. These data are consistent with the observation that the RBK1 coding region is present on a single exonic domain in the rat genome. (Note the presence of a 1.9kb hybridization signal in the rat genomic DNA sample treated with Hind Ill.)

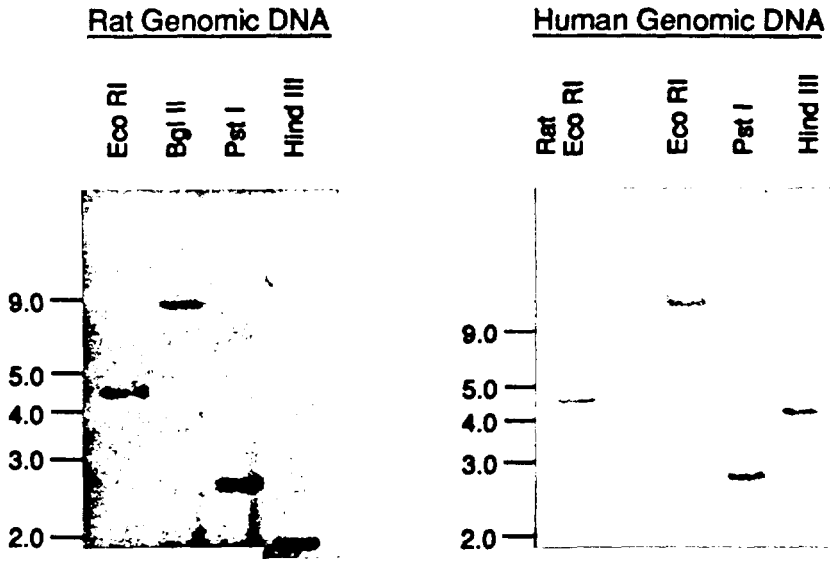



**FIGURE 3.** *Southern blot analysis of rat and human genomic DNA hybridized with $^{32}$P-labeled RBK1 cDNA under stringent conditions*

NOTE: Rat and human genomic DNA was treated with the noted restriction endonucleases (Eco RI, Bgl II, Pst I, and Hind Ill) before electrophoresis on a 1 -percent agarose gel and subsequent transfer to nylon membranes. The hybridization probe was a 1.7kb RBK1 cDNA fragment radiolabeled by random-priming with $^{32}$P-dCTP. Hybridization conditions were 50 percent formamide/42°C. DNA molecular weight markers are shown to the left of each autoradiograph.

Similar profiles (i.e., single hybridization signals) are observed for human genomic DNA analyzed under identical conditions. The data suggest that the human RBK1 gene equivalent is highly homologous to rat RBK1 at the nucleotide level, that it is present as a single copy gene in the human genome, and that the majority of the coding region probably also is contained on a single exonic domain.

The same Southern blots were reprobed with the identical radiolabeled RBK1 cDNA fragment, but under slightly less stringent hybridization conditions (40 percent formamide/42ºC). The resulting autoradiographs are shown in figure 4. In rat and human DNA sample lanes, additional hybridizing bands now are

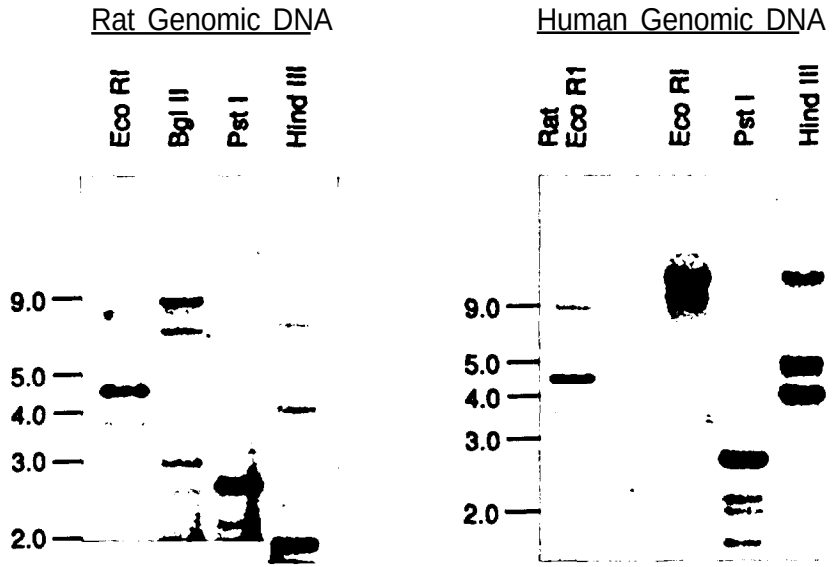



**FIGURE 4.** *Southern blot analysis of rat and human genomic DNA hybridized with $^{32}$P-labeled RBK1 under semistringent conditions*

NOTE: The experiment is identical to that described in figure 3, only the hybridization conditions were 40 percent formamide/42ºC.

observed. Anywhere from two to six additional bands are observed in each lane. These data suggest that the additional bands may encode other potassium channel genes, a result consistent with the hypothesis that the variety of mammalian potassium channels that have been characterized electrophysiologically share common structural features and are encoded by different structural genes.

The recent cloning of additional voltage-gated potassium channels further suggests that this is indeed the case. Low-stringency hybridization screening of rat brain cDNA libraries has resulted in the cloning of rat voltage-gated potassium channels RBK2 (McKinnon 1989), RCK1 (RBK1), RCK3, RCK4, and RCK5 (Baumann et al. 1988; Christie et al. 1989; Stuhmer et al. 1989). Low-stringency screening of a rat genomic DNA library has afforded the characterization of the potassium channel RGK5 (Douglass et al. 1990). Expression cloning also has identified a novel rat brain potassium channel, DRKl (Frech et al. 1989). Amino acid and nucleotide sequence analysis of

these potassium channels confirms that distinct genes encode all the aforementioned channels.

## EXPRESSION OF CLONED RAT POTASSIUM CHANNELS IN *XENOPUS* OOCYTES

At least 30 distinguishable types of potassium channels have been characterized *(in vivo)* to date, showing diversity in their gating and regulation (Yellen 1987). They vary remarkably with regard to activation threshold and kinetics; inactivation rate; and sensitivity to blockers such as 4-aminopyridine (4-AP), tetraethylammonium (TEA), and toxins (for review, see Rudy 1988). To attempt to correlate cloned mammalian potassium channels with those characterized *in vivo,* it is important to determine the properties of the former channel types. Expression of cloned channels in *Xenopus oocytes* has provided an excellent means of analysis.

Figure 5 shows the potassium currents generated in *Xenopus* oocytes following the injection of in vitro synthesized, capped RNA encoding the RBK1 (Baumann et al. 1988; Christie et al. 1989), RBK2 (McKinnon 1989), and RGK5 (Douglass et al. 1990; RCK3 [Stuhmer et al. 1989]) rat voltage-gated potassium channels. Oocytes (Dumont stages V-VI) were harvested from adult *Xenopus laevis,* and theca/follicular layers were removed by collagenase treatment. Oocytes were injected within 10 hours of harvest with the aforementioned RNAs (usually 1 ng RNA). Recording of membrane currents were made 1 to 3 days after injection of RNA. Oocytes were voltage-clamped at various potentials using two microelectrodes.

Currents were evoked by depolarizing voltage-command pulses from a holding potential of -80 mV (records are single sweeps, leak subtracted). The depolarizing steps were 50 ms in duration, to the potential (in mV) indicated beside each trace. Large outward currents were produced in all RNA-injected oocytes, activating at approximately -40 mV for RBK1, -20 mV for RBK2, and -30 mV for RGK5. No such currents were observed in uninjected oocytes. Measurement of RBK1, RBK2, and RGK5 tail current reversal potentials indicated that the currents were carried exclusively by potassium ions.

These channel activation properties alone are not sufficient to assign a particular cloned potassium channel to one that has been characterized in vivo. However, channel inactivation kinetics as well as sensitivity profiles to various potassium channel blockers can be used to functionally distinguish one cloned channel from another. For example, the channels described above have dramatically different profiles of sensitivity to the potassium channel blocker TEA. The $IC_{50}$s for the expressed channels are 0.3 mM, RBK1; 10 mM, RGK5;

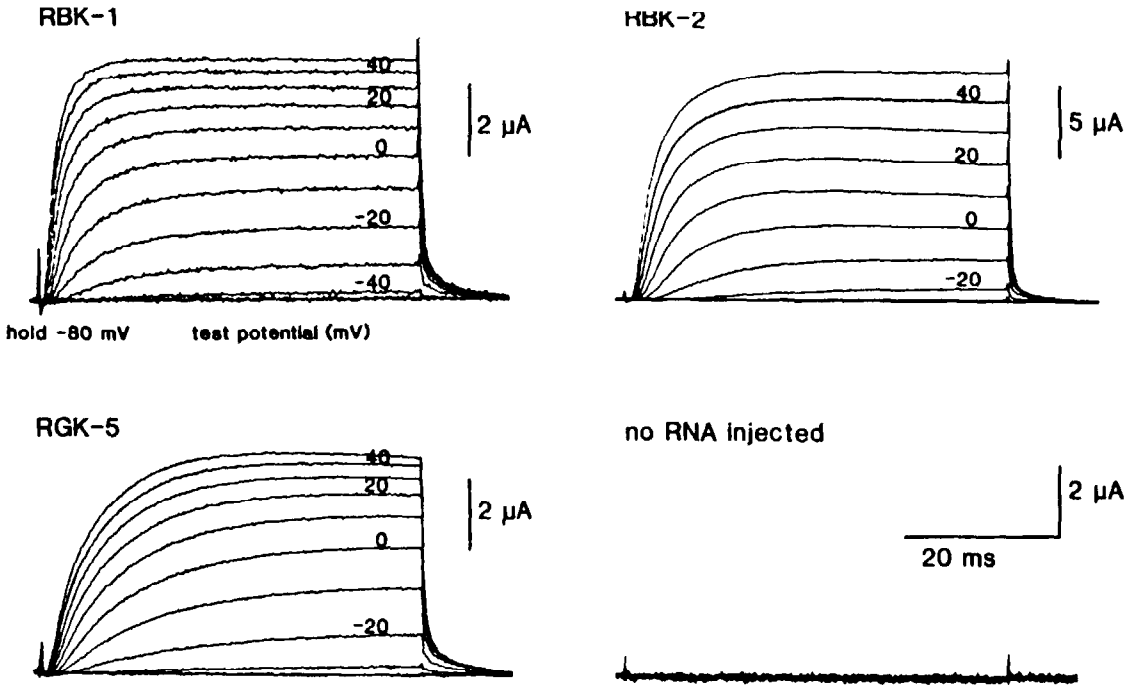



**FIGURE 5.** *Generation of transient outward currents in Xenopus oocytes injected with in vitro synthesized RBK1, RBK2, and RGK5 potassium channel RNA*

NOTE: Currents were evoked by depolarizing voltage command pulses from a holding potential of -80 mV. Test potentials, in mV, are noted beside each trace. The depolarizing step was 50 ms in duration. Generation of current is dependent on the injection of potassium channel-encoding RNA (for additional details, see Christie et al. 1989).

and >= 100 mM, RBK2 (Christie et al. 1990; Stuhmer et al. 1989). Unique sensitivity profiles to other channel blockers, such as 4-AP, dendrotoxin, and charybdotoxin, in addition to distinguishable channel inactivation kinetics, provide these cloned channels with a unique compilation of functional characteristics (Stuhmer et al. 1989).

In at least one instance, channel properties characterized in the *Xenopus* oocyte system, coupled with Northern blot analysis, has allowed for a cloned rat potassium channel to be correlated with an *in vivo*-characterized channel (Douglass et al. 1990). The RGK5 rat potassium channel, described above, appears to represent the *n*-type potassium channel, which has been characterized extensively in mammalian lymphocytes (Cahalan et al. 1987; DeCoursey et al. 1985; Lewis and Cahalan 1988). The RGK5 sensitivity profile to channel blockers, kinetics of inactivation, and modulation of inactivation by divalent cations (as determined in the *Xenopus* oocyte system) are nearly identical to the properties of the *n*-channel, as characterized in murine and human T-lymphocytes. In addition, the presence of RGK5 mRNA in thymus is consistent with the notion that the RGK5 channel is the T-lymphocyte n-channel. Similar studies, no doubt, will serve to provide additional functional assignments in the future.

## CONCLUSIONS

Potassium channels are membrane proteins that are selectively permeable to potassium ions. They are present in virtually all animal cells but take on particularly important signaling functions in excitable tissues. Potassium channels have been classified on the basis of the factors that open (or close) them; some are opened by changes in the membrane potential, others by the binding of intracellular ligands to the molecule. The classes do not exclude each other. Roles for voltage-dependent channels are well developed in neurons and include maintaining a resting potential, action potential repolarization, patterning of spike bursts, and limiting synaptic depolarizations. To understand the molecular mechanisms by which these channels regulate such a variety of cellular functions, it is necessary to determine the structure of these proteins and their corresponding genes.

Studies utilizing Drosophila as a model system have allowed for the molecular characterization of voltage-gated potassium channels to begin. In the fly, transcription from a huge genomic DNA locus results in the production of multiple species of mRNAs encoding unique potassium channel proteins. These multiple mRNAs are produced as a result of alternate splicing of precursor mRNA. The resulting potassium channels share a common core sequence, with different amino and carboxyl termini present on the different

channel proteins. Different species of *Shaker* channels have been expressed in *Xenopus* oocytes, and unique kinetics of inactivation are seen for each channel type, indicating that the *Shaker* products may contribute to kinetic diversity in A-channels of the fly.

*Shaker* DNA sequences have been used to isolate potassium channel cDNA clones from rat brain libraries. One such clone encodes the rat brain potassium channel RBK1, The nucleotide sequence of the clone predicts the generation of a protein 495 amino acids in length. Computer analysis of the RBK1 amino acid sequence suggests the presence of six transmembrane domains, with the fourth domain possibly acting as the channel voltage sensor. Individual subunits may associate as a tetramer to form functional potassium channels.

Rat cDNA and genomic DNA fragments encoding voltage-gated potassium channels have been used as templates for the *in vitro* generation of capped RNA. This RNA has been injected into *Xenopus* oocytes to functionally characterize these cloned channels, Each channel reveals unique kinetics of activation and inactivation as well as unique profiles of sensitivity to a range of potassium channel blockers, This analysis has served to demonstrate functional diversity among the cloned rat voltage-gated potassium channels and also has led to the correlation of at least one cloned channel (RGK5) with a potassium channel previously characterized in murine and human T-lymphocytes (the rechannel).

The presence in the mammalian genome of multiple genes encoding unique species of potassium channels appears to be the major basis for diversity of mammalian voltage-gated potassium channels. Southern blot analysis of rat and human genomic DNA reveals single hybridizing bands when a radiolabeled rat potassium channel-encoding cDNA fragment is used as a hybridization probe under stringent hybridization conditions. Under slightly less stringent hybridization conditions, however, multiple hybridizing bands are observed. Thus, voltage-gated potassium channels in mammals appear to represent a relatively large gene family. The continued isolation and characterization of mammalian potassium channel cDNA and genomic clones, along with functional characterization of the cloned channels in *Xenopus* oocytes, certainly will improve our understanding of the molecular basis underlying diversity of this protein family.

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## ACKNOWLEDGMENTS

The studies described were supported by the following U.S. Public Health Service grants: National Institute on Drug Abuse (NIDA) grant DA-04154 (JOD); National Institute of Child Health and Human Development grant HD-24562 (JPA); NIDA grants DA-03160, DA-03161, DK-32979; and National Institute of Mental Health grant MH-40416 (RAN).

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# Problems and Approaches in Studying Membrane Opioid Receptors

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## INTRODUCTION

Opioid receptors were identified first in mammalian brain in the early 1970s (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973), and their pharmacological properties have been characterized extensively since that time (for reviews, see Chang 1984; lwamoto and Martin 1981; Smith and Loh 1981; Wood 1982). However, full structural and functional characterization of a receptor requires its purification in a form retaining the ability to bind ligands. Such a purified preparation can be reconstituted into a membrane environment and tested for the ability to mediate some biochemical function. Its amino acid sequence can be determined and its conformation deduced. Antibodies to the purified receptor can be prepared and used to map its tissue and cellular localizations, to determine the role of various portions of the protein in ligand binding and receptor-mediated function, and to prepare affinity columns capable of purifying more receptor.

The conventional procedure for purifying membrane opioid receptors involves solubilization, followed by fractionation such as affinity chromatography and gel filtration. Recently, several laboratories have reported some success using this procedure. Bidlack and coworkers (1981) solubilized rat brain membranes with Triton X-100, then isolated opioid-binding material from the solubilized preparation by means of affinity chromatography using 14-ß bromacetamido-morphine. The resulting preparation bound dihydromorphine and several other opioids with nM affinity, but the specific binding activity (cpm/mg protein) was at least an order of magnitude lower than theoretically expected for a pure receptor. SDS gel analysis revealed three polypeptides of molecular weights 25 to 50 kD.

Simon and colleagues solubilized opioid receptors from toad and, later, from rat and other mammalian brain preparations with digitonin (Howells et al. 1982). Using opioid ligand affinity chromatography coupled with lectin affinity chromatography, they were able to purify an opioid-binding protein from bovine

striatum to theoretical homogeneity (Gioannini et al. 1985). The opioid-binding material had a molecular weight of 300 to 350 kD under nondenaturing conditions and 65 kD on SDS gels. This material was able to bind the l-specific ligand DAGO and ß-endorphin.

Maneckjee and coworkers (1985) reported partial purification (500-fold) of a p-specific opioid receptor from rat brain using 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS) as solubilizer; SDS gel electrophoresis indicated the presence of three peptides, of molecular weights 94 kD, 42 kD, and 35 kD. Subsequently, they prepared a more highly purified receptor preparation from bovine striatum (Maneckjee et al. 1987) using a combination of affinity chromatography and hydroxyapatite chromatography. Studies with polyclonal antibodies prepared to this protein indicated that the 94 kD component was involved in opioid binding.

Cho and coworkers (1986) reported purification from bovine brain of a protein selective for opioid alkaloid ligands using a combination of affinity chromatography, lectin chromatography, and gel filtration. A novel feature of this protein was that it required acidic lipids possessing unsaturated fatty acids to manifest binding activity; neither the protein nor the lipids alone possessed significant opioid binding (Hasegawa et al. 1987). The binding affinities of ligands to this reconstituted rnaterial were lower than the corresponding values for binding to brain membranes, but the rank order of binding affinities to the two preparations were highly correlated (Cho et al. 1986; Hasegawa et al. 1987).

Ueda and coworkers (1987, 1988), using a similar method, also obtained a protein consisting primarily of a 58 kD band. When this preparation was reconstituted with the purified G-proteins $G_1$ or $G_0$, a large increase in displacement of $^3$H-naloxone binding by the p-agonist DAGO was observed, and this increase was sensitive to GTP. µ-Agonists, but not δ- or κ-agonists, also stimulated GTP binding and GTPase activity in this preparation. Thus, this work suggests that µ-opioid receptors may exert their *in vivo* effects through a G-protein,

Many of the solubilized opioid preparations initially reported did not bind k-ligands. However, Chow and Zukin (1983) reported that CHAPS-solubilized rat brain membranes containedκ-as well as p-binding species, as assayed by $^3$H-bremazocine binding. The material eluted as two peaks of 50 and about 250 kD. ltzhak and colleagues (Itzhak et al. 1984; ltzhak and Pasternak 1986) solubilized guinea pig brain membranes using digitonin in the presence of high NaCl concentrations and found that δ-binding was associated with material of 750-875 kD, while κ-binding was associated with material of about 400 kD.

DeMoliou-Mason and Barnard (1984), using digitonin extraction in the presence of 10 mM $MgCl_2$, were able to solubilize material that bound dynorphin-(1-9), a putative k-ligand, as well as μ- and δ-selective ligands. Subsequently, they characterized this binding material by gel filtration (DeMoliou-Mason and Barnard 1986). δ-Ligands were found to be associated with material over 500 kD, while both μ-alkaloids and dynorphin-(1-9) were associated with both this high-molecular-weight material and with lower-molecular-weight material.

Simon and coworkers (1987) purified k-opioid receptors from digitonin-solubilized frog brain membranes, An affinity column consisting of D-ala$^2$-D-leu$^5$enkephalin (DADLE) coupled to Sepharose-6B was used to isolate μ, δ, and κ-receptors from the solubilized preparation; and k-receptors were separated from the other two by gel filtration. The extent of purification was over 4,000, based on pmol of $^3$H-EKC bound/mg protein. SDS gel analysis revealed two bands of 65 and 58 kD.

This approach has had limited success with opioid receptors, however, for several reasons. First, these receptors have proved to be very difficult to solubilize in a form that retains binding activity. Ligand binding of opioid receptors is eliminated or greatly reduced by low concentrations of most nonionic detergents that have been used successfully to solubilize other cell surface receptors. At least part of this problem may be due to the more stringent criteria necessary to establish the existence of opioid receptors. The activity of most cell surface receptors is measured by specific, or displaceable, binding, which is defined as the difference between the amount of radioactive ligand bound in the presence and absence of a large amount of the same unlabeled ligand. A genuine opioid receptor, in contrast, must exhibit not only specific but also stereospecific binding-that which is selective for the 1-forms of stereoisomeric agonists such as levorphanol and levallorphan.

Most laboratories that have reported solubilization of opioid receptors have demonstrated the existence only of specific binding in their preparations. Stereospecific binding either is very low or is not reported at all. While it is conceivable that opioid receptors lose stereospecificity upon solubilization, obviously the inability to demonstrate it casts doubt on the pharmacological relevance of the preparation.

A second obstacle to purification of opioid receptors is that no simple biochemical process mediating opioid receptor function has as yet been identified. Opioid receptors have been classically defined as those promoting antinociception in mammals or, in some cases, by their effects in certain *in vivo* tissue systems. Recent work has indicated an association between opioid receptors and several other functional molecules in cell membranes, including

adenylate cyclase (Law et al. 1981; Sharma et al. 1975) and ion channels (North and Williams 1983; Werz and MacDonald 1984, 1985; Williams et al. 1982). However, none of these molecules has been demonstrated to play a role in the process of antinociception. Thus, even if a purified protein has opioid-binding activity, there is no definitive biochemical test that can confirm its functional relevance.

Finally, opioid receptors are heterogeneous. At least three different classes are present in brain: μ, δ, and κ, which differ in their ligand selectivity and in their pharmacological effects (Lord et al. 1977; Martin et al. 1976). Other receptor types may exist, in brain or in peripheral tissues (Grevel and Sadee 1983; Schulz et al. 1981), as well as subtypes for p-receptors (Loew et al. 1986; Nishimura et al. 1984) and κ receptors (lyengar et al. 1986). Because most opioid ligands are not completely selective for a single receptor type, it is difficult to isolate a single type, which complicates pharmacological characterization.

For these reasons, some investigators have sought alternative ways of purifying opioid receptors. This chapter discusses and evaluates some of these procedures.

## PURIFICATION OF COVALENTLY LABELED OPIOID RECEPTORS

Since solubilization of opioid receptors in a form retaining ligand-binding properties has proved to be so difficult, some investigators have used an alternative approach in which the receptor is labeled with a radioactive, covalently bound ligand. The receptor then can be solubilized with a denaturing detergent, and purification followed by means of the radioactive label. That many opioid ligands are peptides has made this approach especially attractive, because, in addition to synthesizing and testing compounds capable of reacting covalently with opioid receptors, some researchers have covalently attached opioid peptides to the receptor by means of a variety of bifunctional cross-linking reagents.

Zukin and Kream (1979) first applied the latter technique using DADLE as ligand. The material so labeled had a molecular weight of 380 kD under nondenaturing conditions and 35 kD on SDS gels. However, as the only available group for cross-linking on DADLE is the terminal amino group, which presumably is involved directly in binding, it could be argued that during cross-linking DADLE was moved from its binding site.

We cross-linked $^{3}$H-ß-endorphin to brain membranes that previously had been solubilized with a nonionic detergent (Brij 36-T). This opioid peptide contains several internal amino groups that could serve as the site of covalent

attachment without interfering with the binding process. SDS gel analysis of the solubilized, cross-linked material revealed a broad spectrum of species, from 2 to 200 kD (Smith and Loh 1979).

Howard and coworkers (1985, 1986) covalently labeled opioid receptors in brain membranes by cross-linking $^{125}$I-ß-endorphin with the bifunctional reagent bis[2-succinimido-oxycarbonyl-oxy-O-ethyl] sulfone. Membranes were incubated with the radioactive ligand, then centrifuged to remove free ligand, and incubated with the cross-linking reagent. By carrying out the original incubation with ß-endorphin in the presence of unlabeled ligands selective for µ-, δ-, or ς-receptors, and analyzing the cross-linked products on SDS gels, they were able to conclude that ß-endorphin labeled both µ and δ–receptors and that the latter could be distinguished by molecular weight. The µ-receptors, identified as bands cross-linked in the presence of δ- and κ-agonists, were associated with a major 65 kD band and a minor 38 kD band, while the B-receptors consisted of a major 53 kD band and a minor 25 kD band. The 53 kD band also was observed in cross-linked membranes from NG 108-15 hybrid cells, which contain only δ-opioid receptors.

Bero and coworkers (1988), using a similar technique, found that $^{125}$I-ß-endorphin labeled three peptides of molecular weight 108, 73, and 49 kD. However, the relationship of these three species to µ-, δ-,and κ-receptors was not clear. Preincubation with unlabeled ß-endorphin was capable of blocking much or most of the cross-linking to each of these species, but other ligands were much less effective. Thus, etorphine, which binds to µ-, δ-,and κ-opioid receptors, blocked 60 percent of the cross-linking to the 73 kD band but less than 40 percent to either of the other two bands. The µ-selective ligand DAGO and the κ-selective U-50.488H blocked 30 to 50 percent of the binding to the 10 and 73 kD bands, while the S-selective ligand D-Pen$^2$-D-Pen$^5$-enkephalin (DPDPE) was ineffective in blocking cross-linking to any of the species.

Other investigators have analyzed opioid receptors labeled with ligands that directly attach themselves to the binding site. Newman and Barnard (1984) synthesized DALECK, an enkephalin derivative possessing a chloromethyl ketone group at its C-terminus. This ligand was capable of binding both reversibly and irreversibly to opioid receptors, depending on the pH. Reversible binding occurred to both µ- and δ--receptors, while the irreversible binding was highly specific for preceptors. Analysis of the irreversibly bound material on SDS gels indicated a single species, of molecular weight 58 kD.

Liu-Chen and Phillips (1987) labeled bovine striatal membranes with $^3$H-ß-funaltrexamine and showed that the irreversible portion of this binding was specific for µ-opioid receptors. SDS gel analysis indicated that the label was

associated with material in the molecular weight range 68 to 97 kD. The diffuse migration pattern was presumed to be due to the attachment of variable amounts of carbohydrate, for the labeled material was adsorbed by wheat germ lectin-Sepharose.

Yeung (1987) prepared a photoreactive enkephalin derivative, which was found to label a 46 kD protein in rat brain and spinal cord membranes, Photolabeling was inhibited by μ-, δ-, and κ-ligands, suggesting that the 46 kD band was a peptide shared by all three receptor types. Bochet and coworkers (1988) labeled rat brain and NG 108-15 cell membranes with the photoaffinity δ-ligand azido-DTLET. In rat brain, the ligand labeled two bands of 44 and 34 kD; while in NG cells, a 33 kD band was detected.

## TARGET ANALYSIS

Another approach to studying membrane receptors is target analysis. In this technique, the cell membrane is subjected to electron beam irradiation of various intensities, and the degree of inactivation of the receptor, as measured by ligand binding or some functional assay, is determined as a function of degree of inactivation. If one assumes that bombardment by a single electron is sufficient to inactivate the receptor, then the molecular size of the latter can be calculated from the irradiation vs. inactivation curve. This procedure has the advantage that molecular weight is determined *in* situ without the necessity of extracting the receptor from its membrane environment.

Ott and coworkers (1986) applied target analysis to opioid receptors in rat, guinea pig, and frog brain, as well as in NG 108-15 neuroblastoma-glioma hybrid cells. By determining binding to different ligands, they were able to estimate the molecular weights of μ-, δ-, and κ-opioid receptors. Furthermore, they also conducted assays in the presence or absence of $Na^{+}$, $Mg^{++}$, and GTP, which are required for coupling of receptors to G-proteins. They found that all three opioid receptor types had a molecular weight of 98 kD in the presence of ions and GTP and a molecular weight of 56 kD in their absence. The latter value is in close agreement with the 58 to 65 kD values for purified opioid receptors reported by several other groups (Newman and Barnard 1984; Gioannini et al. 1985). Moreover, the 44-kD increase observed in the presence of ions and GTP is consistent with association with the α-subunit of the inhibitory G-proteins $G_i(\alpha_i)$ which is approximately 40 kD in molecular weight.

On the other hand, other groups employing target analysis have estimated considerably higher values for opioid receptors. McLawhon and coworkers (1983) obtained a value of 200 kD for opioid receptors in NG 108-15 cells, using either $^{3}$H-DADLE or $^{3}$H-nalozone as ligand. Lai and coworkers (1984)

estimated a value of 110 kD for both μ- and B-receptors in rat brain. In another study applying target analysis to rat brain membranes, Tao and coworkers (1986) assayed opioid binding with $^3$H-diprenorphine, which binds to μ-, δ-, and κ-opioid receptors. In the presence of DADLE, NaCI, and Gpp(NH)p, the estimated size was 165 kD. In the presence of Gpp(NH)p, the size was 217 kD, while in the presence of $Mg^{++}$, it was 286 kD.

Tao and coworkers (1986) suggested that the 165 kD value obtained in the presence of NaCl or NaCl and Gpp(NH)p was the size of the uncoupled receptor. In the presence of DADLE, NaCI, and Gpp(NH)p, the receptor was coupled to a GTP-binding component of 96 kD (including not only the α-subunit, but also the ß- and y-subunits) and a $Na^+$ binding component of 52 kD. In the presence of Gpp(NH)p, it was coupled only to the $Na^+$ binding component; while in the presence of $Mg^{++}$, it was coupled only to the GTP-binding component.

The reason for the discrepancies in molecular weight values reported by these various groups is not entirely clear. However, as discussed above, many laboratories have reported that the molecular weight of opioid receptors is considerably greater in nondenaturing detergents than in denaturing ones, suggesting that *in situ* the ligand-binding subunit is associated with other components. Under the conditions used by three research groups (McLawhon et al. 1983; Lai et al. 1984; Tao et al. 1986), the receptor was apparently in this larger form: whereas another group (Ott et al. 1986) appeared to be observing just the binding subunit of the receptor, with or without association of just the a-subunit of $G_1$. Moreover, the study of Tao and coworkers (1986) suggests that opioid receptors may exist in several associated forms, with $Na^+$-binding component involved as well as GTP-binding component.

However, several studies (Lai et al. 1984; Tao et al. 1986; Ott et al. 1986) are consistent with the notion that, under a given set of conditions, different opioid receptor types are approximately the same molecular weight. Ott and coworkers (1986) found identical molecular weights for μ-, δ-, and κ-receptors, whereas Lai and coworkers (1984) reported identical molecular weights for μ- and b-receptors. While Tao and coworkers (1986) assayed opioid binding with $^3$H-diprenorphine, which should bind to all three receptor types, the plot of log inactivation against radiation dose was linear under all conditions, suggesting that the receptors being measured were a single size.

Because opioid receptor types can be inactivated selectively by covalent ligands under conditions in which some types are protected by irreversible ligands (James and Goldstein 1984), it often is concluded that they must represent distinct molecules. However, identical receptor molecules could behave differently if their ligand selectivity were determined by association with

different messenger systems and if, on any particular cell type, the receptor had access to only one type of second messenger system. Thus, one could postulate, for example, that δ-type opioid receptors result when the ligand-binding molecule associates with adenylate cyclase, while μ- and κ-opioid receptors result from association of the same binding molecule with other second messengers.

## ANTIBODIES

In addition to their usefulness in characterizing purified receptors, antibodies also can be used as a rapid and efficient means of purification when other approaches are not successful. To prepare them, it is necessary only to have a partially purified preparation of receptor; moreover, the receptor need not bind ligand. Antibodies can be made to denatured receptors, such as those identified by covalent labeling with a tritiated ligand and partially purified by SDS gel electrophoresis. Once such antibodies are available, they can be screened efficiently by their ability to inhibit opioid binding in vitro. In addition, because of their high specificity, antibodies can be prepared to a single opioid receptor type, if each type is a distinct molecule.

Several recent reports illustrate the power and flexibility of this approach in purifying opioid receptors. Simonds and coworkers (1985) covalently labeled δ-receptors on NG 108-15 neuroblastoma-glioma cells with tritiated FIT, which served as a marker to follow the receptors in subsequent purification steps. Then antibodies to protein-conjugated FIT were prepared and used to construct an affinity column. When detergent-solubilized hybrid cell membranes were applied to this column, the FIT-receptor complex was preferentially bound, thus effecting a major purification step. When this step was combined with an additional lectin affinity column elution, the receptor-FIT complex was purified essentially to homogeneity. It had a molecular weight of 58 kD.

A somewhat different immunological approach to opioid receptor characterization was introduced by Roy and coworkers (1988a). These investigators detected and isolated anti-idiotypic antibodies to ß-endorphin. Binding of the antibodies to brain membranes was inhibited by opioid ligands. In addition, the antiidiotype bound to a 60 kD peptide in Western immunoblots.

Several groups now have prepared antibodies to opioid receptor preparations that previously were purified in their laboratory. As discussed earlier, Bidlack and coworkers (1981) reported purification of an opioid receptor from Triton-solubilized rat brain membranes using affinity chromatography. Subsequently, this group prepared a monoclonal antibody (Mab) to this material, which proved to be directed against the 35 kD band observed on SDS gels. This Mab was

capable of partially inhibiting opioid binding to the solubilized preparation of Bidlack and Denton (1985) although long incubation periods were required. Moreover, Fab fragments prepared from the antibody rapidly and completely inhibited binding of μ- and δ- opioid ligands to brain membranes (Bidlack and O'Malley 1986).

Maneckjee and coworkers (1987) prepared a polyclonal antibody to the highly purified opioid-binding protein they had previously isolated from bovine striatum. This antibody inhibited selectively the binding of -opioids to rat brain membranes and also selectively precipitated a 94 kD band from detergent-solubilized striatal membranes.

Cho and colleagues (1986) and Schofield and coworkers (1989) recently purified to homogeneity an opioid-binding protein (OBCAM) from bovine brain and isolated the cDNA coding for this protein. Both monoclonal and polyclonal antibodies have been prepared to this protein, as well as polyclonal antibodies to peptides corresponding to portions of the predicted amino acid sequence of the cDNA (Roy et al. 1988b; Schofield et al. 1989). The monoclonal antibodies, or the Fab fragments derived from them, inhibit opioid binding to both the purified protein and to brain membranes, with binding of μ-, δ-, and κ-ligands all affected (Roy et al. 1988c). The polyclonal antibodies to the purified protein also inhibit opioid binding to brain membranes (Roy et al. 1988b).

Although OBCAM shows some selectivity toward p-alkaloid ligands, both the monoclonal Fab fragments and the polyclonal antibodies inhibited opioid binding to NG 108-15 cell membranes, which contain exclusively δ-opioid receptors, as well as to guinea pig cerebellum membranes, which are enriched in κ -opioid receptors, This suggests that the three major opioid receptor types in mammalian brain contain common antigenic epitopes. However, Western blot analysis revealed that the polyclonal antibodies interacted with a 63 kD band in guinea pig cerebellum and with a 39 kD band as well as a 58 kD band in NG 108-15 cells. In addition, a polyclonal antibody raised to a peptide corresponding to a portion of OBCAM's predicted amino acid sequence specifically adsorbed proteins of 39 and 58 kD from detergent-solubilized NG 108-15 cell membranes (S. Roy, unpublished data).

To determine whether either of the two bands recognized by polyclonal antibodies to OBCAM in NG 108-15 cells are involved in opioid receptor function, the cells were treated with 10 mM DADLE for 12 to 48 hours. This treatment is known to result in down-regulation, or decrease, of opioid receptors in these cells (Law et al. 1983). Under these conditions, the 39 kD band, but not the 58 kD band, was decreased in a time-dependent, nalozone-reversible fashion.

In view of the evidence discussed above, the result was somewhat surprising: A 58 kD protein binds opioids in NG 108-15 ceils. However, OBCAM is thought to contain carbohydrate and, in fact, its predicted amino acid sequence has a molecular weight of just 39 kD (Schofield et al. 1989). Thus, it is conceivable that the 39 kD protein isolated from NG 108-15 cell is identical in amino acid sequence to OBCAM but has little or no carbohydrate.

**GENE CLONING**

Another approach to purification of cell surface receptors is provided by gene cloning. An ultimate goal of most receptor studies is to clone the receptor in any case, but a particular advantage of this procedure is that it does not require a receptor that retains ligand bind activity; a denatured receptor, such as that obtained from covalent labeling, is sufficient. The amino acid sequence of a portion of the material is determined and, from this, the corresponding oligonucleotide probe (or probes) is synthesized. These probes then can be used to screen a cDNA library prepared from tissue rich in the receptor of interest. When the corresponding cDNA has been identified, it may be possible to transfect it into certain cell lines that can synthesize large quantities of it.

As discussed earlier, Cho and coworkers (1986) purified an opioid-binding protein from bovine brain that was selective for alkaloids. The cDNA coding for this opioid-binding protein then was cloned using standard procedures (Schofield et al. 1989). This sequence was determined and translated to give the amino acid sequence of the opioid-binding protein. The protein consists of 345 amino acids, with a calculated molecular weight of 37.9 kD. This discrepancy between this value and the molecular weight of the originally purified opioid-binding protein (58 kD) suggests that the protein might be glycosylated. This conclusion is supported by two other observations: (1) The opioid-binding protein binds to lectin affinity columns (Cho et al. 1986), and (2) there are six potential glycosylation sites in the protein's amino acid sequence, consisting of an aspartamine residue close to a serine or threonine (Schofield et al. 1989).

Although it has not yet been possible to show that transfection of the cDNA into cells lacking opioid receptors confers opioid-binding activity, other indirect evidence indicates the pharmacological relevance of this opioid-binding protein. An antibody to a portion of the predicted amino acid sequence was used to construct an affinity column. This column was capable of specifically binding protein from solubilized brain membranes that bound opioids in *in vitro* assays (Schofield et al. 1989). Moreover, a monoclonal antibody raised to the purified protein (Roy et al. 1988c) inhibited opioid binding to this affinity-purified material. These results indicate that the cloned sequence indeed codes for the purified opioid-binding protein,.

A search of the NBRF-PIR database revealed that this opioid-binding protein had significant homologies to several proteins, all of which are members of the immunoglobin superfamily (Schofield et al. 1989). This is a group of proteins characterized by repeating domains flanked by cystine residues (Williams 1987). The highest degree of homology was to two cell adhesion molecules, neural cell adhesion molecule (N-CAM) and myelin-associated glycoproteln; the sequences of both these proteins are about 20 percent homologous with that of the opioid-binding protein. Somewhat lower significance values, also suggestive of an evolutionary relationship, were found for several other adhesion molecules, neural adhesion molecule (L1), and fasciculin, as well as for receptors for interleukin-6 (IL-6) and platelet-derived growth factor.

Sequence analysis therefore is consistent with the notion that this protein functions as a neuropeptide receptor, while also suggesting that it could play a role in cell adhesion. Accordingly, it has been named OBCAM, or opioid-binding cell adhesion molecule. A possible role of this molecule in cell adhesion is also consistent with recent evidence that opioids can modulate cell-cell interactions of immune cells (Stefano et al. 1989).

We also have made an attempt to clone the δ-opioid receptor of NG 108-15 hybrid cells without beginning with purified receptor. Total mRNA was isolated from NG 108-15 cells and used to synthesize a "library" of cDNA, that is, DNA containing all of the genomic sequences found in NG 108-15 cells. This cDNA library was fractionated by size, then inserted by means of a plasmid vector into a glioma cell line that expresses no detectable opioid binding.

A key to the success of these studies was the construction of the subtraction probes, which were used to enrich or "prescreen" the initial colonies for opioid receptor cDNA. To make these probes, we took advantage of the fact that several treatments can selectively alter the number of opioid receptors in clonal cells. Prolonged treatment of NG 108-15 hybrid cells with opioid agonist, such as 100 nM DADLE for 24 hours, results in a decrease, or down-regulation, of as much as 80 percent in receptor number (Law et al. 1983). In addition, treatment of the PC cell subtype PC12h, which ordinarily contains undetectable levels of opioid receptors, with nerve growth factor for 14 days results in the appearance of high levels of δ-opioid receptors.

Accordingly, mRNA was prepared from these cells under both high receptor (normal NG 108-15 cells, or NGF-treated PC12h cells) and low receptor (down-regulated NG 108-15 hybrid cells or normal PC12h cells). The mRNA from the high-receptor cells was used to make cDNA's, which then were hybridized with nRNA from low-receptor cells. This hybridization in effect "subtracts" all the common mRNA from the high- and low-receptor conditions and, in theory, leaves cDNA highly enriched in opioid receptor DNA.

After such screening, the colonies that tested positively for both subtraction probes were screened for opioid binding. Several candidate cDNA's that bestowed both opioid binding and opioid-mediated adenylate cyclase inhibition on these cells are currently under investigation.

## SUMMARY AND CONCLUSIONS

Considerable progress has been made in recent years in understanding opioid receptors at the molecular level. Several laboratories have reported purification of opioid receptors by standard procedures involving solubilization of brain membranes in nondenaturing detergent, followed by affinity chromatography and other fractionation methods. In addition, several alternative approaches have been used with some degree of success. Several investigators have labeled receptors with covalent ligands, then characterized them under denaturing conditions such as SDS gel electrophoresis. Antibodies have been prepared to several pure or partially pure opioid receptor preparations that can be used to (1) map the brain regional distribution of opioid receptors, (2) determine the role of specific regions of the receptor molecules in ligand binding and in interaction with other functional molecules, (3) compare the structural features of different opioid receptor types, and (4) purify receptors further. One group also has reported cloning of the cDNA for an opioid-binding protein using antibodies as affinity ligands to purify receptors from solubiiized material and the application of gene cloning techniques.

The successful use of these varied approaches ensures that future progress in this field will be rapid. The purification, reconstitution, and detailed structural characterization of different opioid receptor types appears feasible within a few years, The next challenge in the molecular understanding of these receptors will be to identify and characterize the second messenger systems in the cell membranes that mediate their pharmacological functions.

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## ACKNOWLEDGMENT

The authors were supported by National Institute on Drug Abuse grants DA-00564 and DA-01583 and Research Scientist Award 5-K05-DA-70554.

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# An Approach to the Molecular Biology of Opiate Tolerance: Identification of Opiate-Regulated Transcripts

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## INTRODUCTION

Over the past two decades, much has been learned about cellular adaptation to opiate exposure. Refinement of receptor-binding methodologies and development of highly specific receptor ligands, characterization of opiate-responsive cell lines as model systems, and elucidation of receptor-coupled second messenger systems have greatly facilitated progress in understanding the function and adaptation of opiate receptor-mediated signal transduction. Most current knowledge, however, is restricted to understanding adaptation events that occur at proximal stages in the signal transduction pathway (Clark 1986; Law et al. 1983). These events include opiate-induced changes in number (down-regulation) and affinity of opiate receptors and, at a slightly distal level, opiate-induced changes in coupling of receptor to second messenger system (desensitization). Beyond these stages, little is known about opiate receptor-mediated signal transduction pathways or their adaptation to chronic stimulation.

In some systems, it has become apparent that later events in cellular response to external stimuli involve selective modulation of gene expression (Chang and Bourne 1987). Although such modulation has not been described in opiate receptor-mediated systems, clues that it may occur include observations that the reversal of opiate-induced down-regulation requires new protein synthesis (Law et al. 1985) and that opiates influence the growth properties of certain cells (Berry and Haynes 1989). We have begun to test the hypothesis that cellular adaptation to opiate exposure involves regulation of gene expression by the application of molecular biologic techniques to two model systems, the NG108-15 cell line and the rat brain. This chapter summarizes briefly the current status of our emerging appreciation of opiate-dependent genetic regulation, derived primarily from study of NG108 cells. The data reviewed in this chapter (1) establish that opiate-dependent modulation of gene expression

does occur and identify several classes of opiate-regulated transcript (ORT), (2) indicate that this modulation is limited to a small subset of genes expressed in the cell, and (3) suggest that opiate-dependent modulation of gene expression may be functionally important to the development of the opiate-tolerant phenotype. Following this review, our current focus is described, which is to explore the occurrence of ORTs in rat brain. In view of the structural complexity of brain, a major consideration in these efforts is the development of methodologies that are capable of detecting gene regulation in specified brain regions and from limited amounts of tissue.

## IDENTIFICATION OF OPIATE-RELATED TRANSCRIPTS IN NG108-15 CELLS

The NG108-15 neuroblastoma-glioma cell line expresses delta-subtype opiate receptor coupled to adenylate cyclase via $G_1$—the GTP-binding protein with inhibitory effect on adenylate cyclase-and to calcium channels, probably via $G_0$—the GTP-binding protein present in large amounts in brain and currently thought to be involved in regulating ion channels (Simonds 1988: Hescheler et al. 1987). This cell line has been characterized extensively and used by a variety of investigators to study opiate-dependent modulation of receptor number and affinity as well as desensitization of opiate-dependent inhibition of adenylate cyclase activity (Law et al. 1983). We have pursued two general approaches to explore opiate-modulated gene regulation in this cell line. First, regulation of specific genes 'was tested directly using oligonucleotide probes complementary to specific "candidate" genes. Second, we searched more generally for regulated transcripts by use of differential screening and subtractive hybridization.

The candidate gene approach was taken with various G protein alpha-subunits (for more detail, see Evans et al. 1988; vonZastrow et al., in preparation). The choice of these molecules was motivated by several considerations, including the pivotal role played by G proteins in mediating signal transduction, the availability of extensive nucleotide sequence information, and the indirect evidence that opiate tolerance may involve alteration of relative activities of $G_5$ and $G_1$, GTP-binding proteins with opposing effects on adenylate cyclase activity (see below). No opiate-dependent change in $G_{1\ alpha}$ mRNA level was noted, but surprisingly, an increase in $G_{5\ alpha}$ mRNA level of approximately 30 to 50 percent was induced by opiate agonist (morphine or etorphine). This increase was slow in onset (detected only after hours of opiate exposure), was blocked by naloxone, and was reversible after withdrawal of opiate agonist (figure 1). Attempts to measure changes in $G_{0\ alpha}$ levels have been unsuccessful due to very low levels of hybridization signal detected with oligonucleotide probe. Reprobing Northern blots with actin cDNA revealed no

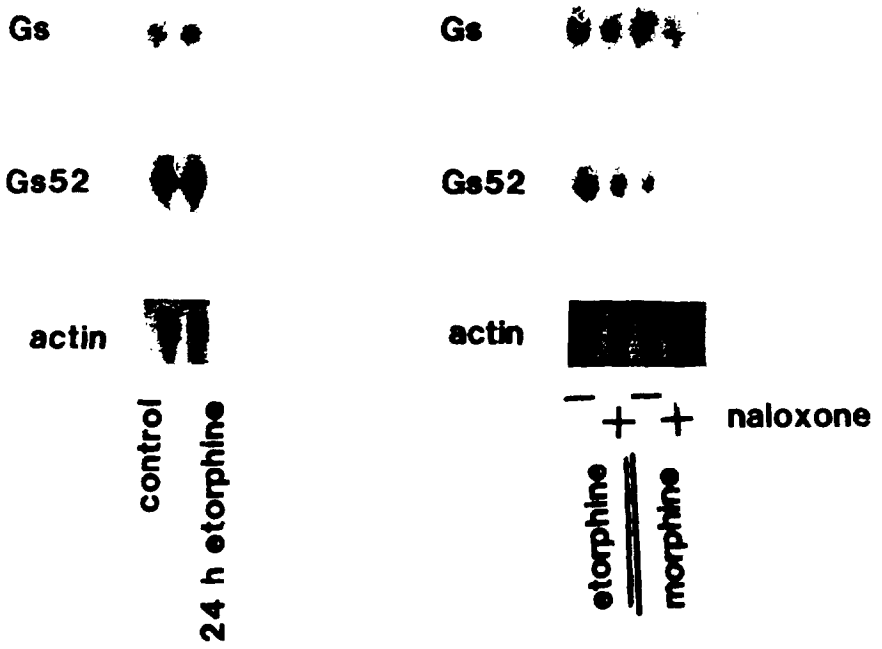


**FIGURE 1.** *Modulation of $G_{s\ alpha}$ mRNA level by opiate agonists*

NOTE: Total RNA, prepared by CsCl density centrifugation from NG1O8-15 cells treated for 24 hours in the presence or absence of opiate drugs, was electrophoresed on 1.2 percent agarose/formaldehyde gels and transferred to nitrocellulose. Transfers were probed with oligonucleotide labeled at the 5'-terminus by gamma-$^{32}$P-ATP catalyzed by polynucleotide kinase (Maniatis et al. 1982). Two $G_{s\ alpha}$ probes were designed, one 42-mer ($G_s$) that is homologous to regions conserved among all murine $G_{s\ alpha}$ sequences reported to date and one ($G_{s52}$) that is homologous to the 42 nt sequence unique to the mRNA encoding the M, 52 k variant cloned from bovine and murine tissues. Blots were stripped and probed sequentially with $G_s$ and $G_{s52}$ oligonucleotides, followed by beta-actin cDNA (cDNA provided by L. Kedes) labeled by random priming with alpha-$^{32}$P-dCTP (Maniatis et al. 1982). The left panel shows increased $G_{s\ alpha}$ signals in response to 24 hours of treatment with 25 nM etorphine. Actin reprobing indicates no regulation of this mRNA, which verifies equivalent loading of the gel lanes transferred. The right panel shows that addition of 100 µM naloxone blocked the increase in $G_{s\ alpha}$ mRNA level induced by either 25 µM etorphine or 25 µM morphine.

detectable opiate-dependent change in actin mRNA level. These data show that prolonged opiate exposure causes a selective and moderate increase in $G_{s\ alpha}$ mRNA level. Therefore, $G_{s\ alpha}$ represents an ORT. The functional importance of this regulation has not been established, but it is intriguing to note that previous observation of "sensitization" of $G_s$-stimulated adenylate cyclase activity during opiate withdrawal suggests differential regulation of G protein activity levels (Griffin et al. 1985), consistent with the present findings of modulation of $G_{s\ alpha}$ mRNA levels.

To approach the problem of identifying ORTs more generally, we have used conventional techniques of "+/-" screening of a cDNA library constructed in lambda-zap II vector from NG108 poly $A^+$ RNA. Alpha-$^{32}$P-labeled cDNA, synthesized from poly A+ RNA purified from control NG108 cells and from etorphine-treated NG108 cells, was then used to screen replicate plaque lifts. Following autoradiography of washed filters, visual comparison of 200,000 plaques screened with both probes indicated 15 plaques whose hybridization intensities were substantially (approximately fivefold) different between the two (control and etorphine-treated) screens. Ten of these "positives" were persistent on second-round screening: Eight showed increased hybridization signal when screened with control cDNA, and two showed increased hybridization signal with etorphine cDNA. The limitations of this technique, with regard to both abundance level detectable and extent of change in abundance necessary to score a definitive "positive," are well known, so the present finding of 10 regulated clones should be taken as a lower estimate. We have not yet fully purified or characterized these opiate-regulated clones; however, it is clear (1) that they are distinct from $G_{s\ alpha}$ according to relative change in hybridization and abundance level in the labeled cDNA preparation and (2) that they represent at least two classes of ORT: those that increase and those that decrease in abundance following opiate exposure.

Because of the limited sensitivity inherent in +/- library screening, we have searched for the less abundant ORTs by subtractive hybridization techniques. Single-stranded cDNA probe, purified from hybridization reactions by hydroxylapatite chromatography, has been used to screen for regulated species. First-round screening of the NG108 phage library indicates numerous positives, but we have not as yet confirmed or purified these clones.

In summary, current results of candidate gene screening and +/- library screening tentatively identify several classes of ORT according to relative abundance level, magnitude, and direction (increase or decrease) of opiate-induced abundance change. While these findings establish clearly the existence of ORTs in NG108-15 cells, the number of ORTs identified may

significantly underestimate the number expressed in the cell due to the limited sensitivity of our methods. Subtractive hybridization may allow the identification of additional ORTs expressed in lower abundance.

## IDENTIFICATION OF OPIATE-REGULATED TRANSCRIPTS IN RAT BRAIN

The NG108 cell line provides a convenient model system for the examination of cellular adaptation to opiates, but our ultimate goal is to understand the molecular biology of opiate tolerance in the intact nervous system. In addition to allowing us to identify ORTs that are regulated in neurons analogously to those in NG108 cells, studying the intact nervous system allows the exploration of gene regulation events that may require interconnected systems of neurons. Toward this goal, we have invested considerable effort in developing tools for the identification of ORTs in rat brain.

A principal limitation associated with studying transcripts in brain results from the tremendous cellular heterogeneity within the central nervous system, making it difficult to examine restricted cell populations independently. In addition, the small amount of tissue available within limited brain regions of interest presents another technical barrier. To approach these limitations, two techniques have been developed in our laboratory over the past several years. First, *in situ* transcription (IST) allows synthesis of cDNA from limited brain regions isolated on a tissue section (Tecott et al. 1988). Second, a novel RNA amplification method allows the synthesis of a population of amplified, antisense RNA (aRNA) that represents a broad population of cDNA molecules in increased copy number (Van Gelder et al. 1990). Furthermore, the aRNA produced is antisense relative to the starting RNA population used to generate the intermediate cDNA. Hence, the aRNA can be used directly as a probe without prior cloning. By using aRNA synthesis in combination with ET, we hope to examine gene expression in restricted brain regions, obtaining enough nucleic acid to be used for subsequent probing and cloning operations. Analogous to our approach to ORTs in NG108 cells, we intend to examine regulation of candidate transcripts and to search for unknown transcripts by differential screening or subtractive methodologies.

Candidate transcripts include $G_{s\ alpha}$ as well as the other ORTs identified in NG108 cells. So far, we have applied the IST-aRNA technology to rat brain sections and have been able to obtain detectable hybridization to $G_{s\ alpha}$, actin, and cyclophillin sequences using aRNA synthesized from several IST sections. However, the hybridization signal detected remains quite low: therefore, it is not clear whether the technique is yet able to detect, with sufficient reliability, regulation of low-abundance mRNA's. We anticipate that further optimization will allow reliable detection of these species and that the technique is currently

able to detect transcripts expressed in somewhat higher abundance, such as those identified in NG108 cells by differential screening.

We have begun to pursue the identification of unknown ORTs in rat brain by using IST-aRNA for +/- library screening. Brain sections prepared from either control (placebo-treated) or opiate (morphine-treated) male Sprague-Dawley rats were used for cDNA synthesis by IST (figure 2). Unlabeled cDNA, pooled from five tissue sections, was used for synthesis of alpha-$^{32}$P-labeled aRNA (figure 3). This aRNA, when used to probe replicate lifts of the NG108 cDNA library, hybridized specifically to a number of clones; first-round screening indicates multiple positives that are detected in either higher or lower amount with IST-aRNA synthesized from brain sections of morphine-treated relative to placebo-treated animals (figure 4); confirmation and purification of these putative ORTs from rat brain is in progress.

**FIGURE 2.** *IST of rat brain sections*

NOTE: Brain sections from placebo- or morphine-treated rats were subjected to IST with the $T_7$57 primer (Van Gelder et al. 1990) in the presence of alpha-$^{32}$P-dCTP (Tecott et al. 1988) to allow subsequent localization of transcribed cDNA by autoradiography. A representative autoradiograph is shown in the figure.

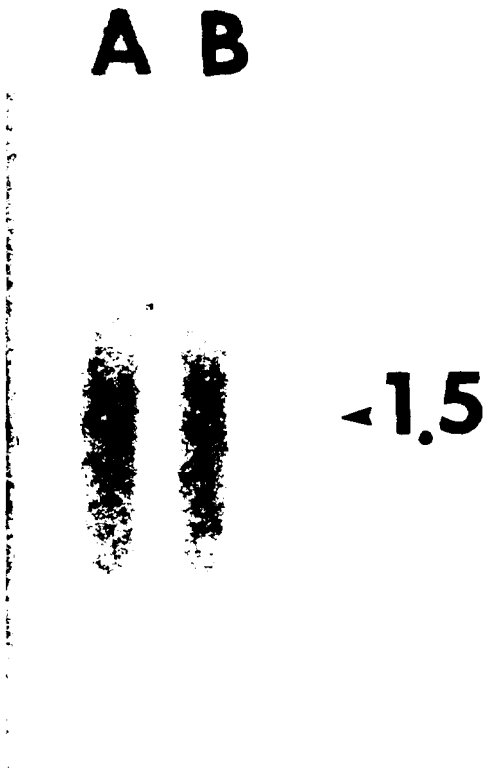


**FIGURE 3.** *Preparation of aRNA probe following IST*

NOTE: Brain sections from placebo- or morphine-treated rats were subjected to IST with $T_7$57 oligonucleotide primer but in the absence of radiolabeled nucleotide. cDNA was eluted from the tissue sections, second-strand synthesis was completed, and aRNA was transcribed with $T_7$ RNA polymerase in the presence of alpha-$^{32}$P-CTP to provide radiolabeled aRNA probe (Van Gelder et al. 1990). The figure shows electrophoretic analysis of aRNA produced (1.2 percent agarose/ formaldehyde gel electrophoresis followed by autoradiography).

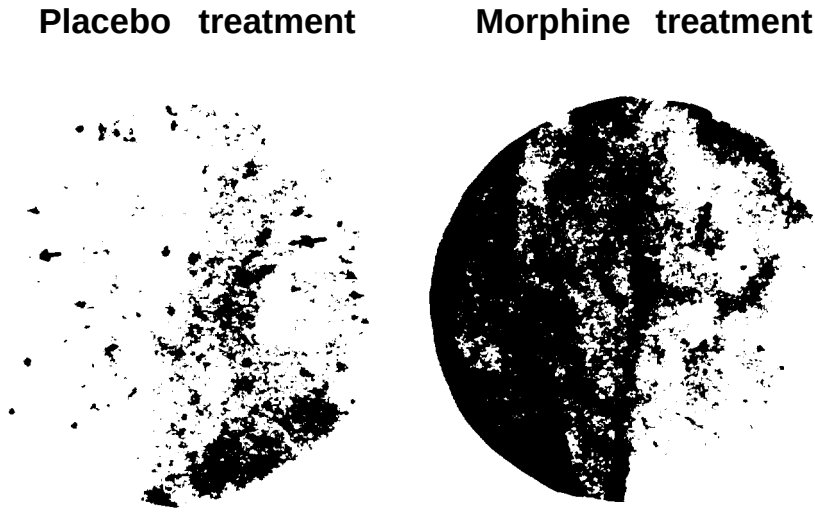


**FIGURE 4.** *Identification of putative ORTs by hybridization of IST-aRNA*

NOTE: aRNA produced following IST of brain sections from placebo- or morphine-treated brain sections was used to screen replicate plaque lifts of the NG108 cDNA library. Comparison of hybridization intensities of individual recombinants indicated putative ORTs that are represented in substantially different amount between IST-aRNA produced from brain sections subjected to the two treatment protocols. The left panel shows a representative hybridization of IST-aRNA from placebo brain sections to a nitrocellulose filter representing 10,000 recombinants. The right panel shows a replicate filter screened with IST-aRNA produced from morphine brain sections. Arrows point out recombinant plaques that differ substantially in hybridization intensity with the two probes and, therefore, represent putative ORTs.

## DISCUSSION

The results summarized in this chapter provide direct evidence that opiates cause modulation in the abundance of a limited subset of cellular genes, which we term ORTs. Several classes of ORT can be identified in NG108 cells according to apparent abundance, magnitude, and direction of opiate-induced variation. Preliminary results from subtractive hybridization experiments suggest that the number of ORTs expressed in NG108 cells may exceed that identified so far, due to the limited sensitivity of +/- library screening. By

combining IST and aRNA methodologies, we have begun to examine opiate-regulated gene expression in rat brain at the level of individual tissue sections, and initial results suggest the occurrence of multiple ORTs in brain. Further refinement should allow such analysis to be accomplished with increasingly limited brain regions, resulting in the identification and spatial resolution of ORTs involved in specific molecular biologic changes associated with the opiate-tolerant phenotype.

The functional roles played by various ORTs in opiate tolerance remain to be determined. In the case of $G_{s\ alpha}$, we suggest that its modulation may serve to balance signal transduction in response to opiate-induced perturbation of $G_1$-mediated pathways. In addition to the receptor-based events of down-regulation and desensitization, then, selective modulation of G protein abundance may contribute to the maintenance of cellular homeostasis. The interdependence of different G proteins in controlling cellular responses is well known; furthermore, $G_s$ may be expressed in limiting amounts (Gilman 1987)) thereby making the adjustment of its abundance a reasonable control point. This view of $G_s$ as a control point suggests that modulation of $G_{s\ alpha}$ may be involved in cellular adaptation to other perturbations. In this regard, it is interesting to note that ethanol, among multiple other cellular effects, causes a decrease in G,a,ph. mRNA level of similar magnitude to the increase observed in response to opiates (Mochly-Rosen et al. 1988; Charness et al. 1988).

By studying opiate-dependent modulation of gene expression, we aim to elucidate regulation events that occur at more distal stages of receptor-mediated signal transduction pathways. In this way, we hope to shed light on previously unknown aspects of opiate tolerance and addiction. In addition, by identifying control points shared by multiple signal transduction systems, we may be able to devise pharmacologic or even behavioral manipulations that will influence the development of drug tolerance, addiction, and withdrawal.

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## ACKNOWLEDGMENTS

Russel N. Van Gelder assisted in the preparation of aRNA probes. The research discussed in this chapter was supported by National Institute on Drug Abuse grant DA-0500 (JDB) and a Giannini Foundation Medical Research Fellowship (MvZ).

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# Prenatal Expression of Pro-Opiomelanocortin mRNA, POMC-Derived Peptides, and μ-Opiate Receptors in the Mouse Embryo

*Y. Peng Loh, R. Adrian Rius, Stela Eikabes, Wojciech Bern, and Carmine J. Coscia*

## INTRODUCTION

Pro-opiomelanocortin (POMC) is a $M_r$=30,000 prohormone that is synthesized primarily in brain and the anterior and intermediate lobes of the pituitary (Liotta and Krieger 1983). Small amounts are also present in adrenals, testes, ovaries, placenta, and pancreas (Liotta and Krieger 1983). This prohormone is processed differentially in brain and the two lobes of the pituitary to yield at least five biologically active peptides (figure 1). In the adult rodent brain, POMC is processed to des-acetyl-α-melanocyte stimulating hormone (MSH), ß-endorphrn,γ-lMSH, and N-$POMC_{1-49}$ (Emeson and Eipper 1986; Loh et al. 1980). Similar products are formed in the intermediate pituitary, except that ß-endorphin is acetylated and much of the α-MSH is diacetylated (Liotta and Krieger 1983; Rudman et al. 1979; Eipper et al. 1983; Zakarian and Smyth 1982). In contrast, in the anterior pituitary, N-$POMC_{1-74}$ adrenocorticotropin $(ACTH)_{1-39}$ and ß-lipotropin (LPH) are the major products, with a small amount of ß-endorphin also present (Seger and Bennett 1986; Eipper and Mains 1978). ß-Endorphin has been shown to have opioid-like activity (Loh et al. 1976) and γ-MSH and α-MSH have been demonstrated to have effects on the central nervous system (CNS) (van Ree et al. 1981; van Wimersma-Greidanus et al. 1981). ACTH stimulates steroidogenesis, which is potentiated by tryptic fragments of the N-terminal $M_r$=16,000 glycopeptide (Pederson and Brownie 1980). In addition to these effects studied in adult rodent tissue, recent data indicate that some of these peptides are mitogenic and have influences on cellular differentiation, suggesting that they may play a role in embryonic development. For example, administration of naloxone, an opiate antagonist, in infant rats resulted in an increase in cellular proliferation in germinal cell layers

in the CNS (Zagon and McLaughlin 1987). In sympathetic ganglia and spinal cord cells, ß-endorphin was shown to stimulate neurite outgrowth (Ilyinsky et al. 1987). More convincing is the *in vivo* inhibition of neonatal brain DNA synthesis by morphine (Kornblum et al. 1987a) and the inhibition of brain ornithine decarboxylase activity by intracisternally administered ß-endorphin (Bartolome et al. 1986). $ACTH_{1-39}$ has been shown to be a specific mitogen for fetal and adult mammalian myogenic cells; α-MSH increased the state of commitment of neural crest cells to melanogenic differentiation *in vitro* (Satoh and Ide 1987) and stimulated embryonic growth *in vivo* (Swaab and Martin 1981). $N\text{-}POMC_{1-49}$ has been shown to be a mitogen for adrenal cells in culture (Estivariz et al. 1988). Other effects of the POMC-derived peptides that may be relevant to embryogenesis and CNS development have been reviewed extensively elsewhere (Berry and Haynes 1989).

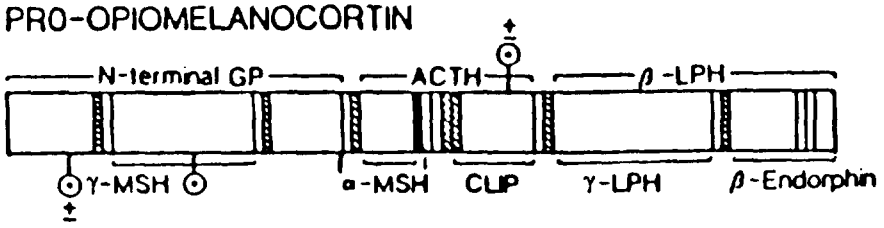


**FIGURE 1.** *Diagrammatic representation of mouse POMC. The pairs of basic amino acid residues (open bars=Arg; hatched bars=Lys; solid bars=Gly), which* are *cleaved to yield various peptide products, are indicated.*

Until recently, little was known about the distribution of POMC mRNA, the pattern of processing of the prohormone, and the subtypes of opioid receptors present during early embryonic development. Knowledge of the distribution of different POMC-derived peptide forms and their receptors in the embryo would facilitate in assaying for a functional role of these peptides in development. We have used *in situ* hybridization histochemistry and immunocytochemistry to map the first onset of expression of POMC mRNA and POMC in developing mouse embryos. The prenatal processing pattern of POMC in the brain and pituitary also has been determined. Finally, the embryonic expression of μ-opioid receptors, a receptor for ß-endorphin, was investigated to determine the time at which the POMC opioid system becomes functional during development. The characterization of opioid subtypes during prenatal development would facilitate the understanding of abnormal fetal development arising from maternal drug addiction.

**TEMPORAL-SPATIAL DISTRIBUTION OF POMC mRNA AND POMC IMMUNOREACTIVITY DURING FETAL DEVELOPMENT**

The temporal-spatial distribution of POMC mRNA was studied in the mouse embryo by in situ hybridization using a 48 mer POMC oligonucleotide probe directed at the **α-**MSH region of the POMC molecule as described previously (Elkabes et al. 1989). To determine if there is a lag time between the expression of the POMC mRNA and its translation, the distribution of POMC immunoreactivity was analyzed by immunocytochemistry using an ACTH antibody, $DP_4$, that cross-reacts with POMC and ACTH. Figure 2, panel b, shows the first detection of POMC mRNA at embryonic day 10.5 (E10.5) in the ventral diencephalon, in the region of the presumptive arcuate nucleus (figure 2, panel a). At this stage, POMC immunoreactivity also was detected in the same region, indicating that translation occurred soon after expression of the POMC mRNA (figure 2, panel c). At E11.5, there was a significant increase in the expression of POMC mRNA and POMC immunoreactivity in the region between the infundibulum and the optic recess in the ventral diencephalon (figure 3, panels a and b). On E12.5, the distribution, POMC mRNA content, and intensity of immunostaining of the POMC neurons in the presumptive arcuate nucleus were similar to that seen on E11.5 (data not shown). From E13.5 to El 4.5, POMC-containing neurons occupied a large area due to the rapid growth of the diencephalon/presumptive arcuate nucleus (figure 3, panels c and d).

POMC mRNA first was expressed in the pituitary gland on E12.5 in the embryonic area that corresponds to the pars distalis (anterior pituitary, figure 4, panel a). POMC immunoreactivity also was observed at E12.5 in the region furthest away from the infundibulum process (figure 4, panel b). On E13.5, the number of POMC-expressing cells increased (data not shown). By E14.5 there was a great increase in the number of heavily labeled and intensely immunostained cells located in the edges of the pars distalis, forming a narrow band, while other immunopositive cells extended toward the center of the lobe (figure 4, panels c and d). Also at E14.5, the first POMC-expressing cells were observed in the pars intermedia (intermediate lobe, figure 4, panels c and d).

lmmunostained POMC fibers were detected as early as E10.5. Axons arising from the ventral diencephalon extended toward the lateral surface of the diencephalon where they formed fine fiber tracts and continued toward the dorsal diencephalon. On E11.5, a dense network of thick fibers with varicosities coursed along the roof of the diencephalon and extended to the mesencephalon. On E12.5, these fibers descended into the dorsal metencephalon, and fiber bundles also were seen in the floor of the myencephalon. A considerable number of processes continued around the pontine flexure (figure 5). Growth-cone-like structures were seen at the end of

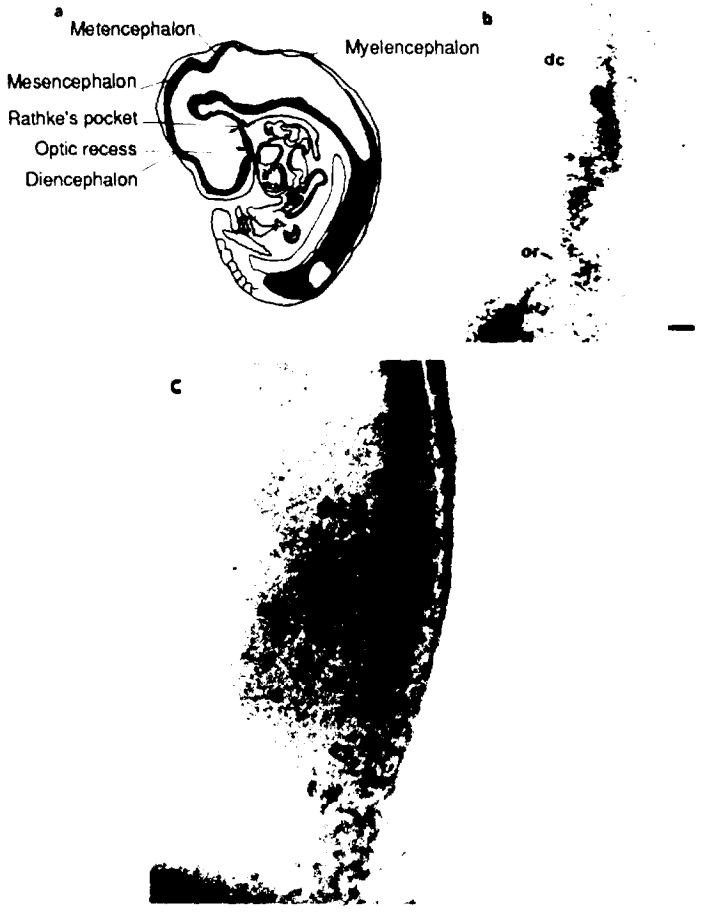


**FIGURE 2.** *Expression of POMC in the diencephalon at E10.5. (Panel a) Schematic diagram showing the region of the presumptive arcuate nucleus (arrows) where POMC-expressing cells are located. (Panel b) Low-magnification, bright-field photomicrograph of a sagittal section in the region containing the presumptive arcuate nucleus. This section was hybridized with a synthetic probe for POMC mRNA. Arrows point to some cells with autoradiographic silver grains (or=optic recess; dc=diocoel; bar=8 µm). (Panel c) Sagittal section of an E10.5 embryo immunostained for POMC/ACTH using an antiserum $DP_4$, which recognizes POMC and ACTH.*

SOURCE: Elkabes et al. 1989, copyright 1989, Elsevier Science Publishers


**FIGURE 3.** *Sagittal sections of 11.5 and 14.5 day-old embryos hybridized with a synthetic probe to POMC mRNA and immunostained for POMC/ACTH in the region containing the presumptive arcuate nucleus. (Panel a) Bright-field photomicrograph of a frozen section of an embryo at 11.5 hybridized with a $^{35}$S-labeled oligodeoxyribonucleotide POMC probe. (Panel b) 11.5 day-old embryo immunostained for POMC/ACTH. tmmunoreactive cells are clustered in the ventral diencephalon but also are present in the region close to the infundibulum. Note the increase in autoradiographic silver grains and immunostaining compared with E10.5. (Panels c and d) E14.5 showing adult-like pattern of distribution of oligonucleotide-labeled (Panel c) and immunoreactive cells (Panel d).*

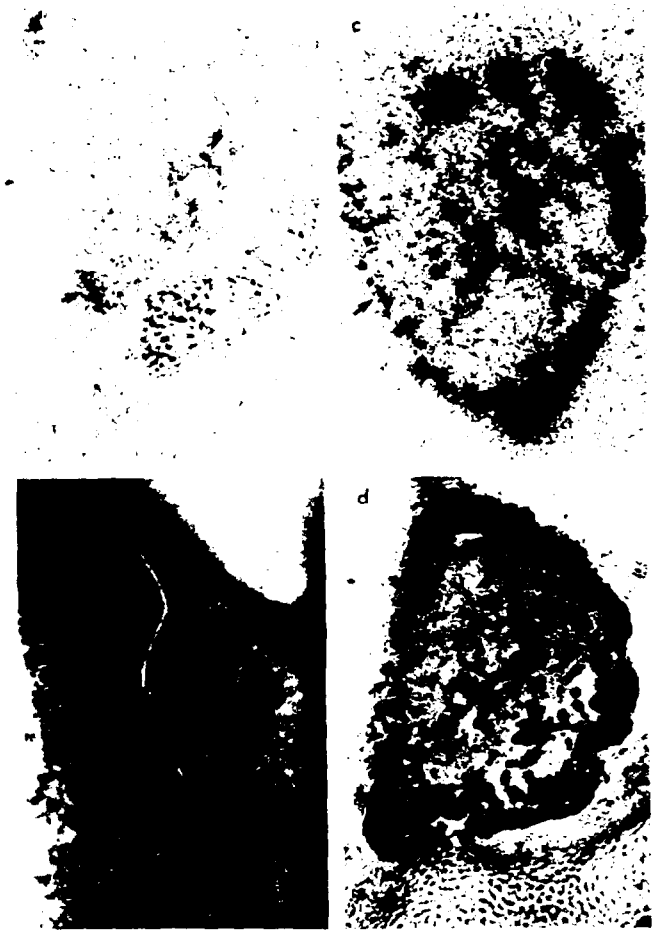


**FIGURE 4.** *Rathke's pouch of 12.5- and 14.5-day-old embryos hybridized with a $^{35}$S-labeled POMC probe and immunostained for POMC/ ACTH. Autoradiographic silver grains (Panel a arrows) and immunostaining (Panel b arrows) in the embryonic area corresponding to the pars distalis of the pituitary gland at E12.5. labeled (Panel c) and immunostained (Panel d) cells in the pars intermedia (black arrows) and distalis (white arrows) of a 14.5-day-old embryo. Note the increase in POMC-expressing cells in the pars distalis.*

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**FIGURE 5.** *POMC fibers with growth-cone-like structures (arrows) along the pontine flexure of a 12.5-day-old embryo*

these fibers. By E14.5, the fiber distribution and appearance changed to the adult-like pattern.

The prenatal expression of POMC mRNA also has been studied in the rat, but only in the pituitary gland (Lugo et al. 1989). In that study, it was reported that POMC mRNA expression first was detected in the ventral aspect of the Rathke's pouch at E13 and in the intermediate lobe at E15. This is consistent with our observations because there is a 1- to 2-day shorter gestation period in the mouse vs. the rat.

## PROCESSING OF POMC DURING FETAL DEVELOPMENT

Mouse embryos were analyzed for POMC and for processed products at El 0.5, When the POMC immunoreactivity first appeared. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis separation of embryo extracts from E10.5, followed by radioimmunoassay using the ACTH antibody $DP_4$, revealed the presence of POMC, but no ACTH processed products, at this stage of development (figure 6). However, 1 day later, at E11.5, only processed products were visible, and the POMC peak had disappeared (figure 6),

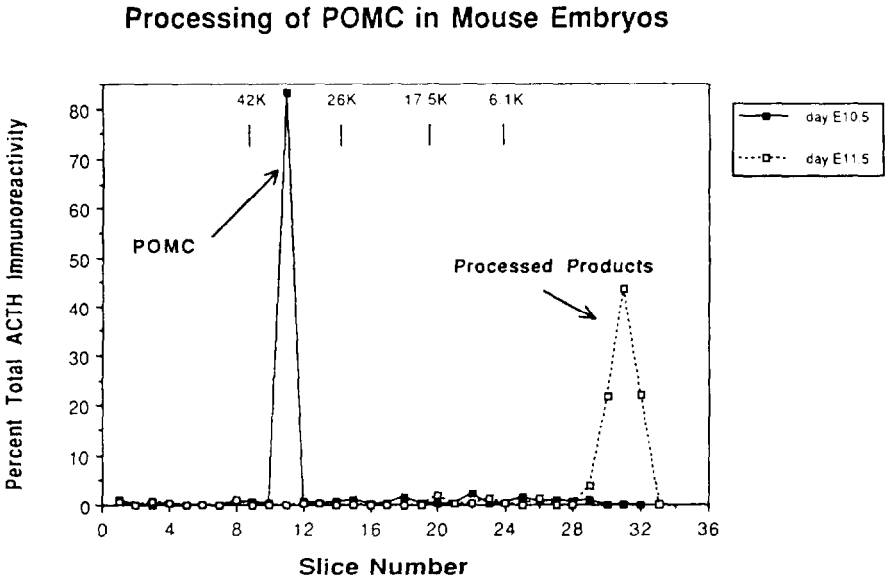


**FIGURE 6.** *SDS-polyacrylamide gel electrophoresis of 10.5 and 11.5-day-old embryo* extracts *followed by radioimmunoassay using an antibody* $DP_4$*,which recognizes POMC and* $ACTH_{1-39}$*, Only POMC, the first translated product,* was *seen at E10.5; posttranslational* cleavage *of the prohormone occurred at E11.5.*

SOURCE: Rius et al. 1991 a, copyright 1991, Elsevier Science Publishers

indicating that the processing machinery was activated at this point. Analysis of the processed products by HPLC and radioimmunoassay revealed the presence of two ACTH-related peptide forms: des-acetyl-α-MSH, which was the major form, and $ACTH_{1-39}$ as well as immunoreactive ß-endorphin (table 1) at both E11.5 and E12.5 (Rius et al. 1991a). At E14.5, there was approximately a fourfold Increase in $ACTH_{1-39}$ corresponding to the massive proliferation of anterior pituitary cells. Similarly, ß-endorphin also increased fourfold at E14.5 No acetylated -endorphin was observed. The presence of these peptides indicates that, during early development, the first POMC peptides that appear are the smaller forms (figure 1) characteristic of proteolytic processing in adult brain and intermediate pituitary. Moreover, although amldation appears to be an early event in development, giving rise to des-acetyl-α--MSH ($ACTH_{1-13}NH_2$) acetylation occurs later in development, primarily in the intermediate lobe (Leenders et al. 1986). Anterior pituitary POMC processing also undergoes a change, from producing ß-endorphin during early fetal development to

**TABLE 1.** *Developmental* expression of ACTH/MSH-related peptides and *ß-endorphin between E11.5 and 14.5*

| | fmoles/embryo | | |
|---|---|---|---|
| | Des-acetyl-α-MSH | $ACTH_{1-39}$ | ß-endorphin |
| E11.5 | 17.28±5.8 | 18.81±2.4 | 41.81±9.0 |
| E12.5 | 50.13±4.5 | 18.93±0.6 | 50.52±1.0 |
| E14.5 | 41.43±4.4 | 72.77±9.2 | 159.93±6.3 |

NOTE: Values shown are the mean±SEM of three experiments. Ten embryos were pooled for each determination at the different embryonic ages.

producing ±-LPH postnatally (Roberts et al. 1978). Similar results also have been obtained for anterior pituitary POMC processing in rat fetuses and neonates (Pintar and Lugo 1987; Sato and Mains 1985, 1987; Allen et al. 1984). In the rat, it also was observed that there was a greater production of des-acetyl-α-MSH in the fetal vs. adult anterior pituitary. The change in pattern of processing of POMC in the fetal anterior pituitary appears to be gradual and extends through postnatal development. Moreover, in the rat, the curtailment of processing of ß-LPH to ß-endorphin appears to lag behind the switch-over of processing from des-acetyl-α-MSH to $ACTH_{1-39}$. Studies on human anterior pituitary also indicate a greater production of des-acetyl-α-MSH in fetuses than in adults (Coates et al. 1989). The production of smaller, nonacetylated forms of peptides, such as des-acetyl-α-MSH and ß-endorphin in early embryos, suggests that these peptides may be important in embryogenesis and CNS development.

## ONTOGENY OF MAMMALIAN BRAIN OPIOID RECEPTORS

The ontogeny of opioid binding sites has been studied extensively in rat brain (Clendeninn et al. 1976; Coyle and Pert 1976; Garcin and Coyle 1976). Antagonists ($^3$H-naltrexone and $^3$H-naloxone) were the first radioligands used to determine specific binding and binding parameter estimates in membranes. In the prenatal period, specific binding, regardless of whether expressed in relation to total brain or to protein, increased with age. Although the absolute number of opioid binding sites increased until adulthood, their concentration, following a quiescent period a few days after birth, increased at a slower rate in the postnatal period than in the prenatal period. Similarly, Kirby (1981) found that $^3$H-diprenorphine binding to rat spinal cord opioid sites increased with prenatal and postnatal development but reached a maximal concentration at P15, which

is well before that in brain. Because diprenorphine binds to μ-, δ- and κ-receptors, it provides a better assessment of total opioid binding than the μ-selective antagonists, naloxone or naltrexone (Barg et al. 1989).

Receptor autoradiography permitted a refinement in anatomical detail of opioid binding in fetal and postnatal rat brain (Kent et al. 1982; Unnerstall et al. 1983; Edley and Herkenham 1984; van der Kooy 1984; Recht et al. 1985; Kornblum et al. 1987b). Although most embryonic brain regions demonstrated diffuse distributions of opioid receptors, in some (e.g., striatum and telencephalon) subsequent dense labeling was observed just before birth. In the postnatal period, the same dense regions reorganize to form a heterogeneous mosaic of patches. It is clear from the autoradiography that there are brain regions from which receptor labeling seems to disappear with time. In light of the difficulties in reproducing sequential sectioning and in quantitation of receptor autoradiography noted by Recht and colleagues (1985), the significance of this transient expression is difficult to assess. Nevertheless, it may be indicative of specific alterations of opioid receptor density and transient expression of opioid sites in brain regions such as cerebellum, which also has been detected using standard membrane binding assays (Tsang et al. 1982; Barg and Simantov 1989).

With the recognition of multiple opioid binding sites, differences in the developmental profiles were observed for high- and low-affinity naloxone (Koch et al. 1980; Tsang et al. 1982) and morphine (Pasternak et al. 1980) binding sites. More recently, the introduction of subtype-specific radioligands facilitated the examination of the differential prenatal and postnatal development of μ-, δ-, and κ- receptors (Wohltmann et al. 1982; Leslie et al. 1982; Spain et al. 1985; Tavani et al. 1985; Dunlop et al. 1986; Szijcs et al. 1986, 1987; Petrillo et al. 1987; McDowell and Kitchen 1986, 1987; Milligan et al. 1987; Barg et al. 1989; Barg and Simantov 1989; Magnan and Tiberi 1989). Studies in prenatal (rat), perinatal (human), and postnatal (rat and mouse) developing species revealed that brain p-sites were the first to appear, as early as El 5 (Barg et al. 1989); δ-sites were always the last to appear. In rat, δ- sites emerged about 5 days after birth in the forebrain (Sziics and Coscia 1990). Autoradiography with selective ligands revealed a myriad of different regional developmental patterns for opioid subtypes in postnatal rat brain, including frequent apparent transient expression (Kornblum et al. 1987b).

Although most of the rat studies were done during the postnatal period, we have focused on the early prenatal developmental expression of p-binding sites in fetal mouse brain, beginning from El 1.5 to E18, using the specific μ-radiolabeled ligand [$^3$H] D-Ala$^2$-Me-Phe$^4$-Gly-ol$^5$-enkephalin (DAMGE). μ-Sites first were detected at E12.5, a day after the processing of POMC had begun (figure 7). Subsequent to E12.5, ligand binding increased in parallel with

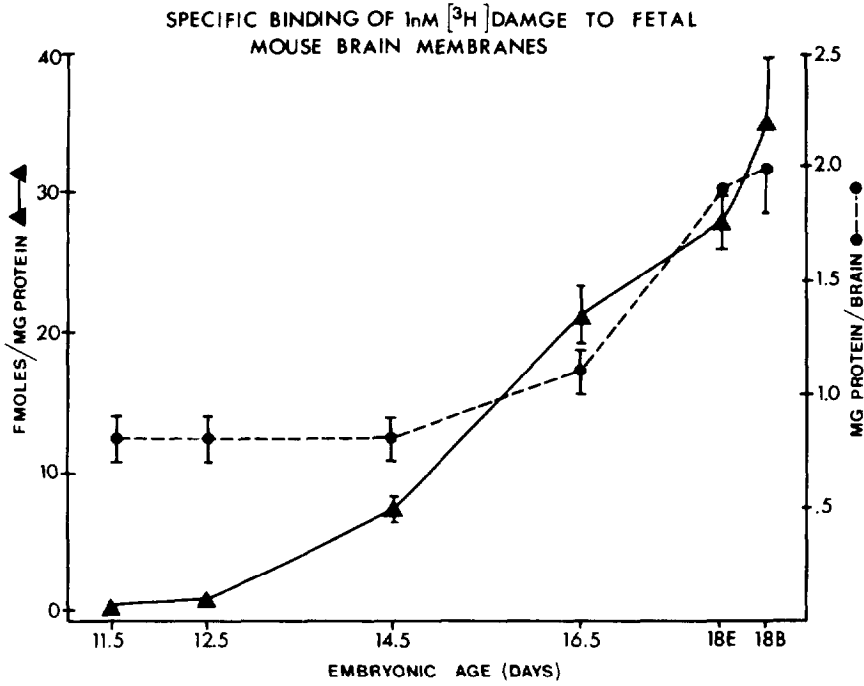


**FIGURE 7.** *Prenatal development of specific binding of [$^3$H]-DAMGE and membrane protein in mouse brain. Mouse brains of various embryonic age were pooled and used to prepare cell-free homogenates from which 20,000 g sediments* were *obtained. Binding assays were performed with these membranes in the presence of 1 nM [$^3$H]-DAMGE as previously described (Spain et al. 1985).*

Values are the means±SEM of three experiments carried out in duplicate.

the increase in protein (figure 7) and ß-endorphin content (table 1). The embryonic µ-binding sites at E14.5 have a Kd of 2.9±0.8 nM similar to that of the adult. Displacement studies with ß-endorphin showed that the [3H]DAMGE binding was displaced by this peptide, indicating that the embryonic µ-receptors present will bind ß-endorphin (Rius et al. 1991b). These findings indicate that by E12.5, the POMC system is fully functional, because the processed peptides and at least one of the receptors (µ-receptor) are present at this time.

## CONCLUSION

The findings reviewed in this chapter strongly support a role of the POMC-derived peptides in embryonic development. First, in the mouse, POMC mRNA and POMC-derived peptides are present by E11.5, and ß-endorphin receptors were detected in the CNS by E12.5, at the beginning of neurogenesis. In addition, adrenal blastemas are evident at El 1.5. Because N-$POMC_{1-49}$ is a potent mitogen for adrenal cells (Estivariz et al. 1988), the CNS source of this peptide may be involved in adrenal development and perhaps the organogenesis of other tissues as well. Second, analysis of the processing of POMC in early embryos reveals that the major peptides present were des-acetyl-α-MSH and ß-endorphin in several species. Acetylation of these peptides appears to occur primarily in the intermediate lobe during the perinatal period and continues postnatally (Leenders et al. 1986). In the anterior lobe, there was a change in the proteolytic cleavage of POMC from producing the embryonic peptides, des-acetyl-α-MSH and ß-endorphin, to the larger peptides, $ACTH_{1-39}$ and ß-LPH, further indicating that the smaller embryonic peptides may be functionally more important during embryogenesis. Finally, the prenatal appearance of opiate p-receptors before κ- (Rius et al. 1991b) and δ-receptors in the brain suggests that the POMC-ß-endorphin system is the opioid system likely to play a role in early developmental events. Future studies using transgenic mice with altered POMC *gene* expression, *as* well as *in vitro* assays for effects of POMC peptides on various embryonic tissue cultured cells, should shed light on the mechanisms of action of POMC peptides in fetal CNS development. Such knowledge will enhance the understanding of the pharmacopathology associated with fetuses born to mothers addicted to opiate drugs.

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## ACKNOWLEDGMENT

This work was supported in part by National Institutes of Health grant DA-05412 to CJC.

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# Regulation of ß-Endorphin Biosynthesis in the Brain: Different Effects of Morphine Pelleting and Repeated Stress

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## INTRODUCTION

In the past 15 years, there has been a tremendous growth in understanding the anatomy and biochemistry of endogenous opioid peptides, both in endocrine and in neural tissues. However, regulatory questions about peptide systems, particularly in the brain, have been difficult to address: thus, much less currently is known about this aspect of opioid peptide physiology. In fact, understanding Of the regulation Of peptide biosynthesis derives primarily from studies of pituitary tissue. An important regulatory principle learned from these studies is that biosynthetic activity usually is coupled fairly tightly to the rate of secretion. Thus, stimuli that cause the release of a peptide product from a cell generally induce biochemical mechanisms that lead to compensatory increases in peptide biosynthesis, whereas decreased peptide release causes biosynthesis to decline. Although there are obvious major differences between endocrine and neural systems, information about how peptide biosynthesis is regulated in the pituitary gland lays a groundwork for conceptualizing how these systems may be regulated in the brain. The usual strategy of regulatory studies in the brain has been to measure the possible changes in steady-state peptide concentrations following exposure to an experimental manipulation. It is now clear that all opioid peptides have a number of immunologically related forms, each with its own spectrum of biological properties. It is important to measure the concentrations of each of the biologically relevant peptide forms following an experimental treatment because alterations in total immunoreactive concentrations may not necessarily be related to behavioral or physiological changes. (Conversely, physiologically relevant changes may occur even though the total amount of immunoreactive peptides does not change.) Another point to consider when studying peptide regulation is that steady-state concentrations provide no information about the “turnover rate” of the peptide of interest. It is now clear that additional biochemical parameters are needed to

help discriminate whether alterations in peptide levels are due to changes in peptide release or biosynthesis. For example, a manipulation that causes peptide levels to rise may result from increased peptide biosynthesis induced by increased peptide release or may be due to an intracellular accumulation of peptide subsequent to reduced release. Now, thanks to recent technological advances in molecular biology that allow the measurement of low levels of specific mRNAs in discrete brain regions, and in conjunction with the knowledge gained from regulatory studies in the pituitary gland, it is feasible to begin to examine how peptide systems in the brain are regulated. This chapter describes the results of brain studies examining the potent endogenous opioid peptide, ß-endorphin (ßE). The goal of these studies was to determine some of the regulatory mechanisms employed by ßE-containing neurons in response to behavioral and pharmacological treatments that alter peptide release and biosynthesis.

## PRO-OPIOMELANOCORTIN STRUCTURE, ANATOMY, AND BIOSYNTHESIS

The biosynthesis of ßE begins with the transcription of the gene coding for the precursor protein pro-opiomelanocortin (POMC) (for a general review of peptide biosynthesis, see Sherman et al. 1989). Within the nucleus, introns of the primary transcript (or heteronuclear RNA) are spliced out, and mature mRNA is transported to the cytoplasm. Translation of the nucleic acid message into its corresponding amino acid sequence occurs as mRNA molecules interact with a ribosomal complex of RNA and protein molecules. The pre-POMC protein contains at its amino terminus a signal peptide sequence that is critical for directing the nascent protein toward the endoplasmic reticulum. The translation process is temporarily arrested as the newly synthesized POMC chain is translocated to the inside of the endoplasmic reticulum, Where the signal peptide is cleaved off. With the resumption of translation, synthesis of the precursor, including its glycosylation, is completed. Prohormone molecules then are shuttled to the Goigi apparatus and packaged into vesicles along with several enzymes important for precursor protein cleavage and processing. In addition to ßE, other biologically important peptides contained within the amino acid structure of POMC include adrenocorticotrophic hormone (ACTH); ß-lipotropin (ß-LPH); and α-, ß-, and γ-melanocyte-stimulating hormones. Peptides are liberated from the precursor molecule by enzymatic cleavage at pairs of dibasic amino acids (i.e., lysine and arginine), which are found at the amino- and the carboxy-termini of all these peptide products. That there are tissue-specific differences in the POMC-derived peptides produced in ceils indicates that enzymatic cleavage of the POMC precursor is not uniform across all tissues. However, because little is known about the precise nature of the enzyme(s) that cleave at the dibasic sites, it is not clear whether some tissues

lack the proper enzyme(s) for fully processing POMC or whether other factors are responsible for the tissue-specific distribution of POMC peptides. In addition to the trypsin-like enzymes that cleave POMC at paired dibasic sites, vesicles often contain other enzymes that can further trim (e.g., carboxypeptidases) or chemically modify (e.g., phosphorylate, acetylate, or amidate) specific peptide products. Post-translational modifications of POMC-derived peptides can dramatically alter the biological characteristics of the original peptide. For example, carboxy-terminal cleavage or N-terminal acetylation of $ßE_{1-31}$, abolishes its analgesic properties and greatly reduces its ability to displace opiate binding (Akil et al. 1961 a; Deakin et al. 1980). More dramatically, there is also evidence to suggest that $ßE_{1-27}$, is antagonistic to the opiate effects of $ßE_{1-31}$ (Bals-Kubik et al. 1988; Nicolas et al. 1964). Tissue--specific (and perhaps vesicle-specific) distribution of these cleaving and modifying enzymes may account for the different peptide products detected in different tissues. Following appropriate stimulation, the peptide contents of vesicles are released from the cell, usually in a $Ca^{2+}$-dependent manner. When considering the question of how POMC cells respond and adapt to incoming stlmuli, one should keep in mind that every step in the pathway from gene transcription through protein translation and processing to peptide release, in theory, may be regulatable. The potential to regulate discrete steps differentially in the biosynthetic pathway thereby provides cells with a rangeof possible responses when challenged.

Given the large number of possibilities for cleavage and posttranslational modifications, it is not surprising that the precise composition of POMC peptides synthesized in a cel is variable, depending on the tissue examined and on the physiological status of the cell (i.e., whether it is in a “resting” basal state or has been recently activated or inhibited). The highest concentrations of ßE-immunoreactive (ßE-ir) peptides are found in the pituitary gland. In the rat anterior pituitary gland (AP), ß-LPH and full-length ßE (i.e., ßE) are the-primary peptide products derived from the carboxy-terminal third of POMC. On the other hand, in the intermediate lobe of the pituitary (IP), virtually no ß-LPH is detected because it is all processed to $ßE_{1-31}$ most $ßE_{1-31}$ is then further cleaved at its carboxy terminus to produce $ßE_{1-27}$ or $BE_{1-26}$ in addition, the majority of all ßE-ir species in the IP is acetylated at the amino termini. In the central nervous system (CNS), all ßE-ir is derived from one of two clusters of POMC cell bodies. The major POMC cell group, situated in the arcuate nucleus of the hypothalamus, has axonal projections that terminate in a diverse number of forebrain and midbrain structures—including various hypothalamic nuclei, the periventricular thalamic nucleus, the periaqueductal gray (PAG) through the diencephalon and midbrain-and such limbic structures as the amygdala and septum (Bloom et al. 1978; Joseph 1980; Khachaturian et al. 1985; Watson et al. 1977). A much smaller and more diffuse cluster of POMC cell bodies has

been localized in the nucleus of the solitary tract (NTS) and nucleus commisuralis in the caudal medulla (Khachaturian et al. 1983; Schwartzberg and Nakane 1983). POMC fiber projections from the medullar cell group have not been definitively described. Although they do not appear to project extensively to rostral structures (Palkovits et al. 1987), it is possible that NTS and arcuate POMC neurons share some common terminal fields. In all rostral brain regions studied, $ßE_{1-31}$, is the major ßE-ir species found, with smaller amounts of $ßE_{1-27}$, and little or no ß-LPH present. In areas innervated primarily by the arcuate cell group (i.e., forebrain and midbrain structures), the great majority of BE-ir peptides is not acetylated, although a substantial degree of acetylation appears to occur in the NTS region (Dares et al. 1986).

## REGULATION OF POMC BIOSYNTHESIS IN THE PITUITARY GLAND

The best illustration of how POMC biosynthesis is regulated at multiple levels is provided by studies of pituitary tissue. A variety of stressors induce the release of ßE-ir peptides and ACTH from corticotrophs of the anterior pituitary. Results from pulse labeling studies indicate that one of the earliest cellular responses to the increased secretion of POMC products is an increase in the rate at which POMC precursor is translated and in the rate at which the precursor protein is converted into peptide products (Shiomi et al. 1986). Thus, Under conditions of acute stress, increases in POMC translation and processing allow pools of ßE-ir peptideS and ACTH to be replenished much faster than normal. If animals are stressed daily for an extended period (i.e., 1 to 2 weeks), changes in other POMC biochemical parameters are observed: There are small but reliable increases in POMC mRNA and larger (fourfold to fivefold) increases in ßE-ir concentrations in the AP (Shiomi et al. 1986). Thus, corticotroph cells adapt to chronic stimulation by increasing the amounts of stored peptide products as well as increasing their overall biosynthetic capacity by producing more POMC mRNA. Interestingly, rates of POMC translation and processing, which increase following acute stress, return to near normal levels in repeatedly stressed animals and do not change even when chronically stressed animals are acutely rechallenged. Increasing translation and processing rates apparently are only short-term mechanisms for increasing peptide products in anterior lobe corticotrophs and are no longer required once more long-term mechanisms are invoked (e.g., increased mRNA).

In addition to regulating discrete steps in the POMC biosynthetic pathway, there is also evidence that corticotrophs possess the capacity to differentially regulate the release of specific ßE-ir peptides. For example, whereas ß-LPH and $ßE_{1-31}$ are stored in the anterior pituitary gland in a ratio of 2:1, stress or a corticotropin-releasing hormone (CRH) challenge causes these peptides to be secreted into the plasma in a ratio of 1:2 (Watson et al. 1988; Young et al.

1986). This implies that $ßE_{1-31}$ is roughly fourfold more likely than ß-LPH to be released from corticotrophs. On the other hand, adrenalectomy, which removes steroid inhibition of POMC production in the AP and causes total ßE-ir in the plasma to increase dramatically, results in sixfold higher ratios of ß-LPH:ßE in plasma relative to normal levels (Young 1989). Thus, while ßE is preferentially secreted following acute stress, ß-LPH appears to be preferentially released following chronic activation by adrenalectomy. These data support the notion that which peptide products are released may depend on the length of time they remain in the vesicle, increasingly susceptible to further enzymatic modifications. For example, when secretion from corticotrophs is chronically stimulated by adrenalectomy, it is likely that POMC-derived peptides are stored in vesicles for shorter periods, and one sees a relative increase in the release of the less processed product, ß-LPH. At the present time, it is not known whether the mechanisms by which different peptide products from the same precursor are differentially released from the AP might be related to heterogeneity in corticotroph ceils, to intracellular differences in storage vesicles, or to other factors. Similar results to those described for the AP also have been observed in studies of the POMC-containing melanotrophs in the IP (Akil et al. 1985; Meador-Woodruff et al. 1990). Taken together, these results clearly illustrate the multiplicity and complexity of mechanisms that POMC cells in the pituitary gland use to regulate ßE biosynthesis and release in response to changes in cellular activiation. We were interested in whether some of the regulatory mechanisms found in pituitary POMC ceils also might be utilized by POMC cells in the brain. In the remainder of this chapter, we present data describing several changes in POMC biosynthesis that take place following exposure to either stress or morphine, treatments that we believe activate or inhibit, respectively, central ßE-containing cells.

## EFFECTS OF REPEATED STRESS ON POMC BIOSYNTHESIS IN BRAIN

The effects of stress on the release and regulation of POMC-derived peptides in the pituitary, both in the anterior and the intermediate lobes, have been characterized in detail (for example, see Akil et al. 1988). In addition to its well-known effects on the pituitary, there is also evidence that stress activates POMC systems in the brain. A variety of stressors (e.g,, footshock, cold-swim, learned helplessness) have been shown to elicit analgesia that appears to be at least partially opioid-mediated (Akil et al. 1976, 1984). That the endogenous opioid mediating this effect might be ßE is supported by data showing that hypothalamic levels of ßE-ir declined significantly following acute footshock stress (Akil et al. 1981b; Millan et al. 1981). More recent data also support the possibility that stress might affect hypothalamic POMC neurons in a manner similar to its effects on anterior lobe corticotrophs. CRH and arginine vasopressin, both of which are well established as mediating stress-induced

secretion of ßE and ACTH from the anterior lobe, recently have been shown to stimulate ßE-ir peptide release from the hypothalamus *in vivo* (Nikolarakis et al. 1988) and *In vitro* (Bronstein and Akil 1990; Burns et al. 1989; Sweep and Wiegant 1989). Therefore, it was reasonable to examine the effects of acute and repeated exposure to a stressor on POMC peptide and mRNA levels in the brain in an attempt to uncover some aspects of ßE biosynthetic regulation.

The stress paradigm involved the use of intermittent footshock (1.5 mA, 1 sec duration, every 5 sec) for a 30-minute period. Four different experimental groups were employed: (1) Control (Con)—animals were unhandled; (2) Acute Stress (AS)—animals received a single, 30-minute stress session immediately before sacrifice; (3) Chronic Stress/Rested (CS/R)animals were stressed for 30 minutes a day for 14 days and rested for 24 hours prior to sacrifice; (4) Chronic Stress/Acute Stress (CS/AS)—animals were stressed daily for 14 days and received a final 30-minute stress session on the 15th day immediately prior to sacrifice. The inclusion of the two chronic stress groups provided us with the opportunity not only to measure peptide and mRNA changes following repeated challenges but also to detect possible peptide effects of acute stress in chronically stressed animals, something difficult to do in previously unstressed animals. Two components of the arcuate nucleus POMC group were examined: (1) a hypothalamic region containing POMC cell bodies as well as ßE-ir nerve terminals and fibers of passage and (2) a midbrain region, including the PAG, which is rich in ßE-ir terminals derived primarily, if not exclusively, from the arcuate nucleus (Joseph 1980; Millan et al. 1984).

The effects of the different stress paradigms on ßE-ir peptide levels in the hypothalamus and midbrain are shown in table 1. In the hypothalamus, acute stress caused ßE-ir content to increase in both naive (AS) and repeatedly stressed (CS/AS) animals, whereas CS/R animals showed only a slight increase in hypothalamic peptide stores compared to unstressed controls. Although these data are consistent with our previous report on the effects of acute stress on total opiate-like factors in brain (Madden et al. 1977) and with increased occupancy of opioid receptors following acute stress (Christie et al. 1981), they do not agree with earlier results from this (Akil et al. 1981 b) and other (Millan et al. 1981) laboratories. Decreases in peptide content following acute manipulations generally have been interpreted to be the result of peptide release. It is difficult to understand how a stimulus that purportedly induces transmitter/hormone release could acutely cause tissue levels of that substance to increase. One explanation for the paradoxical acute increase in ßE-ir content in the hypothalamus may be related to this tissue containing both POMC cell bodies and nerve terminals. If POMC translation and processing in the arcuate nucleus increased following acute stress, as it does in the pituitary gland, and

ßE were degraded at a slow rate following release from nerve terminals, then a mixed cell body/nerve terminal brain region might be expected to exhibit increased ßE-ir content following acute activation,

**TABLE 1.** *Effects of acute and chronic stress on ßE-ir peptides in brain*

| | Con | AS | CS/R | CS/AS |
|---|---|---|---|---|
| Hypothalamus | 4.42±.28 (100%) | 5.61±.32 (127%) | 4.84±.25 (109%) | 5.34±.39 (120%) |
| Midbrain | 0.81±.08 (100%) | 0.99±.12 (122%) | 1.24±.21 (154%) | 0.87±.44 (108%) |

KEY: Con-animals were unhandled; AS-animals received 30 minutes of electric footshock immediately before sacrifice; CS/R—animals were stressed once a day for 14 days and sacrificed on the 15th day; CS/AS—identical to CS/R group except that animals received a final stress session immediately prior to sacrifice on the 15th day.

NOTE: Values represent the means±SEM of total ßE-ir (pmoles/tissue) measured in different brain regions. Numbers in parentheses represent ßE-ir values expressed as a percent of control levels.

More pronounced effects of stress on total ßE-ir content were observed in the midbrain (table 1). In naive animals, acute stress tended to increase ßE-ir levels in the midbrain, as it did in the hypothalamus, although this effect was not statistically significant. On the other hand, there was a dramatic decline in ßE-ir peptide levels immediately following acute stress of chronically stressed animals. Furthermore, in chronically stressed animals that were allowed to rest 24 hours before sacrifice, ßE-ir content in the midbrain increased by approximately 50 percent over control levels. The changes in midbrain ßE-ir content in chronically stressed animals are compatible with the hypothesis that stress stimulates release and biosynthesis in central ßE-containing neurons. Chromatographic analyses of midbrain extracts revealed two majorßE-ir peaks, corresponding to $ßE_{1\text{-}31}$ and $ßE_{1\text{-}27}$/$ßE_{1\text{-}26}$ (which could not be resolved from each other under these chromatographic conditions). These two ßE-ir forms were found in relative concentrations of 1:0.6 in control animals (figure 1). Acutely stressed animals showed no changes in the relative amounts of the different ßE-ir forms. In chronic stress/rested animals, however, the proportion of smaller size ßE-ir peptides was greatly increased such that the ratio of

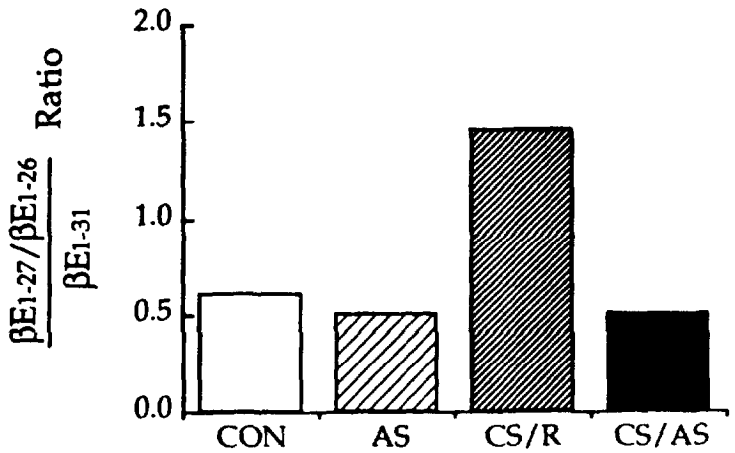


**FIGURE 1.** *Changes in the relative amounts of ßE$_{1-31}$/ß$_{1-26}$ and ßE$_{1-31}$* peptides *following* acute and *repeated* footshock **stress.** *ßE$_{1-27}$/ßE$_{1-26}$ concentrations are expressed* as *a ratio of the ßE$_{1-31}$ concentration. Pooled aliquots from the four experimental groups* (Con; AS; CS/R; and (CS/AS, see table 1 legend) were *chromatographed on a Sephadex G-50 column and collected fractions were assayed for ßE-ir. Total immunoreactivity was determined in the* peaks *that co-eluted with authentic ßE$_{1-31}$ and ßE$_{1-27}$ standards (since ßE$_{1-26}$ and ßE$_{1-27}$ could not be resolved on this column, the ßE$_{1-27}$ peak includes both of these peptides).*

ßE$_{1-31}$:ßE$_{1-27}$/ßE$_{1-26}$ was 1:15. The amounts of ßE$_{1-31}$ in control and chronically stressed/rested animals were comparable, indicating that the increase in total ßE-ir in the chronically stressed animal was due to a selective increase in carboxy-terminal cleaved products of ßE$_{1-31}$. In chronic/acute stress animals, the ßE$_{1-31}$:ßE/ßE$_{1-26}$ ratio returns to near normal levels (i.e., 1:0.5), suggesting that more processed ßE-ir forms may have been released more readily than full-length ßE$_{1-31}$ This implies that less opioid active material is liberated by a stress challenge in animals with a history of chronic stress compared to acutely stressed naive animals. This conclusion is supported by behavioral data showing that chronically stressed animals that are rechallenged acutely do not display the analgesic responses that are apparent in acutely stressed naive animals; that is, they have developed tolerance (Akil et al. 1976). Keeping in mind that ßE$_{1-27}$/ßE$_{1-26}$ are much less potent opioids than or may be antagonistic to ßE$_{1-31}$ (Akil et al. 1981a; Bals-Kubik et al. 1988; Deakin et al. 1980; Nicolas et al. 1984), the analgesic tolerance observed following repeated

stress may have resulted from a shift in the ßE-ir products released, with $ßE_{1-31}$ being released following the Initial stress exposure and $ßE_{1-27}$/$ßE_{1·26}$ being released following repeated stress exposures.

As mentioned earlier, one cannot determine *a priori* whether the chronic stress-induced increases in midbrain ßE-ir result from an increase or decrease in neural activity and secretion. Hence, a time course of the effects of repeated stress on POMC mRNA levels was carried out. Animals were stressed for 30 minutes a day for 0 (controls), 3, 5, 8, 15, or 22 days and were sacrificed immediately after the final stress session. Total hypothalamic RNA was extracted by a guanidium isothiocyanate/CsCl method, separated on 1.4 percent agarose gels, and transferred to nylon membranes. The membranes were hybridized overnight with a 420-base radiolabeled riboprobe complementary to a mouse POMC cDNA sequence (courtesy of Dr. James Roberts). Following treatment with RNase A (to digest unhybridired riboprobe), the membrane was washed, air dried, and exposed to x-ray film. As shown in figure 2, POMC mRNA levels were elevated following 3 to 5 days of stress but returned toward control levels after 8 to 22 days. Densitometric quantitation of the labeled bands revealed that the maximal effects at 3 and 5 days were 196 percent and 158 percent, respectively, of control values, suggesting elevated POMC biosynthetic capacity at these times. If one assumes that changes in brain levels of mRNA are linked to changes in secretion, as they are in the pituitary gland, then one may conclude that stress activates ßE-ir secretion from brain POMC cells. Interestingly, POMC mRNA levels return to control levels during the second and third weeks of stress exposure. Perhaps it is no longer necessary for cells to maintain elevated message levels once they accumulate sufficiently high peptide pools. Alternatively, animals may have adapted to the stressor with repeated exposure, making it progressively less stressful with subsequent exposures.

At this point, the regulatory responses of POMC neurons can be compared with those of corticotrophic and melanotrophic cells of the pituitary. Both types of POMC cells alter the rate and/or extent of POMC precursor processing to produce different mixtures of ßE-ir products under appropriate conditions. Following repeated activation, there are compensatory increases in ßE-ir peptide and POMC mRNA levels, thereby providing cells with a ready reserve of peptide transmitter and an increased biosynthetic capacity with which to respond to future challenges. There is also evidence that, under certain circumstances, endocrine and central POMC cells have the capacity to differentially regulate the release of the various ßE-ir peptides, in effect altering the precise signal emitted into the synapse or blood. Taken together, it seems fair to say that there are several similarities in POMC regulation in the brain and

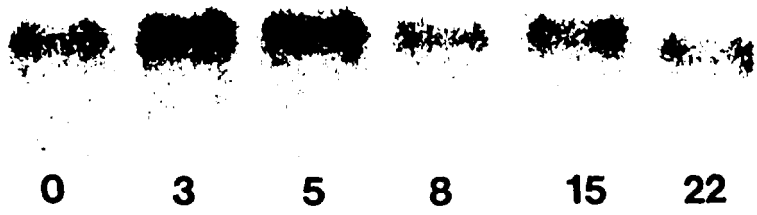


**FIGURE 2.** *Northern analysis of the effects of repeated stress on POMC mRNA in the hypothalamus. Animals were footshock stressed for 30 minutes* a *day for 3, 5, 8, 15, or 22 days (unstressed controls* are designated *by* 0). Total hypothalamic RNA was *separated* on *a* 1.5 *percent araose gel, transferred to a nylon membrane, and hybridized with a radiolabeled riboprobe complementary to a mouse POMC cDNA sequence. The membrane then* was treated *with RNase, washed, air dried, and exposed to x-ray film.*

pituitary gland following acute or repeated stimulation, although the onset and duration of these responses may differ widely. In the next section, we will examine some of the changes in POMC biosynthesis that occur when ßE neurons are chronically inhibited.

## EFFECTS OF MORPHINE TREATMENT ON POMC BIOSYNTHESIS IN THE BRAIN

One of the most obvious questions raised after the enkephalins were first discovered was whether the phenomenon of opiate tolerance and/or dependence might be related to alterations in endogenous opioid systems in the brain, Kosterlitz and Hughes (1975) hypothesized that chronic morphine treatment may result in agonist-induced feedback inhibition of endogenous opioid biosynthesis. However, to date, no changes in the steady-state levels of any of the opioid peptides have been detected following relatively short-term morphine treatments (3 to 10 days), which produce morphine tolerance and dependence (Berglund et al. 1989; Childers et al. 1977; Fratta et al. 1977; Hollt et al. 1978; Wesche et al. 1977). More recently, there have been conflicting reports about whether chronic morphine pelleting (5 to 7 days) affects POMC mRNA levels In the hypothalamus, with Costa and colleagues reporting morphine-induced decreases in POMC message (Mocchetti and Costa 1986; Mocchetti et al. 1989), while Hollt and coworkers (1989) found no effect. We reexamined the question of morphine's effects on POMC biosynthesis (Bronstein et al. 1990), attempting in these studies to obtain measures that might reflect the dynamics of ßE biosynthesis. Hence, in addition to steady

skate levels of total ßE-ir, concentrations of different ßE-ir species (i.e., ß-LPH, $ßE_{1-31}$ and $ßE_{1-27}$/$ßE_{1-28}$) in different brain regions as well as POMC mRNA levels in the arcuate nucleus were measured at several time points during the course of 7 days of morphine treatment.

Male Sprague-Dawley rats were implanted with morphine (75 mg/pellet) or placebo pellets (provided courtesy of the National Institute on Drug Abuse). Some animals received one pellet on day 1 and were killed 24 or 72 hours later. Other animals were implanted with one pellet on day 1 and three additional pellets on day 4 and were killed on day 7. As in the stress experiments, we examined a POMC cell body/nerve terminal region in the hypothalamus and an exclusively nerve terminal midbrain region, containing the PAG.

In accordance with previous reports, we found that 7 days of morphine treatment had no effect on ßE-ir concentrations in the hypothalamus or midbrain, Gel chromatographic analyses then were carried out, followed by radioimmunoassay to measure different size ßE-ir species. Although morphine had no effect on total ßE-ir, it was conceivable that it produced changes in POMC processing that may have altered the relative concentrations of the different ßE-ir species. Our results with repeated stress had revealed that tolerance to stress-induced analgesia was associated with a selective increase in $ßE_{1-27}$/$ßE_{1-26}$, peptides that have much less opiate activity than or may be antagonistic to $ßE_{1-31}$. It was attractive to consider the possibility that a similar mechanism might mediate the tolerance induced by chronic opiate administration. Chromatographic analyses indicated that the relative amounts of $ßE_{1-31}$ and $ßE_{1-27}$/$ßE_{1-26}$-size material in the hypothalamus or midbrain were similar in placebo- and morphine-pelleted animals; in addition, morphine-pelleted animals injected with naloxone (to produce precipitated withdrawal) showed no changes in their chromatographic ßE-ir profiles (table 2). These data, indicating that brain concentrations of different ßE-ir peptides are not altered by 7 days of morphine pelleting, suggest that changes in POMC processing may not play a significant role in mechanisms of opiate tolerance or dependence.

Although opiate treatment does not appear to affect steady-state concentrations of different ßE-ir peptides, there have been conflicting reports on whether this treatment alters POMC mRNA levels in the hypothalamus. Hence, we assessed the effects of morphine pelleting on POMC mRNA levels using the sensitive solution phase hybridization/RNase protection technique. Total nucleic acids were extracted from hypothalami in LET buffer (Lithium dodecyl sulfate, EDTA, Tris) and hybridized with a radiolabeled riboprobe complementary to a coding region of the POMC mRNA sequence. Nonhybridized single-stranded RNAs then were digested by RNase

**TABLE 2.** *Effect of morphine pelleting and precipitated withdrawal on ßE-ir peptides in brain*

| | Plac-Sal | Plac-Nal | Mor-Sal | Mor-Nal |
|---|---|---|---|---|
| Hypothalamus | | | | |
| $ßE_{1-31}$ | 4.41 | 4.65 | 4.69 | 4.70 |
| $ßE_{1-27}/ßE_{1-26}$ | 3.32 | 3.8 | 3.41 | 3.41 |
| Midbrain | | | | |
| $ßE_{1-31}$ | 3.39 | 3.11 | 2.75 | 3.26 |
| $ßE_{1-27}ßE_{1-26}$ | 1.35 | 1.34 | 1.14 | 1.16 |

KEY: Plac-Sal—animals were implanted with four placebo pellets over a 7-day period and received an injection of saline vehicle 30 minutes before sacrifice; Plac-Nal—placebo-pelleted animals received an injection of naloxone (2.5 mg/kg) 30 minutes before sacrifice; Mor-Sal-animals were implanted with four morphine pellets (75 mg) over a 7-day period and were injected with saline vehicle 30 minutes before sacrifice; Mor-Nal-morphine-pelleted animals received an injection of naloxone 30 minutes before sacrifice.

NOTE: Values represent total immunoreactivity associated with the $ßE_{1-31}$ and $ßE_{1-27}/ßE_{1-26}$ peaks of chromatographed pools of hypothalamus or midbrain.

treatment, leaving intact the double-stranded hybrids of POMC mRNA and complementary riboprobe. Protected hybrids were fractionated on polyacrylamide gels, visualized by autoradiography, and quantitated by densitometry. In an initial study, using a mouse POMC cRNA that was not completely homologous to the rat sequence, we found a significant 30- to 40-percent decrease in POMC mRNA levels following 7 days of morphine treatment. In a subsequent study, using the homologous rat POMC riboprobe (courtesy of Dr. Jim Eberwine), a decrease in POMC message also was observed following morphine treatment. However, the magnitude of the decrease (about 20 percent) was smaller than that observed in the initial study, and POMC mRNA levels in morphine- and placebo-treated animals in the second study were not statistically different, No changes in POMC mRNA levels were detected following 3 days of morphine pelleting. The reasons for obtaining a significant morphine-induced decrease in POMC message in one study but not in the other may be related to the use of different riboprobes or, perhaps, to our failure to control other factors consistently (e.g., stress, which

previously has been shown to affect central POMC systems). Although variable, these data tend to support those of Mochetti and coworkers (Mocchetti and Costa 1986; Mocchetti et al. 1989) and suggest that 7 days of morphine pelleting apparently causes an overall decrease in ßE biosynthetic capacity by reducing levels of POMC message.

If one extends the notion derived from studies in the pituitary to the brain, the observed decrease in POMC biosynthetic capacity would be the result of decreased ßE-ir secretion. We reasoned that if morphine does in fact inhibit ßE-ir release from nerve terminals, then at some time point during the 7-day morphine treatment we might be able to detect an accumulation of these peptides in tissue. Hence, we carried out a time-course study in which we compared animals implanted with morphine or placebo pellets for 1, 3, or 7 days. Morphine treatment had no effect on total ßE-ir in the hypothalamus or midbrain following 1 or 7 days of pelleting. However, 3 days after morphine pellet implantation, there was a significant increase in ßE-ir in the hypothalamus (figure 3). This result is consistent with the hypothesis that morphine inhibits the release of ßE-ir peptides, causing the gradual accumulation of these peptides intraneuronally. This conclusion also is supported by chromatographic analyses that revealed that the increase in ßE-ir at 3 days was primarily due to a selective increase in more processed ßE-ir forms (i.e., $ßE_{1\text{-}27}$/$ßE_{1\text{-}26}$) with little effect on the concentrations of full-length $ßE_{1\text{-}31}$ (Bronstein et al. 1990). It is generally believed that peptide maturation is a time-dependent process; thus, posttranslational modifications that occur later in processing (such as the COOH-terminal cleavage of $ßE_{1\text{-}31}$) may not proceed fully to completion (for example, see Akil et al. 1985). Hence, the elevated levels of $ßE_{1\text{-}27}$/$ßE_{1\text{-}26}$ are likely the result of increased storage time in granules, as a result of decreased release, leading to more complete processing. Given that opiate tolerance and dependence have developed following 3 days of morphine pelleting, it is tempting to speculate that the selective accumulation of carboxy-terminal-cleaved products of $ßE_{1\text{-}31}$ has some role in the development or expression of these phenomena (see discussion below).

The data described above illustrate the importance of measuring several parameters of biosynthetic activity at multiple time points to detect possible regulatory changes in peptide biosynthesis. If only ßE-ir peptide concentrations were measured, one would have concluded that opiate administration had no effect on POMC biosynthesis. By measuring POMC mRNA, peptide levels, and peptide forms at several different times during the morphine treatment, we observed changes in hypothalamic POMC neurons that have led us to suggest a sequence of cellular events that unfold as a result of chronic exposure to morphine. We propose that the initial effect of morphine is to reduce neural

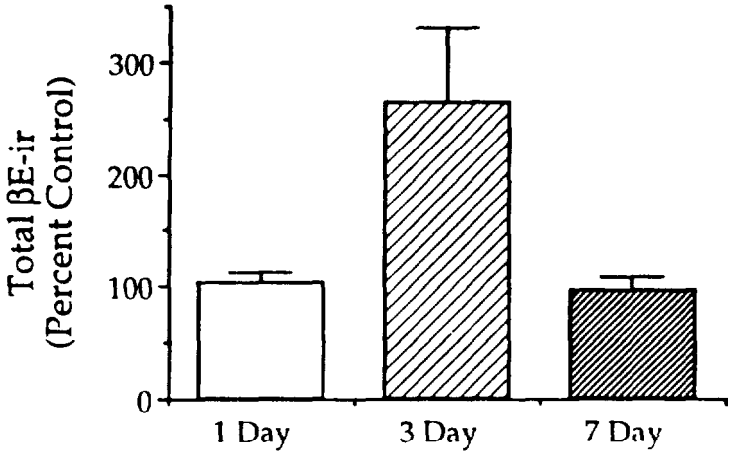


**FIGURE 3.** *Effect of chronic morphine pelleting on total ßE-ir in the hypothalamus. Rats* were *implanted* subcutaneously *with either placebo or morphine pellets for 1, 3, or 7 days, and total ßE-ir in hypothalamic extracts was determined* by *radioimmunoassay. Data represent the means±SEM of morphine-treated animals expressed as a percent of placebo control values.*

activity and ßE release from POMC neurons. During the initial period of morphine pelleting, POMC formation and processing in hypothalamic cells continue at a relatively normal rate despite the reduced rate of ßE-ir peptide release, thereby causing an eventual accumulation of ßE-ir after several days. As the peptides remain longer in secretory granules, they undergo the relatively slow process of conversion from $ßE_{1-31}$ to the opiate antagonist $ßE_{1-27}$. (This conversion step has been shown to be slow in the IP by Seizinger and Hollt [1980] and may be rate-limiting under some circumstances, such as stress [Akil et al. 1985]). This leads to the observed shift in ratio toward the $ßE_{1-27}/ßE_{1-26}$ forms following 3 days of pelleting. With continued exposure to the opiate, neurons exhibit a reduction in the amount of POMC message present, as cells apparently strive to reduce overall ßE-ir biosynthetic capacity. (The decrease in message levels could be due to either a decrease in the transcription rate of the POMC gene or to an increase in the degradative rate of its mRNA.) Presumably, homeostatic pressures would continue to reduce POMC mRNA levels until the rate of ßE biosynthesis once again matches the rate of ßE release. Thus, following 7 days of treatment, the new equilibrium between synthesis and secretion results in placebo- and morphine-treated animals

having similar steady-state peptide levels. Preliminary results from *in vitro* release studies, indicating that basal and stimulated release of ßE-ir were reduced in morphine-pelleted animals compared with placebo-pelleted controls, further support the hypothesis that morphine inhibits release from ßE-containing neurons (Bronstein and Akil, unpublished observation).

If morphine treatment did cause the sequelae of molecular events in POMC biosynthesis just described, it could have profound effects when the opiate is removed (i.e., withdrawal). At earlier stages of morphine treatment, when there is a relative increase in the levels of $ßE_{1\text{-}27}/ßE_{1\text{-}26}$, removal of the exogenous opiate would leave the organism with relatively lower levels of endogenous opiolds as well as increased amounts of opiate inactive or antagonistic materials. At later stages of morphine pelleting, opiate withdrawal still would result in decreased ß-endorphinergic tone, as overall biosynthesis and release both appear to be reduced. From these data, it is not unreasonable to suggest that the alterations in POMC biosynthesis, processing, and release that occur following chronic morphine treatment may play a role in mediating some of the effects of opiate withdrawal.

Obviously, the intricacies involved in the regulation of opioid peptide systems in the brain are just beginning to be understood. Although several techniques exist that permit the expansion of understanding of regulatory processes in the pituitary gland (e.g., pulse labeling, transcription run-on assays, heteronuclear RNA quantitation), there are some limitations on the kinds of regulatory questions that can be asked about neural systems since these systems are usually quantitatively (and qualitatively) more difficult than their endocrine counterparts. Several of these biochemical and molecular biological techniques need to be adapted to studying the CNS. In addition, the anatomical complexity of this tissue needs to be respected, along with the range of timeframes in which it operates (from nanoseconds to hours, days, and months). By studying cellular regulation-from gene transcription to secretion-in the context of known circuits and in conjunction with specific behavioral manifestations, we can begin to understand the intrinsic function of endogenous opioids and their modulation by drugs of abuse.

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## ACKNOWLEDGMENTS

This research was supported by a National Institute on Drug Abuse grant DA-02265 (HA), a National Institutes of Health-funded pilot project granted through the University of Michigan Gastrointestinal Peptide Research Center, and postdoctoral fellowships from the Medical Research Council of Canada and the National Institutes of Health training grant in Endocrinology, Metabolism, and Aging (DMB).

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# Transcriptional Regulation of the Rat Prodynorphin Gene

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## INTRODUCTION

cDNA cloning techniques have been used to characterize a variety of neuroendocrine peptide precursor molecules encoding small biologically active peptides that act as neurotransmitters and neuromodulators in the central nervous system (CNS) and hormones in the endocrine system (Douglass et al. 1984). One such precursor, prodynorphin, encodes the dynorphin family of opioid peptides (Kakidani et al. 1982; Horikawa et al. 1983; Civelli et al. 1985). The prodynorphin precursor contains three leucine-enkephalin moieties, with at least six additional biologically active opioids (Dyn A 1-8, Dyn A 1-17, α-neoendorphin, ß-reoendorphin, Dyn B 1-13, and Dyn B 1-28) representing carboxy-terminal extended forms of the leucine-enkephalin sequences. Northern blot and in situ histochemical techniques have been used to determine the rat tissues and cell types in which the prodynorphin gene is transcriptionally active (Civelli et al. 1985; Akil et al. 1984). Such regions include numerous brain structures (e.g., the hypothalamus, striatum, hippocampus, midbrain, brain stem, and cerebral cortex), as well as spinal cord neurons. In endocrine tissues, the prodynorphin gene is transcriptionally active in the anterior pituitary, adrenal gland, testis, ovary, and uterus (Civelli et al. 1985; Douglass et al. 1987). Numerous studies have suggested that the prodynorphin opioids serve to regulate a variety of physiological and behavioral responses such as pain perception, intestinal peristalsis, motor function, and reproductive function(s). A role for dynorphin peptides in narcotic tolerance mechanisms also has been suggested (Lee 1984).

Cloning of rat prodynorphin cDNA and genomic DNA (Civelli et al. 1985; Douglass et al. 1989) has afforded the opportunity to study regulated patterns of prodynorphin mRNA levels in specific neuronal cell types following surgical or pharmacological manipulations. Two such studies described here represent those in which the most dramatic alterations in prodynorphin mRNA levels have been observed to date. Proenkephalin mRNA levels, which also were

measured in these studies, exhibited regulatory profiles distinct from prodynorphin mRNA. The isolation and characterization of the rat prodynorphin gene has enabled us to begin dissecting the regions of genomic DNA involved in the regulated production of prodynorphin transcripts, Both transcriptional and posttranscriptional events may regulate the ultimate production of prodynorphin-derived peptides. In the testis, alternate processing of prodynorphin (and proenkephalin) precursor mRNA results in the generation of mature transcripts containing unique 5' untranslated regions, perhaps affecting the translational status of the mRNA. The characterization of the rat prodynorphin promoter region has allowed us to initiate prelude studies aimed at determining the cis-acting nucleotide sequence elements controlling the production of prodynorphin mRNA.

## *IN VIVO* REGULATION OF PRODYNORPHIN mRNA LEVELS

### Prodynorphln Gene Regulation In the Rat Spinal Cord

The prodynorphin gene is transcriptionally active in the mammalian spinal cord. *In situ* hybridization histochemistry/immunohistochemistry has localized the majority of rat spinal cord prodynorphin transcripts and peptides to laminae I-II and V-VI (Ruda et al. 1988). Other studies have localized proenkephalin-derived peptides to similar regions of the rat spinal cord (Basbaum and Fields 1984). The localization of both prodynorphin- and proenkephalin-derived opioid peptides to these specific regions of the spinal cord suggests that these peptides play a role in the endogenous pain recognition/control system. Numerous experimental paradigms have served to strengthen this belief (ladarola et al. 1988: Ruda et al. 1988; Millan et al. 1985, 1987).

In the study described here, prodynorphin mRNA and peptide (Dyn A 1-8) levels were measured in the rat lumbar spinal cord at various times following inflammation of the hindpaw (ladarola et al. 1988). An experimental model of unilateral inflammation of the hindlimb (induced by a single, intraplantar injection of Freund's adjuvant) produced hyperalgesia to both mechanical and radiant thermal stimulation, which was rapid in onset. During this period, prodynorphin biosynthesis was substantially elevated in the region of the spinal cord receiving sensory input from the affected limb (figure 1, panel A). Prodynorphin mRNA levels rose substantially within the first 24-hour period, and maximal stimulation (eightfold to ninefold increase) was observed between 3 to 5 days after injection. By day 14, prodynorphin mRNA levels approached control values. This time course of induction and subsequent decline closely paralleled that of hindpaw edema and hyperalgesia. Spinal cord Dyn A 1-8 levels also rose approximately threefold during the inflammatory period. This pattern is consistent with an increase in both the rate of synthesis and the

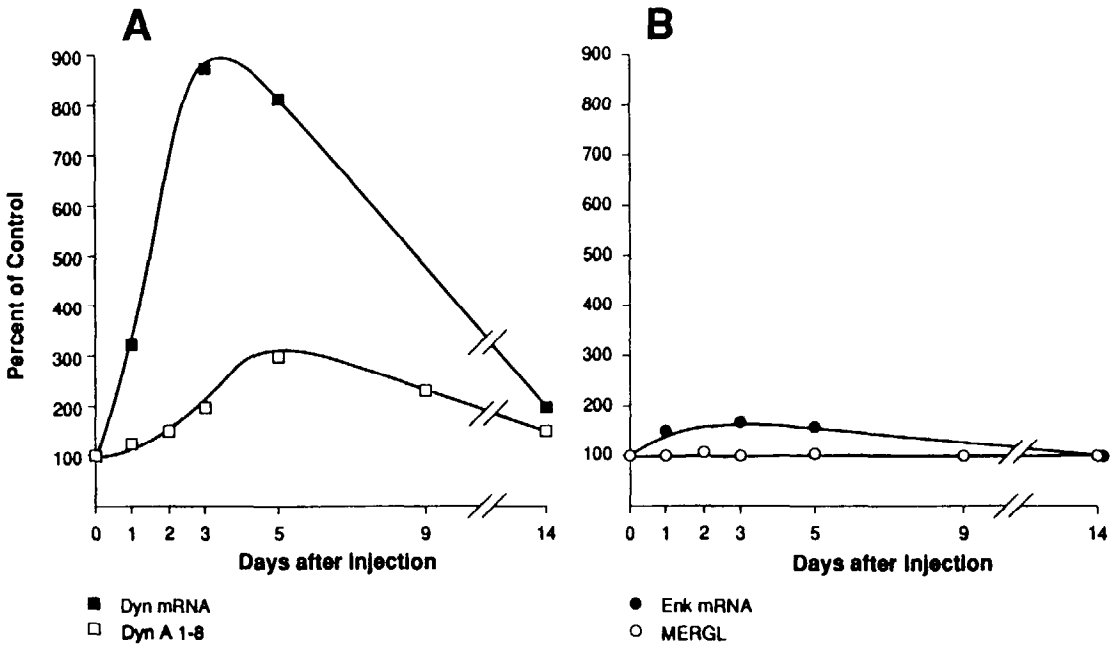



**FIGURE 1.** *Differential regulation of spinal cord prodynorphin and proenkephalin mRNA and peptide levels following the induction of unilateral peripheral inflammation*

NOTE: Prodynorphin mRNA/Dyn A 1-8 peptide levels (panel A), and proenkephalin mRNA/MERGL peptide levels (panel B) in the rat lumbar spinal cord were measured at various times after the administration of Freund's adjuvant in a rear footpad. Values are represented as percent of control (saline-injected animals). (For specific details of the procedure, see ladarola et al. 1988.)

release of dynorphin peptides from spinal cord neurons. In contrast, the spinal cord proenkephalin system was relatively unresponsive (figure 1, panel B). Proenkephalin mRNA levels rose a modest twofold, with met-enkephalin-Arg-Gly-Leu (MERGL) levels remaining relatively unchanged. These data suggest the active participation of dynorphin-containing spinal cord neurons in the modulation of sensory afferent input during peripheral inflammatory pain states. The study is also of interest in that both the prodynorphin and the proenkephalin genes are transcriptionally active in similar regions of the spinal cord; yet, only the prodynorphin gene is transcriptionally responsive to peripheral inflammation.

**Prodynorphln Gene Regulation In the Rat Hippocampus**

Opioid peptides derived from the prodynorphin and proenkephalin precursors are present at high levels in the rodent hippocampus. Enkephalin immunoreactivity is observed mainly in the mossy fiber axons of the dentate gyrus granule cells, with staining also observed in axons bordering the stratum radiatum and the lacunosum of the stratum moleculare of CA1, and in perforant path axons that arise from the lateral entorhinal/perirhinal cortices (Gall et al. 1981). Dynorphin immunoreactivity exhibits a more restricted distribution, being localized to the mossy fiber axonal system (McGinty et al. 1983). Thus, both prodynorphin- and proenkephalin-derived peptides are present in the granule cells of the hippocampal dentate gyrus.

The hippocampus is a highly excitable structure, unusually susceptible to seizure activity. Numerous studies have documented specific alterations in the levels of hippocampal opioid peptides and opioid peptide mRNAs following the induction of seizure activity by electroconvulsive shock (Yoshikawa et al. 1985; Kanamatsu et al. 1986a; Xie et al. 1989a) or kindling (ladarola et al. 1986; Xie et al. 1989b). Kainic acid (KA) is an excitatory amino acid that also elicits the induction of hippocampal epileptiform activity and motor seizures. Over the past decade, a variety of studies have utilized this potent neurotoxin as a model of temporal lobe epilepsy (Ben-Ari 1985). Administration of KA results in the onset of a range of epileptiform behaviors, which also depends on the dose and route of administration of the drug. The hippocampal proenkephalin system is stimulated by intrastriatal administration of KA, with increases in both peptide and mRNA levels observed in a time-dependent manner (Kanamatsu et al. 1986b). In the study described here, hippocampal prodynorphin and proenkephalin mRNA and peptide levels were measured at various times following a single subcutaneous injection of KA (8.0 mg/kg).

Prodynorphin mRNA levels rose remarkably quickly, with a 13 to 14-fold induction observed 3 hours after KA administration (figure 2, panel A). Prodynorphin mRNA levels began to decline at 12 hours, and by 48 hours, the

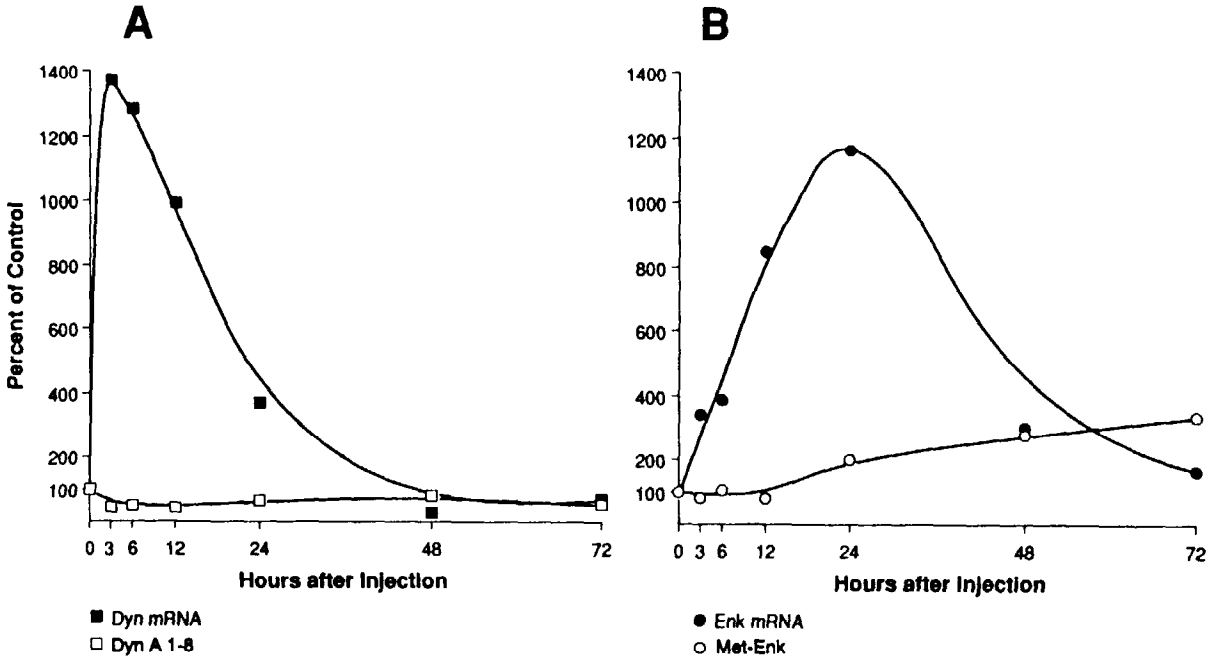



**FIGURE 2.** *Differential regulation of hippocampas prodynorphin and proenkephalin mRNA and peptide levels following subcutaneouss administration of KA*

NOTE: Prodynorphin mRNA/Dyn A 1-8 peptide levels (panel A) and proenkephalin mRNA/met-enkephalin peptide levels (panel B) in the rat hippocampus were measured at various times after the administration of KA (s.c., 8 rng/kg). Values are represented as percent of control (saline-injected animals).

levels were at or below control values. Even though mRNA levels were dramatically stimulated, hippocampal Dyn A 1-8 levels remained at values below control at all time points monitored. This observation suggests that KA treatment also results in a prolonged stimulation of release of dynorphin peptides from hippocampal neurons.

The hippocampal proenkephalin system also responded in dramatic fashion to KA administration (figure 2, panel B). Proenkephalin mRNA levels began to rise within 3 hours, showing a fourfold elevation, and at 24 hours, a maximal 11- to 1 P-fold induction was observed. At the 48-hour time point, proenkephalin mRNA values were still elevated (twofold to threefold) but had returned to control values by 72 hours, Hippocampal met-enkephalin peptide levels also were stimulated following KA administration. Levels remained close to control values for the first 12 hours, then proceeded to increase steadily throughout the 24-to 72-hour period, eventually reaching values 3.0-to 3.5-fold above control levels. This observation is consistent with a KA-induced increase in both synthesis and release of hippocampal met-enkephalin.

Once again, the prodynorphin and proenkephalin systems appear to be regulated differentially. Prodynorphin mRNA levels rose quickly and had returned to control values by 24 hours. Conversely, proenkephalin mRNA levels were stimulated maximally at the 24-hour time point and were still elevated at 48 hours. Both species of opioid peptide mRNAs were present in the granule cells of the hippocampal dentate gyrus, suggesting that the prodynorphin and proenkephalin systems are differentially regulated not only in the same brain region but also in the same neural network.

## CHARACTERIZATION OF THE RAT PRODYNORPHIN GENE

The studies described above document relatively dramatic changes in prodynorphin mRNA levels in neuronal cell types. These changes are presumably the result of alterations in the rate of transcription of the prodynorphin gene. We have isolated and characterized the rat prodynorphin gene to begin to understand the molecular mechanisms that mediate transcriptional regulatory events. Such analysis has led to the identification of the rat prodynorphin mRNA cap site, promoter element, and possible regulatory sequences. In addition, RNase protection studies, using various gene sequences as complementary probes, have enabled us to characterize the alternately spliced forms of both rat prodynorphin and proenkephalin mRNA that are present in the testis, These studies are described in the following section.

**Alternate Splicing of Opioid Peptide mRNAs**

Prodynorphin transcripts are present in both rat brain and testis (Civelli et al. 1985; Douglass et al. 1987; Garrett et al. 1989). Northern blot analysis has determined that the 2,400-base transcript present in brain is approximately 100 bases larger than the 2,300-base transcript present in adult testis. Different sized transcripts encoding the proenkephalin precursor also are seen in these tissues (Douglass et al. 1987; Garrett et al. 1989). In this case, however, the 1,400-base transcript seen in brain is approximately 350 bases smaller than the 1,750-base transcript present in adult testis. In the rat, single copy genes encode both prodynorphin and proenkephalin; therefore, the tissue-specific differences in transcript size must arise from alternative sites of transcription initiation and termination, alternative mRNA splicing patterns, or differing lengths of poly(A) tails, RNase protection mapping studies were undertaken to define precisely the basis for the observed size differences. The results are diagramed in figure 3.

For prodynorphin mRNA, the testicular transcript is smaller than the brain transcript due to the absence of exon 2 in the former species of mRNA. Exon 2 encodes 65 bases of 5' untranslated region, with 15 bases of additional 5' untranslated sequence present on exon 3. Thus, the 5' untranslated region beginning 15 bases from the translational start site is unique in the two species of prodynorphin mRNA. Sucrose gradient poiysome analysis of striatal and testicular prodynorphin mRNA reveals that both species of mRNA are present on high-molecular-weight polysomes, suggesting that the mRNA is translated efficiently in both tissues (for data presentation, see Garrett et al. 1989). The functional significance of any alternate splicing event involving prodynorphin mRNA is currently unknown.

For proenkephalin mRNA, the testicular transcript is larger than the brain transcript due to the retention of approximately 350 bases of intron 1 sequence in the former species of mRNA. Exon 2 contains 3 bases of 5' untranslated sequence; again, the nucleotide sequence immediately adjacent to the translational start codon is unique to each species of mRNA. Furthermore, the intron sequence present in the testicular transcript contains three open reading frames (ORFs) varying from 9 to 156 bases in length (Garrett et al. 1989). Evidence from experimental systems and from naturally occurring examples indicates that the presence of methionine-initiated ORFs 5' to a protein-coding region will severely inhibit translation of the downstream region. Presumably, ribosomes load onto the mRNA at the 5' cap and scan downstream until the first AUG codon in a context favorable for translation initiation is reached. On most mRNA's, subsequent reinitiation at any downstream AUG does not appear to occur at *any* appreciable frequency. The sequence around the initiation codon

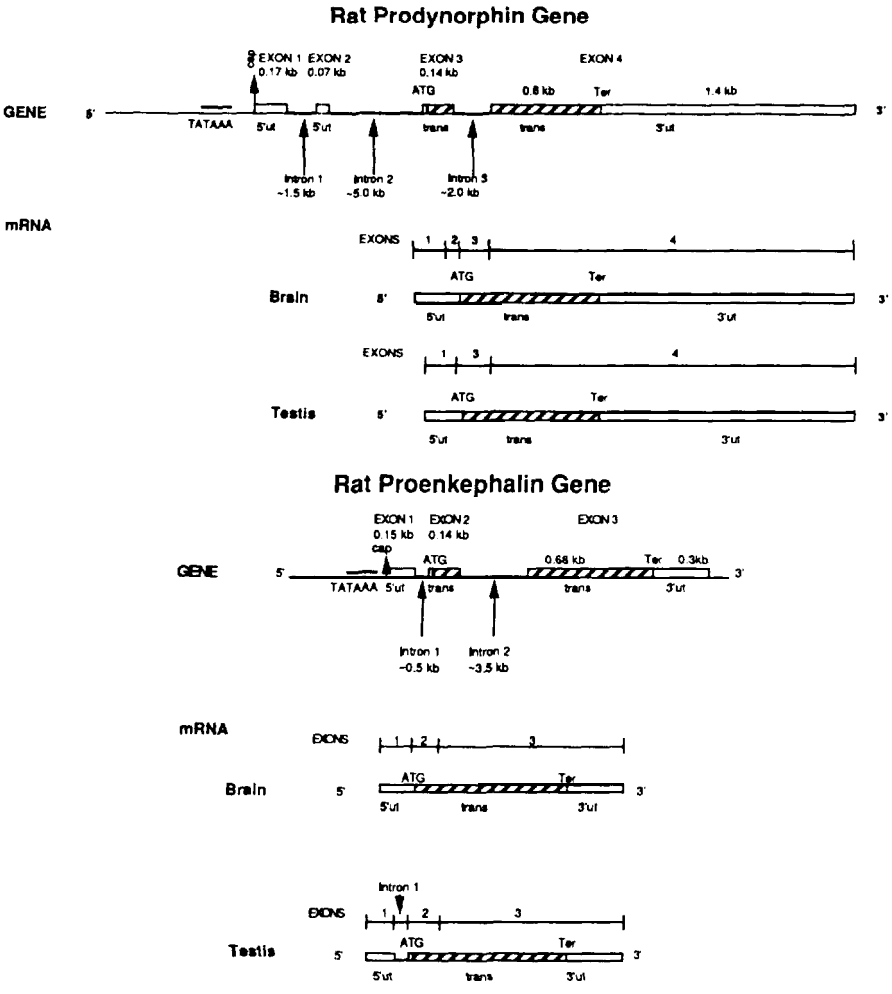



**FIGURE 3.** *Alternate splicing of rat prodynorphin and proenkephalin precursor mRNA to generate different forms of mature mRNA in the brain and testis*

NOTE: For prodynorphin, exons 1-4 are spliced together to generate a 2,400-base transcript present in brain. In the testis, exon 2 is spliced out of the mature prodynorphin transcript. For proenkephalin, exons 1-3 are spliced to generate a 1,400-base brain transcript. In the testis, approximately 350 nucleotides of intron 1 remain in the mature proenkephalin transcript.

of the first intron-encoded ORF of the testicular proenkephalin transcript conforms to the consensus sequence of a strong translation initiation site and therefore may inhibit efficient translation of the mRNA. Sucrose gradient polysome analysis does reveal that the majority of the testicular proenkephalin transcript is unassociated with poiysomes or is present on monosomes or low-molecular-weight poiysomes (Garrett et al. 1989). In contrast, striatal proenkephalin mRNA is present on high-molecular-weight polysomes, suggesting that the mRNA is translated efficiently. Thus, the additional nucieotide sequence present on the testicular form of proenkephalin mRNA may play a role in determining the translational status of the transcript in this tissue.

**Characterization of the Rat Prodynorphin Promoter Region**

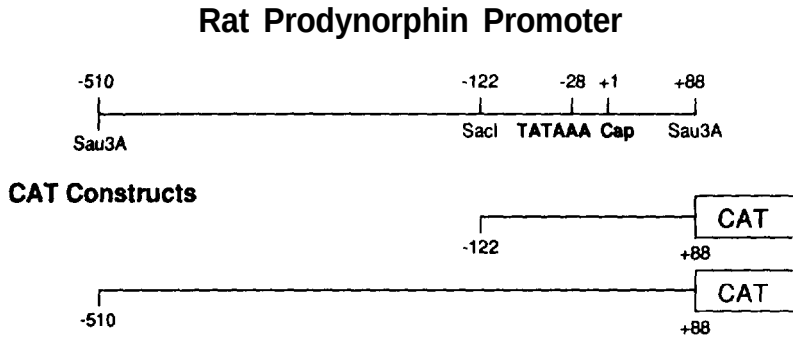
*In vivo* studies have shown that rat prodynorphin mRNA levels in specific neuronai ceil types can be dramatically altered following surgical or pharmacological manipulations. These changes presumably reflect alterations in the rate of prodynorphin mRNA synthesis. Thus, signals received by prodynorphin-expressing neurons must be relayed to the cell nucleus, resulting in altered levels of transcription of the prodynorphin gene.

In many cases, chemical signals received at the neuronal cell membrane are transduced through the plasma membrane via cell surface receptors coupled to G-proteins (for review, see Johnson and Dhanasekaran 1989). Activation of these G-proteins regulates numerous cellular events, including the production of second messenger molecules and the activity of a variety of protein kinases. Second messengers, such as cyclic AMP and diacylglycerol, act to regulate the activity of kinases, including cAMP-dependent protein kinase and protein kinase C, respectively (for review, see Ross 1989). it is believed that one class of protein factors whose activity is regulated at the level of phosphorylation by the aforementioned protein kinases are DNA binding proteins that control the transcriptional activity of specific genes in a tissue- and cell type-specific manner (Mitchell and Tjian 1989; Jones et al. 1988). Thus, the regulation of neuronal gene expression by signals received at the postsynaptic membrane is an important mechanism by which neurons control the synthesis of peptide neurotransmitters specific to that particular cell. Moreover, the presence of a variety of postsynaptic receptors on any given ceil body provides an additional degree of complexity to the phenomenon of transsynaptic regulation of neuronai gene expression. As a first step in characterizing the unique compilation of *cis*-acting nucieotide sequence elements regulating the production of prodynorphin mRNA, we have used gene transfer techniques to begin to functionally characterize the rat prodynorphin promoter region.

Specific regions of rat prodynorphin genomic DNA, containing the mapped mRNA cap site and a consensus RNA poiymerase II promoter sequence (TATAAA), have been ligated 5' to the bacterial chloramphenicol acetyl transferase (CAT) gene (figure 4). One such piasmid contains prodynorphin promoter sequences from -122 to +88 (with the cap site at position +1 representing the point of reference). A second plasmid contains nucleotides from -510 to +88 linked to the CAT structural gene. These plasmids have been introduced via $CaPO_4$ precipitation into three different eukaryotic cell lines (Douglass et al. 1989). The NG 108 cell line is of neural origin and represents a mouse neuroblastoma x rat C6 giioma hybrid. No detectable levels of prodynorphin mRNA are observed in this line. CV1 cells are derived from an African green monkey kidney tumor and do not contain detectable levels of prodynorphin mRNA. R2C cells are derived from a rat testicular Leydig cell tumor and do contain detectable, albeit low, levels of prodynorphin mRNA (McMurray et al. 1989).

The promoter-reporter plasmids were introduced into the various cell lines, and 24 hours later, cellular extracts were assayed for CAT activity (figure 4). CAT activity is represented as percent conversion of $^{14}$C-chloramphenicol to the mono- and di-acetylated forms (McMurray et al. 1989). CAT activity levels are dependent on the cellular levels of CAT mRNA, which presumably are related directly to the relative strength of the upstream promoter element(s). A control piasmid (containing the constitutive Rous sarcoma virus long terminal repeat promoter region linked to the bacterial ß-galactosidase structural gene) was cotransfected into the various ceil lines to ensure that all transfection reactions proceeded with approximately similar efficiencies. Plasmids containing rat prodynorphin 5' flanking DNA were transcriptionally active, resulting in the production of CAT activity. The -122 to +88 construct produced the highest levels of CAT activity, and percent CAT conversion ranged from 14.5 percent in NG 108 cells to only 1.0 percent in R2C cells. This observation is consistent with the presence of a consensus TATAAA element and mapped cap site 5' to the CAT structural gene. The -510 to +88 construct also was transcriptionally active, although CAT levels were an average of fivefold lower than those observed with the -122 to +88 construct. This observation suggests that nucieotide sequences between -510 and -122 may be serving to inhibit transcription from the promoter located at approximately -30. Also, there was no correlation between transient promoter activity and expression of the endogenous prodynorphin gene in the cell lines tested.

In the R2C ceil line, endogenous prodynorphin mRNA levels are elevated approximately fourfold following an 8-hour treatment with 8-bromo-cAMP (McMurray et al. 1989). To determine if this increase in mRNA levels correlates with induction of prodynorphin promoter activity, the -122 to +88 CAT construct




## Cell Lines Transfected

| CAT Constructs | NG108 | CV1 | R2C | R2C+8Br-cAMP | |
|---|---|---|---|---|---|
| -122/+88 CAT | 14.5±6.0 (1.10) | 2.0±1.0 (0.72) | 1.0±0.5 (0.42) | 3.0±0.5 | %conversion CAT ($\beta$-gal/A$_{420}$) |
| -510 /+80 CAT | 2.0±0.2 (0.80) | 0.5±0.3 (0.57) | 0.3±0.2 (0.36) | | |

**FIGURE 4.** *Characterization of the ability of rat prodynorphin genomic DNA fragments* to *serve* as *transient transcriptional promoters*

NOTE: Nucleotides are shown representing 5' and 3' ends of rat prodynorphin genomic DNA fragments (containing the mapped cap site at +1 and the promoter element TATAAA at -28) ligated proximal to the CAT structural gene. The resulting plasmids were transfected into the NG 108, CV1 , and R2C cells lines, and cellular CAT activity was measured. RSV-ß-gal was cotransfected to monitor transfection efficiency, and cellular levels of ß-gal were determined. Some transfected R2C cells were treated with 1 mM 8-Br-cAMP for 8 hours before determination of CAT activity.

was transfected into the R2C cell line, followed by an 8-hour period of treatment with 1.0 mM 8-Br-cAMP. Cellular CAT activity levels were increased approximately threefold following this treatment. Thus, increases in cellular R2C prodynorphin mRNA levels by cAMP analogs are more than likely due to enhanced promoter activity. Furthermore, at least some of the nucleotide sequences involved in mediating this form of regulation appear to be present in the prodynorphin promoter region from -122 to +88.

## CONCLUSIONS

Neurons are constantly receiving and assimilating information represented by chemical messengers; environmental stimuli, internal biological clocks, and signals from endocrine tissues all serve to modulate the biochemical properties that define each neuronal cell type. The introduction of drugs of abuse also dramatically affects neuronal cellular events. The molecular mechanisms underlying narcotic dependence, withdrawal, and tolerance associated with the compulsive use of drugs are poorly understood. The problem of physical dependence and withdrawal is highly complex and probably involves a complex cascade of events that begins with the diverse and widely distributed drug receptor systems and extends to complex arrays of autonomic and sensorimotor neural networks (Abood 1984). These events also are influenced by genetic, species, and environmental factors, which only serve to complicate scientific ventures aimed at characterizing the molecular basis of these narcotic-related syndromes, It is reasonable to assume that the introduction of exogenous drugs results in the activation of specific neuronal G-protein coupled receptors, This activation then alters numerous cellular events, including changes at the genomic DNA level regarding the transcriptional activity of specific sets of genes. Thus, to gain additional insight into the molecular events associated with substance abuse, researchers must understand the basic mechanisms that regulate the expression of transcriptionally active genes in the CNS.

We have been studying specific aspects of opioid peptide gene expression, focusing on the regulated expression of the prodynorphin gene. *In vivo* studies have documented that prodynorphin-expressing neurons are capable of dramatic transcriptional responses, resulting in profound increases in the cellular levels of prodynorphin mRNA. In prodynorphin-expressing spinal cord neurons, inflammation of a peripheral limb results in marked increases in both prodynorphin mRNA and peptide levels. Furthermore, these increases occur in the spinal cord segments that receive sensory imput from the affected limb. The rapid onset of behavioral hyperalgesia may act as a trigger for the activation of dynorphin biosynthesis, with such neurotransmitters as substance P or calcitonin gene-related peptide mediating the response (ladarola et al. 1988). In hippocampal granule cells of the dentate gyrus, prodynorphin mRNA levels are dramatically, positively regulated following the subcutaneous administration of the excitatory amino acid KA. The prodynorphin biosynthetic system responds rapidly to KA administration, with maximal stimulation of prodynorphin mRNA levels seen within 3 hours. In contrast, proenkephalin mRNA levels rise at a much slower rate, with maximal stimulation observed 24 hours after drug administration. Thus, the hippocampal prodynorphin and

proenkephalin systems, presumably active in overlapping sets of neurons, respond differentially to stimulation by KA administration.

Thus far, in Investigations of the molecular events controlling the production of prodynorphin mRNA, the rat prodynorphin gene has been isolated and characterized. The intron/exon architecture has been determined, the mRNA cap site mapped, and the promoter region functionally characterized. Alternate splicing of precursor mRNA results in the generation of different forms of prodynorphin mRNA in the brain and testis. The two species of mRNA contain different 5' untranslated regions beginning 15 nucleotides from the translational start site that are apparently translated at similar efficiencies. Any functional significance resulting from this pattern of alternate mRNA splicing is currently unknown.

Transfection of DNA into various eukaryotic cell lines has allowed for a preliminary characterization of the rat prodynorphin promoter region. The consensus TATAAA promoter element at position -30 appears to be functionally active, with nucleotide *sequences* between -510 and -122 perhaps serving to negatively regulate transcription from the aforementioned promoter. In addition, a CAMP-responsive element may be present in the region from -122 to +88. Additional studies are needed to define more precisely the sequence elements mediating the controlled production of prodynorphin mRNA in neuronal cell types. Such studies will increase understanding of the nuclear events underlying transsynaptic regulation of neuronally active genes.

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## ACKNOWLEDGMENTS

The studies described in this chapter were supported in part by National Institute on Drug Abuse grant DA-04154 (JOD), National Research Service Award fellowship NS-08068 (JEG), and American Cancer Society postdoctoral fellowship PF-2997 (CTM).

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# Mechanisms Underlying Synaptic Regulation of Proenkephalin Transcription

*Michael Comb, Steven E. Hyman, Linda Kobierski, Hung Ming Chu, and Tuong Van Nguyen*

## INTRODUCTION

The molecular mechanisms underlying learning, memory, and other stable/adaptive changes in the nervous system are largely unknown. With the discovery that neurons transduce environmental inputs into changes in gene expression, the hypothesis has emerged that transsynaptic regulation of gene expression may induce the biochemical changes underlying many forms of neural plasticity. To understand these processes, it is necessary to identify both the pathways and the mechanisms mediating the effects of neurotransmitters, psychoactive drugs, learning, and other synaptically mediated processes on gene expression. Neural regulation of the opioid precursor, proenkephalin, is an excellent model system to study mechanisms underlying synaptic regulation of gene expression because (1) its transcription is both activated and repressed by synaptic signals; (2) in the brain, such events as electrical stimulation that induce long-term potentiation (LTP) also induce dramatic and long-lasting changes in proenkephalin gene expression; and (3) the gene products, enkephalins, are important signaling molecules regulating diverse neural pathways in the brain and peripheral nervous system. This chapter discusses approaches and methodologies to define how synaptic activity, intracellular signaling pathways, and nuclear events regulate expression of the human proenkephalin gene.

## NEURAL PLASTICITY AND REGULATION OF PROENKEPHALIN GENE EXPRESSION: A MODEL SYSTEM TO EXPLORE MECHANISMS

Over the past 20 years, a variety of neurotransmitters have been identified that carry out and regulate cell-to-cell communication in the nervous system. Peptide neurotransmitters differ from conventional small molecule transmitters in their extraordinary potency, prolonged actions, and presumed effects on

neural plasticity. The endogenous opioids met- and leu-enkephalin mediate neurotransmission at the opiate receptor, which is also the site of narcotic drug action. These pentapeptides function as important neurotransmitters/ neuromodulators, regulating diverse neural pathways, including analgesia, reward, motivation, and hormone release. Both the synthesis and the release of enkephalins are regulated by synaptic events involving activation of neurotransmitter receptors, second messenger pathways, and membrane depolarization. Regulation of proenkephalin gene expression (mRNA levels) has been studied in a variety of systems, including primary cultures of bovine chromaffin cells (Eiden et al. 1984; Quach et al. 1984; Kiey et al. 1987), tissue culture cells (Yoshikawa and Sabol 1988; Schwartz 1988; Boarder et al. 1986), rat adrenal (Kilpatrick et al. 1984; La Gamma et al. 1985), and various brain regions (Tang et al. 1983; Sabol et al. 1983; Sivam et al. 1986; Shivers et al, 1986).

The influences of synaptic activity and second messengers have been examined in the greatest detail using primary cultures of bovine chromaffin cells. Chromaffin cells have been used to show that agents that increase intracellular cyclic AMP (cAMP) levels, such as forskolin, or stable cAMP analogs increase proenkephalin mRNA levels (Eiden et al. 1984; Quach et al. 1984). In addition, agents that cause membrane depolarization or Cat+ entry, such as nicotine, potassium, veratridine, and barium, all have been shown to increase proenkephalin mRNA levels in bovine chromaffin cells (Kley et al. 1987). Activation of protein kinase C by phorbol esters also will induce proenkephalin mRNA levels in these cells (Kley et al. 1988). *In vivo* experiments have shown that proenkephalin mRNA levels are dramatically elevated by increased impulse intensity of the rat splanchnic nerve (Kanamatsu et al. 1986; Colbrie et al. 1988).

*In situ* hybridization has allowed visualization of changes, at the single cell level, in striatial proenkephalin mRNA levels resulting from neuroleptic drug treatments and specific lesions (Romano et al. 1987; Young et al. 1986). These results suggest that dopamine negatively regulates proenkephalin gene expression in the caudate-putamen. *In situ* hybridization and RNA blot techniques also have been used to study the regulation of opioid gene expression in rat hippocampus, a region in which long-term changes in synaptic efficacy have been readily demonstrated and may play a role in the molecular basis of memory (White et al. 1987; Morris et al. 1988). Granule cells of the rat dentate gyrus contain undetectable levels of proenkephalin mRNA and very high levels of prodynorphin mRNA. Electrical stimulation of this region *in vivo,* using stimulation parameters identical to those that can induce LTP, produces dramatic increases in proenkephalin mRNA levels and decreases prodynorphin mRNA levels (Morris et al. 1988), Enkephalin biosynthesis in the granule cell/

mossy fiber system of the mouse also has been investigated following the induction of seizure induced by placement of a small unilateral lesion within the hilus of the dentate gyrus (Gall 1988; White and Gall 1987). Marked increase in both the enkephalin peptides (14-fold) and the proenkephalin mRNA levels (18-fold) were observed in granule cells 24-hours postseizure induction. These data indicate that recurrent seizure activity stimulates a large increase in proenkephalin biosynthesis in the dentate gyrus cell/mossy fibers of the rodent hippocampus (Gall 1988).

## RECONSTITUTING REGULATION AND DEFINING CONTROL REGIONS

To study the mechanisms underlying synaptic regulation of the proenkephalin gene, a plasmid, pENKAT-12, was constructed in which human proenkephalin control regions were fused to the bacterial chloramphenicol acetyl transferase (CAT) gene (figure 1) (Comb et al. 1986). Expression of this reporter gene can be easily monitored after transfection into tissue culture cells (figure 2). After introduction and expression of this fusion gene in tissue culture cells, we have shown the gene to be regulated by cAMP, TPA, and Ca++ in a fashion similar to the endogenous gene (Comb et al. 1986; Nguyen et al. 1990). Deletion and transfer mapping studies of the 5'-flanking region indicate that regulation by the cAMP, TPA, and Ca++ intracellular signaling pathways is controlled by a short 50-base pair region of proenkephalin DNA that acts as a second messenger-Inducible DNA enhancer (Comb et al. 1986,1988). Effects of TPA and Ca++ are dependent on and synergistic with simultaneous or prior stimulation of the cAMP pathway, indicating that these pathways interact in both a conditional and a synergistic fashion to regulate proenkephalin gene expression (Comb et al. 1986, 1987; Nguyen et al. 1990). The mechanisms underlying these effects are not as yet understood and require further study.

## TRANSCRIPTION IS RAPIDLY ACTIVATED AND RAPIDLY DESENSITIZED TO cAMP

Because proenkephalin mRNA is quite stable in C6-glioma cells, with a half-life of at least 24 hours (Yoshikawa and Sabol 1986), while the RNA transcript produced from the proenkephalin/CAT fusion gene is relatively unstable, analysis of CAT mRNA is a much more sensitive indicator of transcriptional events than is the endogenous gene. To determine the time course of mRNA activation and its dependence on new protein synthesis, RNA isolated from a stable clonal line of C6-glioma cells (C6-D2), which express the proenkephalin/CAT fusion gene stably integrated into their chromosomal genome, was analyzed at various times after treatment with forskolin and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) by S1 or primer

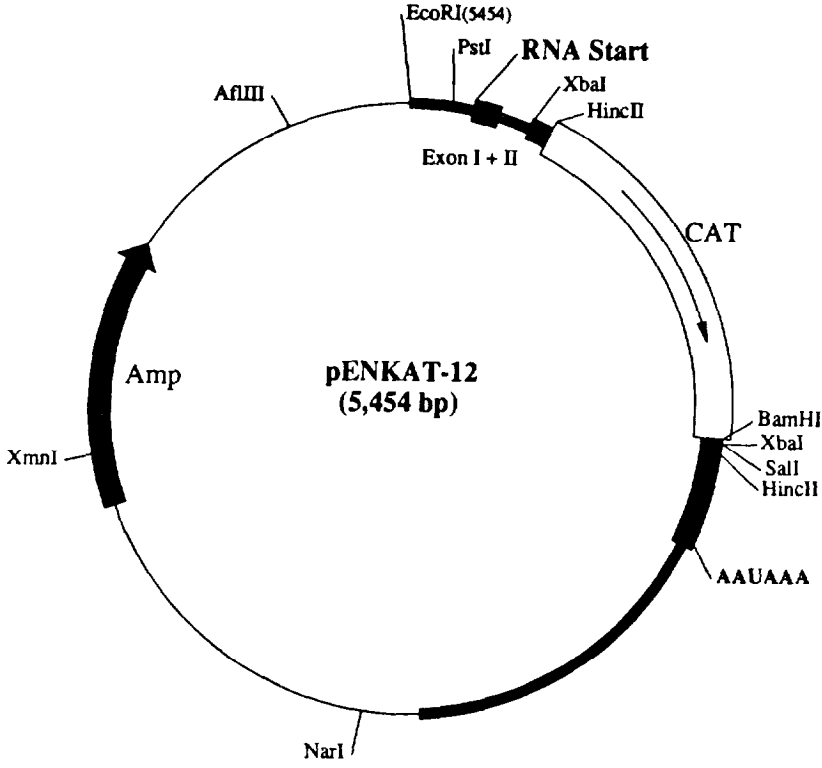


**FIGURE 1.** *Structure of the human proenkephalin/CAT fusion gene. This plasmid directs expression of CAT enzyme under the control of the human proenkephalin promoter and poly (A) regions (Comb et al. 1986). After transfection into tissue culture cells, CAT enzyme activity is proportional to proenkephalin promoter activity, Open box is CAT coding sequence; large black boxes represent proenkephalin noncoding exons; and smaller stippled regions represent proenkephalin 5'-flanking sequence, intron A, and 3'-untranslated sequences.*

extension analysis. Correctly initiated RNA is detected within 15 minutes of forskolin treatment, peaks by 90 to 120 minutes, and rapidly declines to baseline by 8 hours following treatment. Further addition of the stable cAMP analog CPT-CAMP at several times following the peak in mRNA produced no further increase in steady-state mRNA levels, suggesting that transcription had become uncoupled or "desensitized" to intracellular levels of cAMP. The rapid

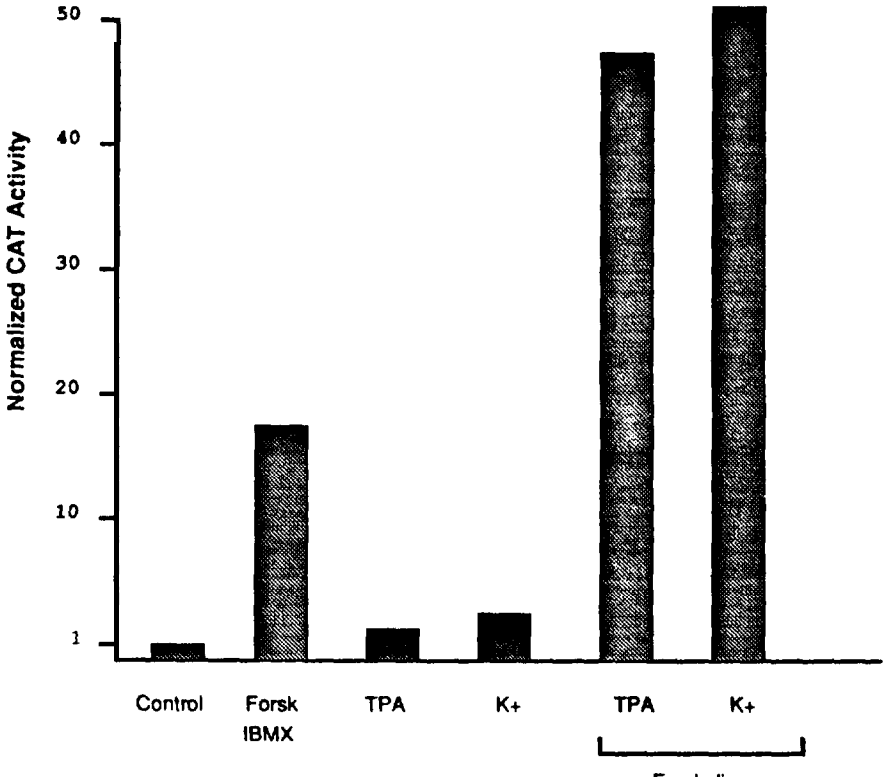


**FIGURE 2.** *Synergistic and conditional effects of cAMP, phorbol esters,* and *K+ on CAT expression directed by the human proenkephalin promoter after treatment of* a stable rat *PC-12* cell *line D2* that *expresses the proenkephalin/CAT fusion plasmid pENKAT-72. Treatments were for 6 hours with 25 µM forskolin, 50 nM TPA, 500 µM IBMX, and 40 µM KCl.*

CAMP-dependent increase in mRNA is not affected by the addition protein synthesis inhibitors cyclohexamide (100 µM) or anisomycin (10 µM) 30 minutes before forskolin addition, consistent with activation being caused by a rapid posttranslational modification of preexisting proteins. In sharp contrast, anisomycin blocked mRNA decline during the refractory phase and caused mRNA levels to accumulate, resulting in superinduction. Neither inhibitor caused induction in the absence of cAMP.

These results suggest that transcriptional activation is due to a posttranslational modification of preexisting factors and that new protein synthesis is required for the "refractory phase" that rapidly follows transcriptional activation. It seems likely that sustained stimulation of the cAMP pathway leads to rapid repression of proenkephalin transcription by mechanisms that require new protein synthesis. Hence, proenkephalin transcription is regulated both positively and negatively by second messenger pathways, resulting in a brief pulse of mRNA synthesis. Desensitization of transcription to cAMP is remarkably similar to neurotransmitter receptor desensitization and may share similar mechanistic features.

### The Second Messenger-Inducible Enhancer Consists of Multiple DNA Elements That Act In a Highly Synergistic Fashion

To further resolve the inducible enhancer into individual elements and to provide regulatory mutations necessary to correlate binding of transcription factors with *in vivo* enhancer function, a series of single-base substitution (SBS) mutants was generated throughout the enhancer region (figure 3). The effect of these point mutations on cAMP-inducible enhancer function was determined by examining transient CAT expression after transfection into CV-1 cells or by examining RNA transcripts from pooled, stably expressing C6 glioma cells (Comb et al. 1986, 1988). Two regions that are sensitive to SBS mutations have been defined: One, called ENKCRE-1, is located between -104 and -98 and spans the sequence TGGCGTA; a second domain, called ENKCRE-2, extends from -93 to -86 and spans the sequence TGCGTCA (Comb et al. 1988). A third region that is sensitive to SBSs has been identified adjacent and downstream from the ENKCRE-2 element and has been identified as an AP-2 element (Hyman et al. 1989). Inactivation of either the ENKCRE-1 or the ENKCRE-2 element by mutation or by alteration of their stereospecific alignment eliminates inducible enhancer activity (Comb et al. 1988). Inactivation of the AP-2 element by point substitutions results in a fivefold reduction of cAMP- and TPA-inducible transcription (Hyman et al. 1989). This analysis clearly demonstrates that each of the three proenkephalin-inducible elements acts in a highly cooperative or synergistic fashion to induce transcription in the presence of either cAMP or TPA. In addition, these results suggest that assembly of a transcription complex, involving proteins bound at the ENKCRE-1 and ENKCRE-2 elements, is required for the transcriptional effects of signals transmitted from cell surface receptors.

## THE ROLE OF EACH ELEMENT IN ISOLATION

The functional activity of each individual element in isolation from the rest of the enhancer was examined to determine which elements respond individually to

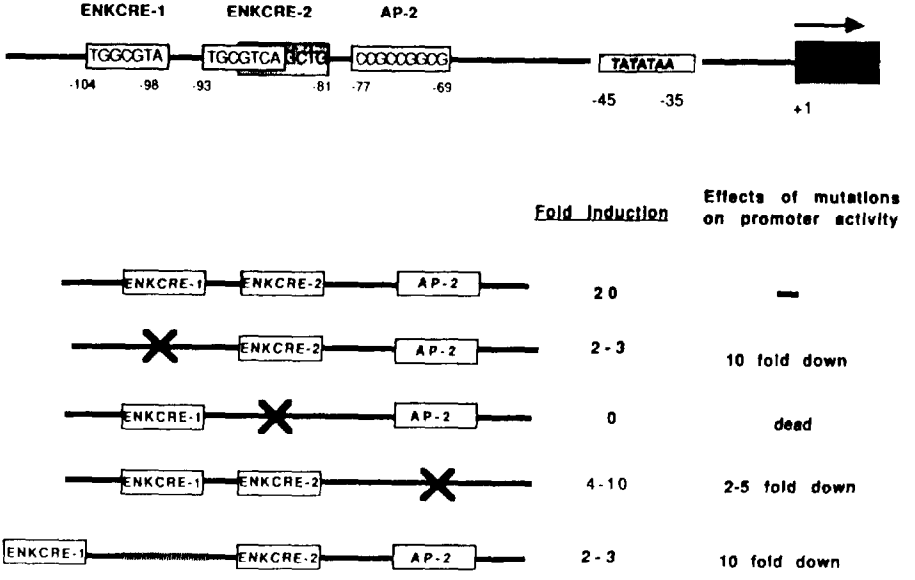


**FIGURE 3.** *The human proenkephalin promoter* contains a *short cAMP-, phorbol ester-, and Ca++-inducible enhancer located between -104 and -65 base pairs upstream from the start site of transcription. The enhancer consists of multiple DNA elements* that *act* cooperatively *to* regulate *transcription in response to* intracellular *signaling* pathways.

cAMP and TPA (figure 4). Individually responsive elements are candidates for direct interactions with the signal transduction pathways. Multiple copies of oligonucleotides containing individual factor binding sites (ENKTF-1, AP-1, AP-4 (Mermod and Tjian 1988], and AP-2) were inserted upstream of a proenkephalin promoter truncated at position -72 that deletes the entire inducible enhancer. The ability of these single-element promoters to respond to cAMP and TPA was measured after transfection into several different cell types. Multiple copies of the ENKCRE-1 element result in small (twofold to threefold inductions) to either cAMP or TPA. Multiple copies of either the AP-2 or the AP-4 element did not confer significant induction, indicating that a promoter consisting solely of multiple copies of these elements is not capable of responding to signals propagated through either the cAMP or the TPA intracellular signaling pathway. In contrast, lmagawa and colleagues (1987) previously reported that multiple copies of an AP-2 element when fused to the B-globin promoter conferred both cAMP- and TPA-inducible expression to this promoter. We do not yet

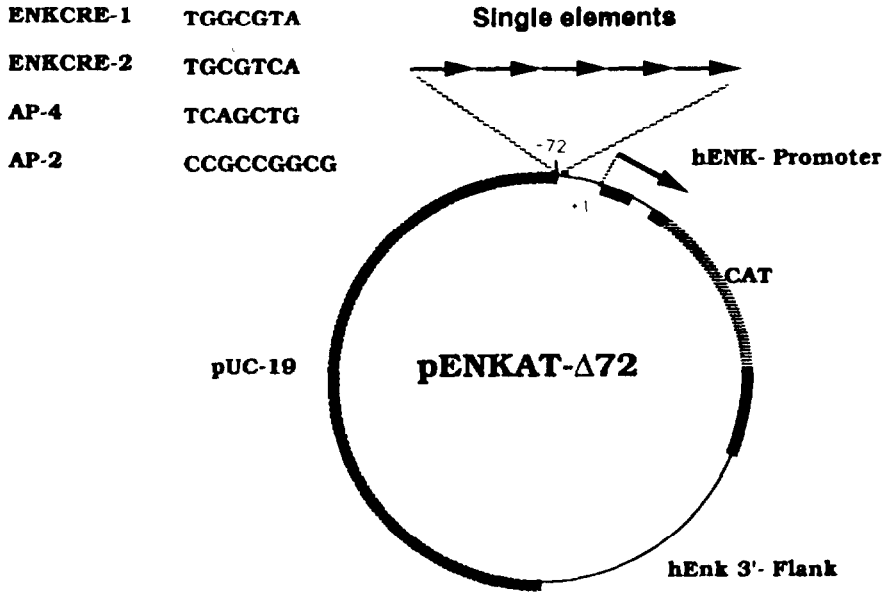


**FIGURE 4a.** *Analysis of individual elements comprising the proenkephalin second messenger-inducible DNA enhancer. Multiple copies of each element were introduced into the enhancerless plasmid PENKAT-72. Each plasmid was introduced into C6-glioma cells, and the ability of individual elements to respond to intracellular signaling pathways was examined. pENKAT-12 is the wild-type promoter/enhancer, and ENKCRE1+2 is a plasmid containing six copies of the ENKCRE-1 and ENKCRE-2 elements.*

understand these differences, but they may be attributed to different AP-2 proteins or to differences between the proenkephalin and B-globin promoters. Multiple copies of the ENKCRE-2 element induce transcription in response to cAMP and TPA, although at levels significantly lower than the wild-type promoter. Multiple copies of the AP-4 element increase basal but not inducible transcription, suggesting that this element may play an important role in basal expression.

## ENKCRE-2 CAN RECEIVE SIGNALS FROM THE cAMP, TPA, AND CA++ PATHWAYS

To further characterize the ability of the ENKCRE-2 element to respond to intracellular signaling pathways, we isolated stably expressing cell lines in which

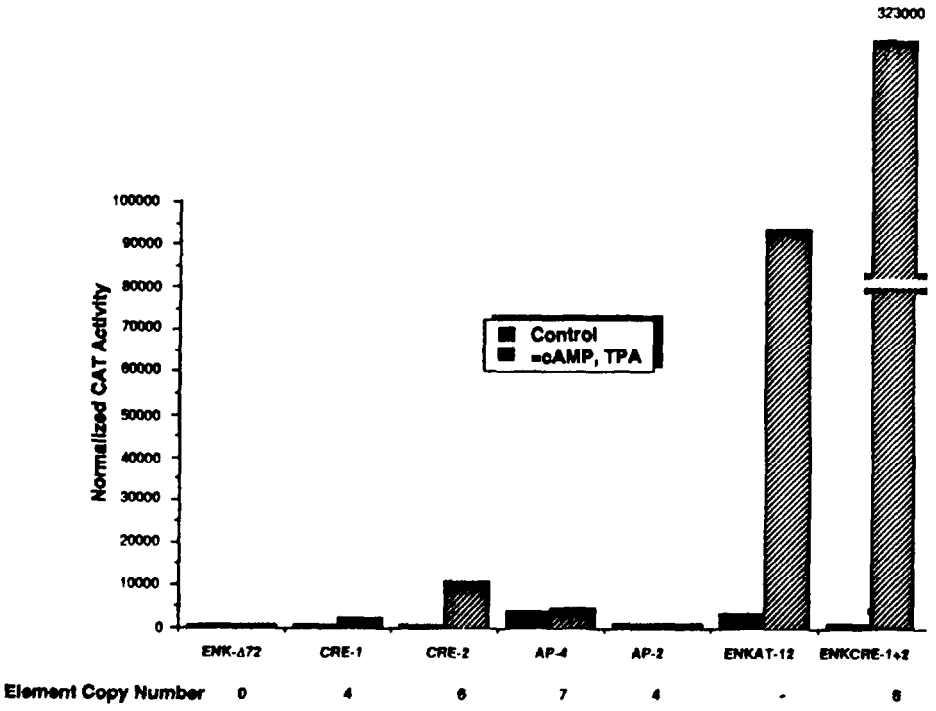


**FIGURE 4b.** *Effects of individual elements*

the plasmid is stably introduced into C6-glioma cell chromosomal DNA. Five independent lines were tested, and a typical response is shown in figure 5. Surprisingly, the multiple copy ENKCRE-2 plasmid exhibited the entire spectrum of responses exhibited by the wild-type enhancer (pENKAT-12). This plasmid is responsive to cAMP, TPA, and depolarizing concentrations of K+ (40 mM). In addition, the additive effects of cAMP and TPA and of cAMP and K+ are reproduced by the ENKCRE-2 element, suggesting that much of the observed regulation may be mediated through this element.

It is important to remember that a single copy of the ENKCRE-2 element is not sufficient, since point mutations that inactivate the ENKCRE-1 element and leave the ENKCRE-2 element intact are unable to regulate transcription in response to activation of second messenger pathways. Therefore, the presence of tandem ENKCRE-2 binding sites is able to compensate for the absence of ENKCRE-1 and AP-2 elements. This suggests that the protein/ protein interactions between proteins bound at the ENKCRE-1 and ENKCRE-2 elements may be replaced by a single protein (complex) bound at multiple ENKCRE-2 elements.

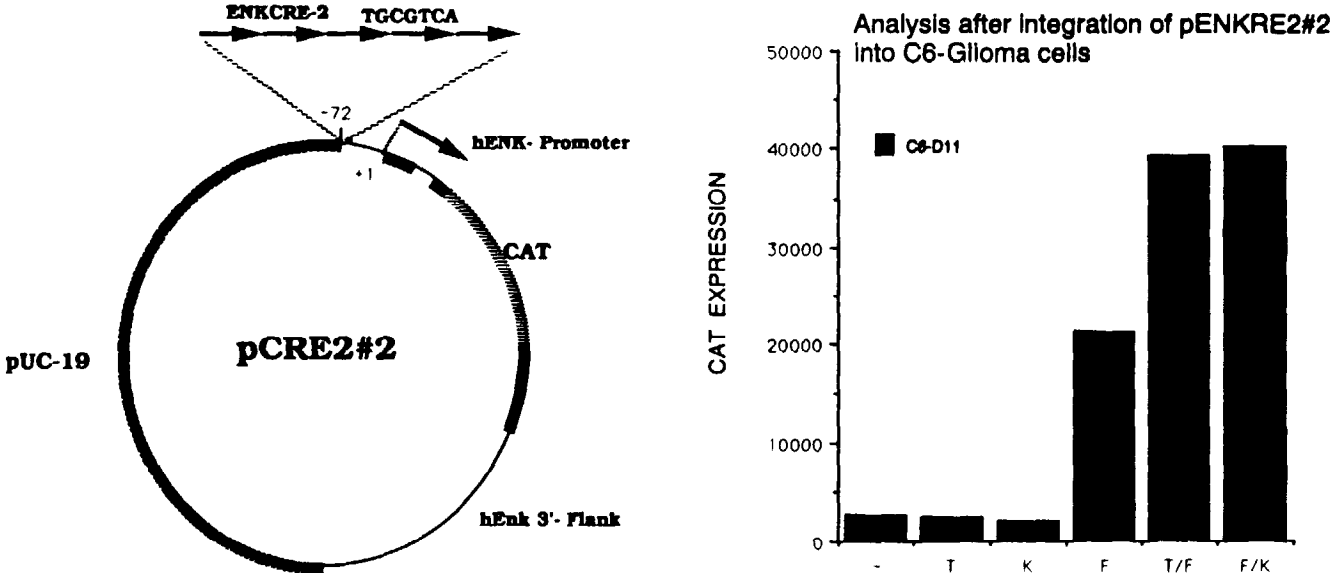


**FIGURE 5.** *Multiple copies of the ENKCRE-2 element reconstitute regulation by cAMP, TPA and Ca++. The plasmid pENKCRE2#2 was cotransfected together with pRSVNE0 into C6-glioma cells and a clonal cell line, C6-D11, was isolated and analyzed. In addition, four other independent lines were tested and gave results similar to those shown above for the C6-D11 line. Cells were treated for 6 hours with 50 nM TPA (T), 25 µM forskolin (F), and 40 mM potassium chloride (K), and CAT activities were determined.*

**PROTEINS BINDING THE PROENKEPHALIN SECOND MESSENGER-INDUCIBLE ENHANCER**

Using DNAase I footprinting protection assay and DNA recognition site affinity chromatography, we have identified and purified four different factors present in Hela cell and bovine brain nuclear extracts that interact with the human proenkephalin enhancer region (figure 6). ENKTF-1, a novel DNA binding protein, binds to the enhancer at the promoter distal ENKCRE-1 element (Chu et al. 1991). The transcription factors AP-1 (Lee et al. 1987; Angel et al. 1987) and AP-4 (Mermod et al. 1988) bind to overlapping sites within the enhancer region spanning ENKCRE-2; and a fourth transcription factor, AP-2 (Mitchell et al. 1987), binds to a site immediately downstream of the ENKCRE-2 element

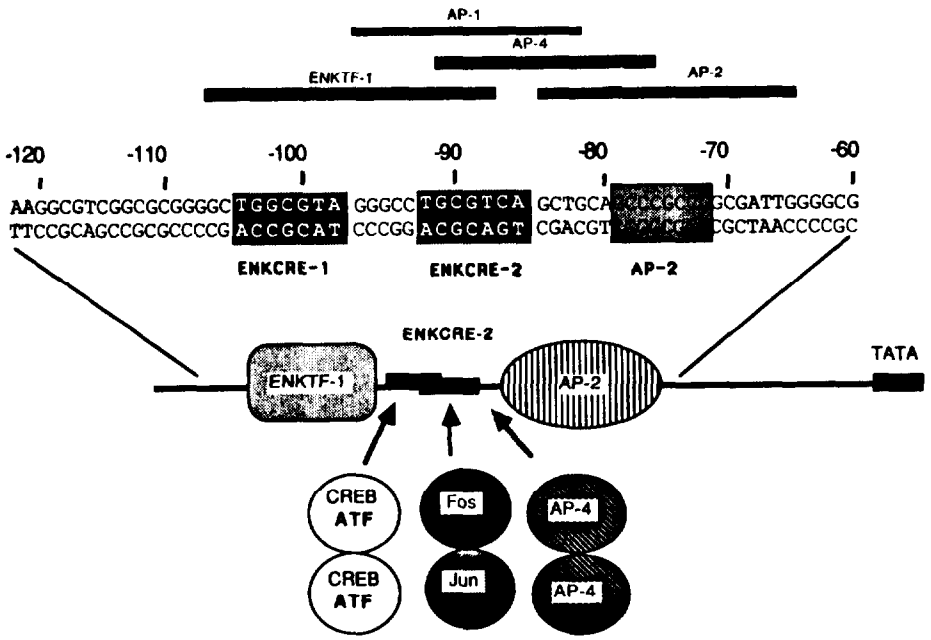


**FIGURE 6.** *Proteins that bind the proenkephalin cAMP-, TPA-, and Ca++-inducible enhancer. ENKTF-1, a family of at least three different proteins purified from total brain, binds the ENKCRE-1 element; AP-1, AP-4, and, possibly, CREB/ATF proteins interact with the ENKCRE-2 element; and AP-2 binds the AP-2 element (Comb et al. 7988; Hyman et al. 1988).*

(Comb et al. 1988; Hyman et al. 1989). The role of the observed *in vitro* binding of these factors to the *in vivo* functioning of the enhancer was assessed by correlating the binding of factors to a series of SBS mutations of known *in vivo* enhancer activity. ENKTF-1 correlates perfectly with the *in vivo* enhancer activity of each mutant, suggesting that binding this factor to the ENKCRE-1 element is the critical regulatory event altered by the various point mutations. Overlapping binding of purified AP-1 and AP-4 to the ENKCRE-2 element suggests a complex regulatory scheme at this site that may involve binding of each factor under different conditions.

The sequence of ENKCRE-2 appears to be a hybrid between the proposed TRE and CRE binding sites that interact with the fos/jun and CREB families of transcription factors (Angel et al. 1987; Bohmann et al. 1987; Montminy and Belezikjian 1987). To examine the *in vivo* role of AP-1 in cAMP- and TPA-inducible enhancer function, we tested the binding of AP-1 to a series of SBS mutants of known activity. Binding correlates perfectly with inducible enhancer activity at 5 of 7 substitutions. However, two different SBS mutations, -92 and -90, severely reduce cAMP- and TPA-inducible enhancer function, yet bind AP-1 with wild-type affinity (-92) or better (-90). This result may be resolved by the observation that AP-1 binds as a c-jun/c-fos heterodimer. Since affinity purified preps contain mostly c-jun with little c-fos (as visualized by silver stains), it is possible that -92, -91, -90 bases interact with c-fos and not c-jun. Alternatively, it is possible that other jun molecules (jun-B or jun-D) and not c-jun mediate regulation at this site *in vivo.*

**PROTEINS BOUND AT ADJACENT DNA ELEMENT: POSITIVE AND NEGATIVE COOPERATIVITY**

Synergistic transcriptional effects between elements could result from (1) cooperative DNA binding interactions (binding of one protein helps the binding of a second protein) between factors bound at each element, (2) cooperative protein/protein interactions, or (3) recruitment of a third protein essential for transcriptional activation. Using DNA recognition site affinity chromatography, four different proteins binding the proenkephalin enhancer—ENKTF-1, AP-1, AP-4, and AP-2—have been highly purified (figure 7). Each of these preparations gives clean footprints over its respective DNA binding elements and is highly purified as determined by gel electrophoresis and silver staining.

The first question was, Which proteins can simultaneously bind the enhancer to form a stable complex? ENKTF-1, AP-4, and AP-2 bind the enhancer simultaneously to form a stable protein/DNA complex as the sum of their individual footprints are observed. Cooperative DNA binding is not observed between ENKTF-1 and AP-4 or AP-2. However, cooperative DNA binding is

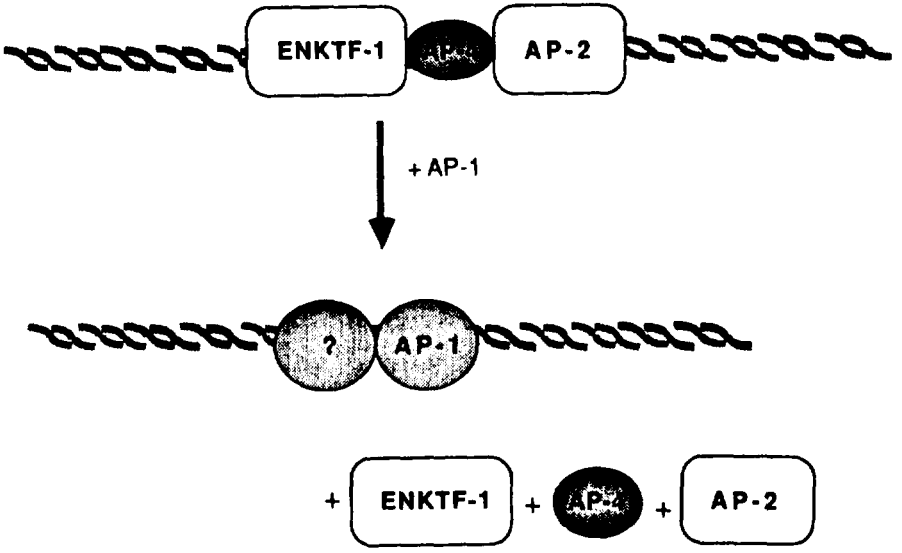


**FIGURE 7.** *In vitro binding studies indicate that ENKTF-1, AP-4, and AP-2 bind the enhancer simultaneously to form* a stable *complex. Conversely, the binding of AP-1 to the ENKCRE-2 element inhibits the binding of ENKTF-1* to *the CRE-1 element, AP-4* to *the ENKCRE-2 element, and AP-2 to the AP-2 element. Hence, in vitro binding studies suggest the formation of two mutually exclusive complexes:* an *AP-1 complex and* a *complex consisting of ENKTF-1, AP-4, and AP-2. The functional role of these complexes in the regulation of proenkephalin transcription is currently unknown.*

observed between AP-2 and AP-4. The physiological significance of this interaction is not yet understood. In contrast, AP-1 preparations inhibit the binding of ENKTF-1, AP-4, and AP-2 to their recognition sites. We believe that this displacement is due to (1) the higher binding affinity of AP-1 for the ENKCRE-2 site and (2) the fact that AP-1 may bind DNA as a large protein complex, which can occlude the binding of nearby factors. This hypothesis is supported by our observation that the repressive effect of AP-1 on the binding of ENKTF-1, AP-4, and AP-2 is completely reversed by competing away AP-1 binding using an ENKCRE-2 oligo. The AP-1 footprint disappears and formation of ENKTF-1, AP-4, AP-2 complex occurs. These results suggest that complex DNA binding interactions at the ENKCRE-2 element are likely to occur *in vivo* and indicate the need for functional *in* vivo assays to determine whether these complexes act to regulate transcription in a positive or negative fashion.

## PROENKEPHALIN AS A NEURAL TARGET GENE REGULATED BY FOS/ JUN NUCLEOPROTEIN COMPLEXES

Because the proenkephalin gene contains a binding site for the transcription factor AP-1 (which consists of fos/jun heterodimers) and because this binding site has been shown to play a critical role in mediating second messenger regulation, this gene represents an excellent model system to explore regulation by fos/jun complexes, In fact, this gene represents the first in vivo target of the fos/jun complex recognized in the nervous system.

Transcription of both fos and jun proteins is rapidly activated *in vivo* by excitation and the activation of neurotransmitter receptors (Greenberg et al. 1986; Hunt et al. 1987; Bartel et al. 1989; Szekely et al. 1988). Newly synthesized fos/jun proteins are thought to act as "third messengers," rapidly activating or repressing transcription of target genes in response to signals transmitted from the cell surface (Morgan et al. 1987). White and Gall (1987) have investigated the regulation of both proenkephalin and c-fos mRNA levels in the hippocampus after dentate gyrus hilus lesion, Shortly after induction of seizures, c-fos mRNA induction peaked at 3 hours followed by a slow decline to control levels by 48 hours, whereas proenkephalin mRNA levels peaked at 18 hours and remained elevated out to 48 hours postlesion. The rapid increase in c-fos RNA observed in granule cells of the dentate gyrus after electrical stimulation or seizure induction suggests that the c-fos/jun nucleoprotein complex may play a role in the subsequent induction of proenkephalin biosynthesis (figure 8).

## COTRANSFECTION STUDIES USING FOS-, JUN-, AND CREB-EXPRESSING PLASMIDS

Cotransfection of a v-jun expression plasmid (cDNA under the control of the CMV promoter) with a proenkephalin/CAT fusion plasmid strongly transactivates CAT expression in the embryonal carcinoma cell line EC-P19. In contrast, cotransfection with c-fos-expressing plasmids produces very little effect (usually a small inhibition is observed) of CAT expression in EC-P19 cells. When c-fos and v-jun expressing plasmids were cotransfected together with pENKAT-12 Into EC-P19 cells, a strong synergistic activation of the proenkephalin enhancer was observed. These results are consistent with a fos/ jun heterodimer strongly activating proenkephalin expression in EC-P19 cells, Cotransfection with a CREB-expressing plasmid also produced strong transactivation of the proenkephalin enhancer. To determine which enhancer elements mediate the effects of jun, fos, and CREB, experiments similar to those described above were performed using the multicopy ENKCRE-2 plasmid as indicator instead of ENKAT-12 (figure 9). These experiments showed that a

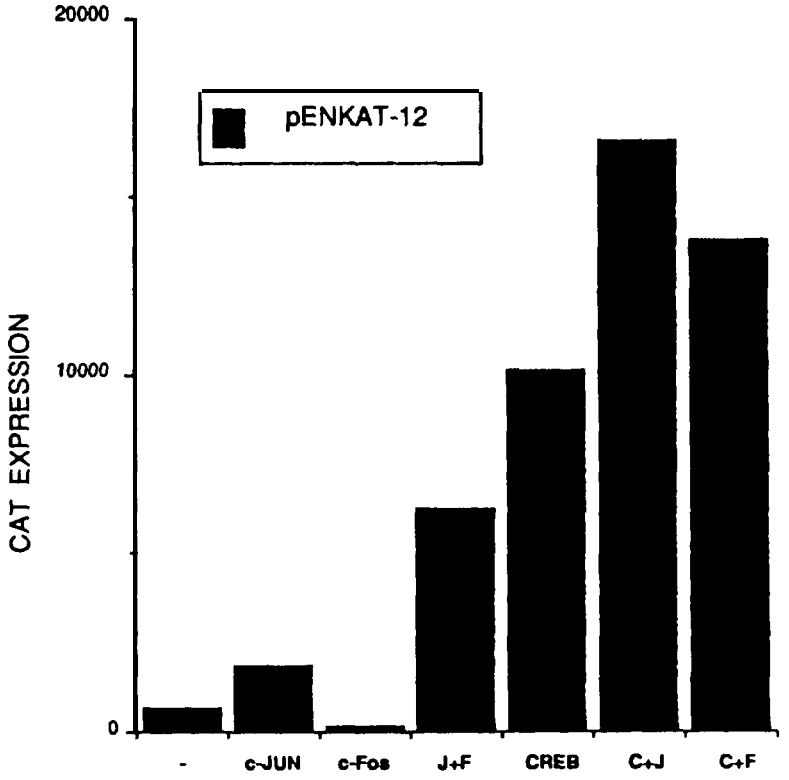


**FIGURE 8.** *Transactivation of the human proenkephalin promoter in vivo by AP-1 and CREB proteins. Cotransfection of embronyal carcinoma cell line EC-P19 with pENKAT-12 (2.5 µg) and plasmids expressing c-jun, c-fos, CREB (10 µg), pRSV-BGAL (10 µg) and pUC-19 to a total of 30 µg DNA/calcium phosphate precipitate. Note that c-fos acts synergistically with c-jun and CREB to promote transcription from the human proenkephalin promoter/enhancer.*

plasmid containing only the ENKCRE-2 with all other elements deleted was able to respond in a fashion very similar to the wild-type enhancer, suggesting that this element alone mediates the above effects of fos, jun, and CREB expression.

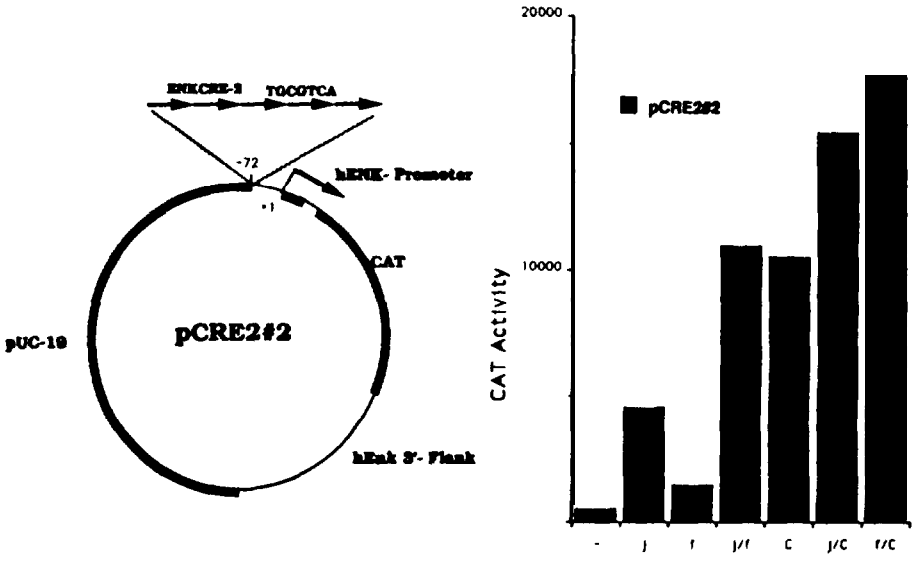


**FIGURE 9.** Transactivation *by c-jun, c-fos, and CREB map to the ENKCRE-2 element. The plasmid pENKCRE2#2, which contains only the ENKCRE-2 element in the absence of all other elements, was cotransfected with c-jun, c-fos, and CREB to determine if this element could mediate the effects of transcription factor cotransfection. As shown, this element alone appears capable, when present in multiple copies, to mediate the effects of c-fos, c-jun, and CREB.*

## SUMMARY AND CONCLUSIONS

It is now apparent that neurotransmitters and drugs that act at neurotransmitter receptors are important regulators of gene expression in the nervous system. These findings suggest that alterations in neural gene expression induced by neurotransmitters or drugs may underlie many stable/adaptive changes in neural signaling. Therefore, it is important to develop an understanding of how neural signaling and synaptic activity influence gene expression and of the role these processes play in normal physiology. The large changes in proenkephalin mRNA levels in both the granule cells of the dentate gyrus and the chromaffin cells of the adrenal medulla in response to electrical or neural stimulation suggest that these responses may have profound effects on neural signaling, on opioid physiology, and ultimately on behavior. Although much has been learned using RNA blotting and *in situ* hybridization techniques to study

changes in mRNA levels in response to physiologic stimuli, these studies are descriptive by nature and cannot address the mechanisms that underlie the observed changes. Investigation of mechanism requires analysis of the intracellular signaling pathways, the responsive DNA regulatory elements, and the proteins transducing synaptic signals into gene regulation (figure 10). In the studies described above, we have defined three different intracellular signaling

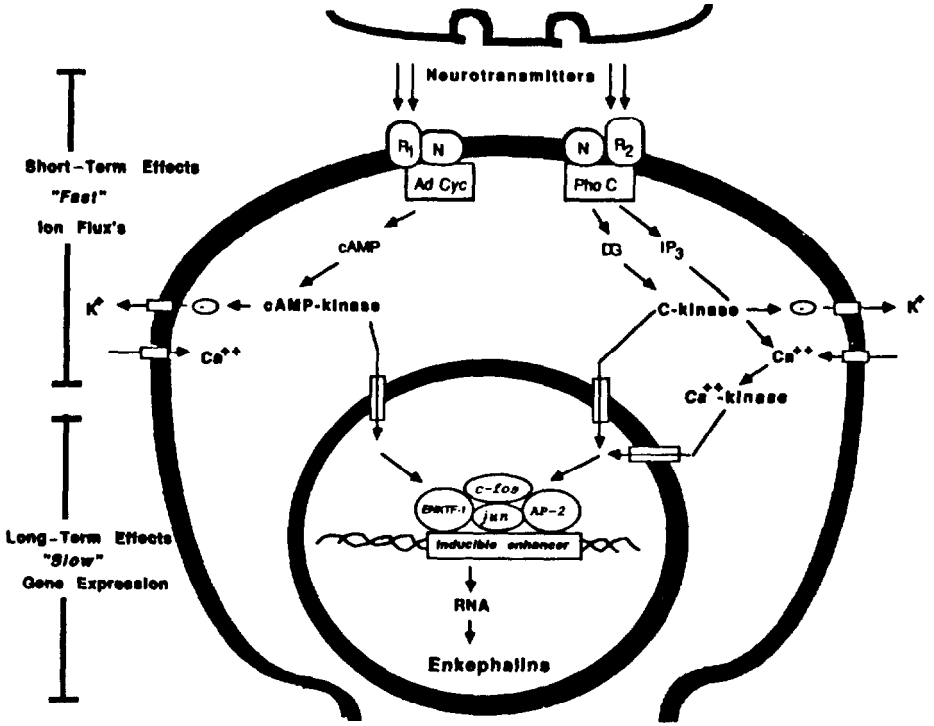


**FIGURE 10.** Synaptic *regulation of proenkephalin gene expression and neural plasticity. Short-term effects of a neurotransmitter binding its cell* surface *receptor include activation of second messenger systems and protein kinases, regulation of ion fiux via channel modification, and modulation of neurotransmitter release. Long-term effects include second messenger- and protein kinase-*mediated *effects on gene expression. These effects serve to regulate the biosynthesis of neurotransmitters and may underly many enviromental and drug-induced stable/adaptive* changes *in neural physiology.*

pathways that, when stimulated, activate transcription of the human proenkephalin gene in a conditional and synergistic fashion. We demonstrate that these three distinct intracellular signaling pathways converge to regulate transcription of the proenkephalin gene via a short DNA enhancer. The protein factors that bind to the enhancer and mediate regulation appear to be complex. At least four different proteins interact with these elements: ENKTF-1, AP-1, AP-4, and AP-2. The recent findings that AP-1 consists of a fos/jun heterodimer; that fos, jun, and CREB all share a common leucine zipper; and that fos, jun, and CREB each exist as a family of proteins further expands the number of potential heterodimers and emphasizes the need to identify family members mediating regulation.

The above complexities suggest a divergence of signals transmitted through a small number of pathways to a large number of regulatory factors (heterodimeric permutations of fos, jun, and CREB proteins) that may differentially regulate and coordinate the neurons' response to stimulation. Because the transcription factors described above are activated by both neurotransmitters and pharmacologic agents, they can be considered as important targets of drug action. Identification and characterization of these factors and determination of how their activities are regulated by synaptic stimulation may lead to the production of specific drugs to activate or block their actions.

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## ACKNOWLEDGMENT

This work was supported in part by National Institute on Drug Abuse grant DA-05706.

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# Regulation of Carboxypeptidase E (Enkephalin Convertase)

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## INTRODUCTION

Many peptide hormones and neurotransmitters are produced initially as large precursors in which the bioactlve peptide is flanked by basic amino acids (Docherty and Steiner 1982). Enzymatic processing by trypsin-like endopeptidases generates intermediates with C-terminal basic residues. These residues then are removed by a carboxypeptidase with a specificity for basic residues. Carboxypeptidase E (CPE) originally was identified as the carboxypeptidase responsible for the formation of met- and leu-enkephalins in the bovine adrenal medulla (Fricker and Snyder 1982; Fricker 1988a). This enzyme initially was designated enkephalin convertase (Fricker and Snyder 1982) and is also known as carboxypeptidase H [EC 3.4.17.10) (Webb 1986). A CPE-like enzyme is present in high levels in several neural and endocrine tissues, such as brain (Flicker and Snyder 1982), pituitary (Fricker et al. 1982), and endocrine pancreas (Docherty and Hutton 1983); CPE mRNA is found in brain, pituitary, and many endocrine tissues (Fricker et al. 1986, 1989; Rodriguez et al. 1989). In adrenal medulla, pituitary, and an insulin-producing tumor, CPE is the only carboxypeptidase activity localized to the peptide-containing secretory granule fraction (Fricker and Snyder 1982; Docherty and Hutton 1983; Hook and Loh 1984). Purified CPE can remove the basic C-terminal residue for a large number of peptide intermediates (Fricker and Snyder 1982; Hook and Loh 1984; Davidson and Hutton 1987; Mackin and Noe 1987; Smyth et al. 1989). These results suggest that CPE is involved in the biosynthesis of numerous peptide hormones and neurotransmitters.

## REGULATION OF CPE ACTIVITY BY pH

The processing of peptide hormones is thought to begin either in the *trans*- Golgi apparatus or in the newly formed secretory granules, depending on the hormone (Orci et al. 1987; Schnabel et al. 1989). Since the pH of these compartments is lower than the neutral pH of the endoplasmic reticulum or

other regions of the Golgi (Anderson and Pathak 1985), it is possible that pH controls the processing enzymes. To investigate whether CPE activity is regulated by pH, we determined $K_{cat}$ and $K_m$ values for dansyl-Phe-Ala-Arg hydrolysis by purified CPE at a variety of pH values. The optimum activity ($K_{cat}/K_m$) is around pH 4.8-5.6 (figure 1), which is consistent with previous studies that measured enzyme activity with only a single substrate concentration (Fricker and Snyder 1982; Docherty and Hutton 1983).

By examining the $K_{cat}K_m$ it is possible to infer the number of ionizing groups that are responsible for the observed pH optimum (Fersht 1983). Typically, a

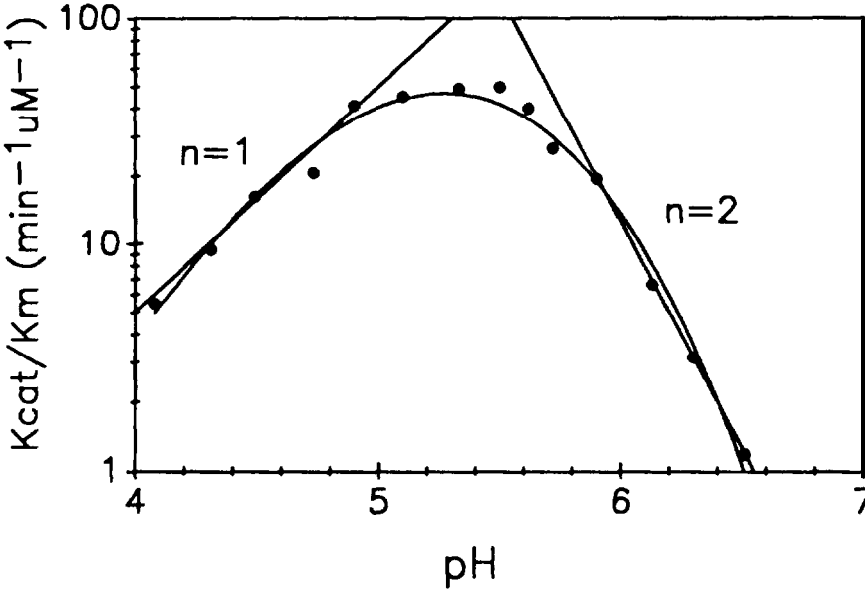


**FIGURE 1.** *Effect of pH on $K_{cat}/K_m$ for dansyl-Phe-Ala-Arg hydrolysis by purified bovine CPE, prepared as described by Fricker and Snyder (1983). The carboxypeptidase assay was performed as described by Fricker and Snyder (1983), using 100 mM sodium* acetate *at the indicated pH. Substrate concentrations for the kinetic analysis were 200, 100, 50, 25, and 12.5 µM, each* assayed *in* triplicate for all *pH* values. Variation within each *triplicate determination was typically less than 10 percent. $K_{cat}$ and Km were* calculated *from Lineweaver-Burke plots.*

change in the ionization of a single amino acid is sufficient to block enzymatic activity, providing that residue is involved with the substrate binding or the catalytic activity. For single ionizations, a plot of log $K_{cat}/K_m$ vs. pH shows a slope of l , which is the case for the ionization of CPE that occurs with a pKa of -5 (figure 1). If two groups are ionizing with similar pKa values and both groups are critically involved with the enzymatic activity, the slope of the plot will be ±2 This is found with CPE for pH values above 5.8 (figure 1). The enzymatic activity of CPE is twice as sensitive to pH values above 5.8, compared with its sensitivity to pH values below 5. Thus, the pH of the environment has a dramatic effect on the activity of CPE.

**MULTIPLE FORMS OF CPE**

Early studies on CPE activity in bovine adrenal medulla chromaffin granules detected both soluble and membrane-associated forms of this enzyme (Fricker and Snyder 1982). Subsequent studies detected soluble and membrane-bound forms of CPE in bovine brain and pituitary (Supattapone et al. 1984); in anglerfish endocrine pancreas (Mackin and Noe 1987); and in neural tissue from *Xenopus,* shark, and *Aplysia* (Fricker and Herbert 1988). In these tissues, one form of CPE activity is soluble at acidic pH values (pH 5-6). Of the membrane-bound CPE activity, 10 to 30 percent can be extracted with 1 M NaCl (Supattapone et al. 1984; Hook 1984; Fricker 1988b). Repeated extractions of the membranes with hlgh salt (at pH 5-6) do not solubilize the majority of the membrane-associated CPE activity. This suggests that at least two distinct membrane-associated forms of CPE are present in these tissues in addition to the soluble form of CPE (S-CPE). One membrane form (M1-CPE) is an extrinsic membrane protein that is released by high salt washes. The other membrane form of CPE (M2-CPE) can be extracted in high yield by a combination of 1 percent Triton X-100 and 1 M NaCI, at pH 5.5 (Supattapone et al. 1984). Detergent alone does not solubilize either membrane form (Supattapone et al. 1984). Interestingly, the membrane-bound forms of CPE are extracted by neutral pH buffers (Mackin and Noe 1987; Fricker 1988b). Since the common mechanism for the attachment of proteins to membranes (such as hydrophobic transmembrane-spanning sequences or phosphatidylinosltol groups) is not known to be dependent on pH in the 5-7 range, it is likely that a novel mechanism is responsible for the membrane association of CPE.

The soluble and membrane forms have been purified to homogeneity using identical chromatographic steps (Supattapone et al. 1984) indicating similar physical properties. The enzymatic properties of the soluble and membrane-associated forms of CPE are also very similar (Supattapone et al. 1984). All forms contain the same partial N-terminal amino acid sequence, and tryptic

maps are almost identical (Fricker et al. 1986). The only reported difference between the forms is the apparent molecular weight-approximately 50 kDa for S-CPE and 52 kDa for M2-CPE (Supattapone et al. 1984).

Results from genomic Southern blot analysis, Northern blot analysis, and nucleotide sequence analysis of bovine and rat CPE cDNA clones suggest that the soluble and membrane-associated forms arise from posttranslational processing of a single precursor and not from different genes or mRNA splicing. The CPE precursor (preproCPE), deduced from the nucleotide sequence of cDNA clones, is 475 (bovine) or 476 (rat) amino acids in length (Fricker 1988c; Fricker et al. 1989; Rodriguez et al. 1989). The precursor contains a short N-terminal peptide 41 amino acids in length that is not present on the forms of CPE that had been partially sequenced (figure 2). The first 20 to 25 residues of the precursor resemble a signal peptide ("pre" sequence). The remainder of the N-terminal extension ("pro" sequence) is presumably removed by a trypsin-like endopeptidase. There are five adjacent Arg residues immediately preceding the known N-terminus of the active forms of CPE. The conversion of proCPE into CPE appears to be fairly rapid since tissue levels of proCPE are extremely low (Fricker and Das, unpublished observation).

Analysis of the predicted sequence of CPE does not reveal any regions of sufficient hydrophobicity and length to form a membrane-spanning helical structure. However, analysis of the C-terminal portion of CPE indicates a propensity of this region to form an amphiphilic helix with eight hydrophobic residues protruding from one face of an $\alpha$-helix (Fricker et al. 1986). Preceding this potential amphiphilic helix is a highly charged region containing nine adjacent basic and acidic amino acids, Antisera raised against C-terminal peptides are able to distinguish between the soluble and membrane-associated forms of CPE (Fricker et al. 1990a, 1990b, 1990c), leading to the model shown in figure 2. The membrane-associated form of CPE that is extractable with a combination of NaCl and detergent (M2-CPE) contains a full-length C-terminus, and it is held to the membrane by a combination of electrostatic and hydrophobic interactions. The membrane form of CPE that can be extracted with NaCl alone (M1-CPE) contains the highly charged portion of the C-terminal region but not the potential amphiphilic region. This form of CPE is held to the membrane primarily through ionic interactions with an unidentified intrinsic membrane protein (or proteins). The soluble form (S-CPE) does not contain either the highly charged region or the potential amphiphilic region. The solubilization of both membrane-associated forms of CPE by neutral pH buffers is presumably the result of a pH-induced conformational change that disrupts the structure of the C-terminal region,

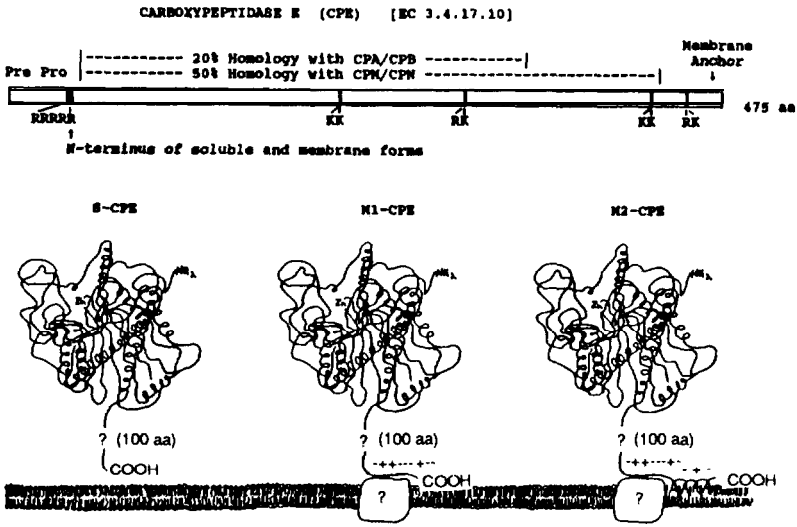


**FIGURE 2.** *Schematic representation of the CPE precursor (top) and the postulated structure of various forms of CPE (bottom). The amino acid sequence of preproCPE* was *deduced from the nucleotide sequence of a bovine cDNA clone (Fricker et* al. 1986): *Rat preproCPE has >90 percent amino acid identity, and* all *the indicated features are* the same *for the two species (Fricker et al. 1989). All sequences of two or more adjacent* basic amino *acids are indicated (K=Lys, R=Arg). The regions showing the 20-percent amino* acid *identity with carboxypeptidases A (CPA) and B (CPB) and the 50-percent amino* acid *identity with carboxypeptidases M (CPM)* and *N (CPN) are indicated. The N-terminus of the forms of CPE that had been purified and partially sequenced (S-CPE and M2-CPE) also is indicated. The hypothetical structures of the soluble form (S-CPE), of the membrane-associated form that is released by high salt* washes *(M1-CPE), and of the membrane-associated form that is released by detergent in the presence of high salt (M2-CPE) are based on the three-dimensional structure of carboxypeptidase B (Schmid and Herriott 7976). A region of CPE without homology to CPA or CPB* is indicated *by* a *"?": The length of this region is 100 amino acids (AA). Another "?" indicates a hypothetical membrane protein that binds the M1 and M2 forms of CPE. The approximate length of the C-terminal portion of the various forms of CPE was determined with antisera directed against* several *C-terminal peptides (Fricker et al. 1990a).*

## EFFECT OF MEMBRANE ASSOCIATION ON THE ACTIVITY OF CPE

The activation state of M2-CPE is influenced by its association with membranes (Hook 1985; Fricker 1988b). Upon extraction with either 1 M NaCl and 1 percent Triton X-100 at pH 5.5 or neutral pH buffers without detergent or salt, the amount of CPE activity recovered in the supernatant and pellet fractions is typically two to three times higher than the amount of CPE activity present before extraction (figure 3, lanes 1-3). Control extractions with 0.1 M NaAc (pH 5.5) do not release any of the membrane-bound CPE activity, nor is the amount of CPE activity altered (figure 3, lane 4). The high pH-induced solubilization and activation of the membrane-associated CPE is partially reversible: If the membranes are treated at high pH but then brought to pH <6 before centrifugation, much less CPE is solubilized and the total amount of CPE activity is reduced (figure 3, lane 5) compared with the samples centrifuged at the high pH (lane 3).

Kinetic analysis of the membrane fractions before and after the extraction revealed that the observed activation is due to both a higher $V_{max}$ and a lower $K_m$ for substrate hydrolysis (Fricker 1988b). It is not likely that the activation results from the conversion of catalytically inactive molecules to active ones since pretreatment of the membranes with an active site-directed irreversible inhibitor of CPE (bromoacetyl-D-arginine) completely blocks the activation (Fricker 1988b). Once M2-CPE is solubilized and purified to homogeneity, the $K_{cat}$ and $K_m$ for substrate hydrolysis are similar to the values with purified S-CPE (Supattapone et al. 1984). Furthermore, the treatment of purified S-CPE or M2-CPE with high pH buffers or 1 percent Triton/1 M NaCl at pH 5.5 does not influence the enzymatic activity (measured at pH 5.5), suggesting that the activation is not a result of intrinsic changes within the CPE molecule (Fricker 1988b).

These results indicate that the association of CPE with membranes lowers the enzymatic activity. The extent of the association with membranes is determined by the ratio of the various forms of CPE (figure 2), as well as the pH. At neutral pH, the majority of CPE is soluble, although there is little enzymatic activity at this pH due to the ionlzation state of sensitive groups. As the pH of the environment drops in the trans-Golgi network or in newly formed secretory vesicles, the ionization of these groups changes, thus activating CPE. However, some of the M1-CPE and M2-CPE will associate with the membranes as the pH drops below 7, and this membrane binding decreases their enzymatic activity. This hypothesis predicts that the membrane forms of CPE will have a broader pH optimum than will S-CPE. To test this, we compared the effect of pH on the CPE activity in soluble and membrane fractions of bovine pituitary (figure 4). The CPE activity in the 0.1 M NaAc, pH 5.5 extracts (S-CPE) shows

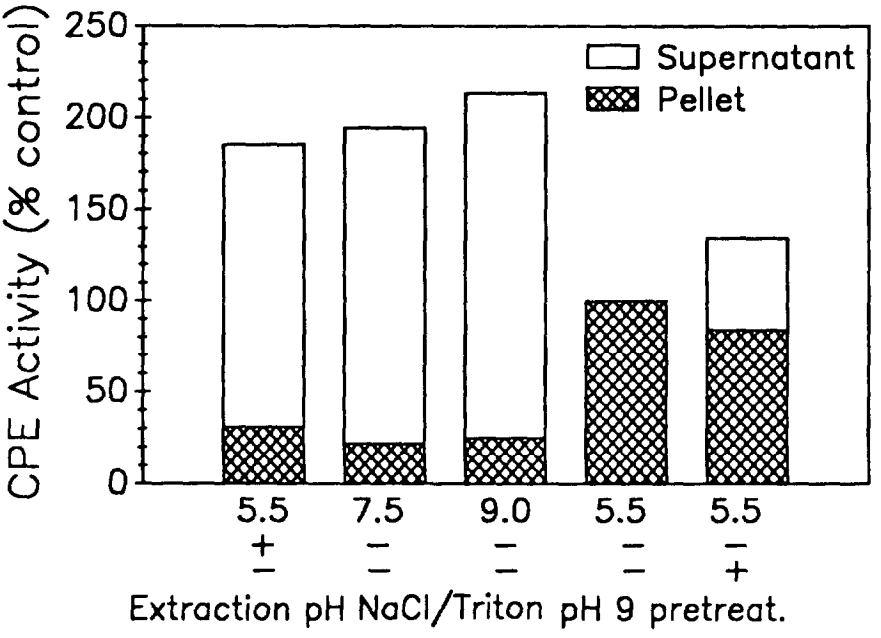


**FIGURE 3.** *Extraction* and activation *of membrane-bound CPE from bovine pituitary membranes. The amount of CPE activity, determined with dansyl-Phe-Ala-Arg, in the soluble* extract *(open bars) or remaining in the pellet fraction (cross-hatched* bars) *is shown relative* to *the amount of CPE activity initially present in the membranes. The experiment was performed as described by Fricker (1988b), using 50 mM NaAc (pH 5.5, 50 mM sodium phosphate (pH 7.5), or 50 mM sodium bicarbonate (pH 9.0).*

a rapid drop in enzymatic activity as the pH is increased above 5.5. In contrast, membrane homogenates containing Ml-CPE and M2-CPE are less sensitive to pH values between 6 and 7 and are approximately three to four times more active than the soluble fraction at pH 6.5 and 7.0. Thus, the activity of CPE can be controlled by the pH and by the extent of the processing of proCPE into the soluble and membrane forms.

## DISTRIBUTION OF SOLUBLE AND MEMBRANE-ASSOCIATED FORMS OF CPE IN RAT TISSUES

Previous studies found CPE activity in rat brain and pituitary but not in many other tissues of the rat (Fricker et al. 1982). In the present study, we have used a substrate that is 20-fold more selective for CPE. As previously found, high

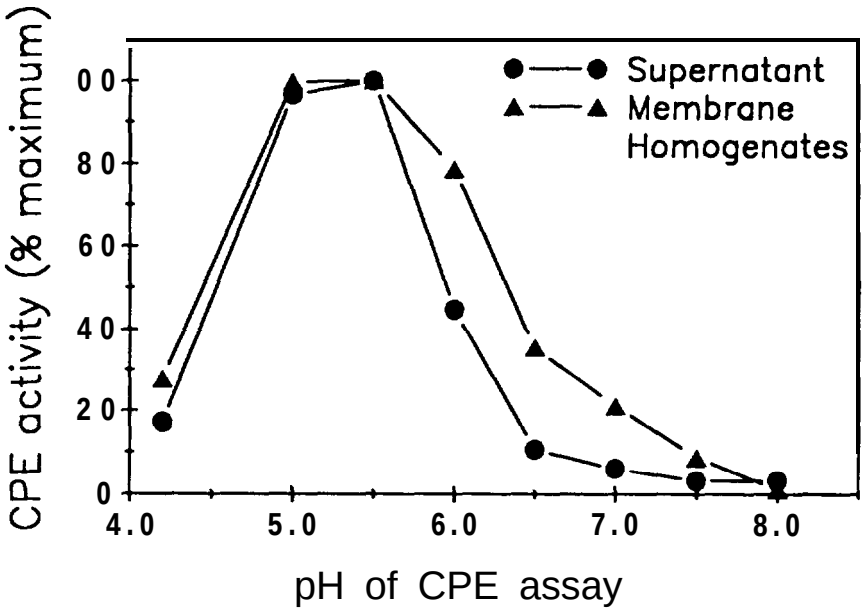


**FIGURE 4.** *Effect of pH on CPE activity in bovine pituitary fractions. One bovine pituitary was homogenized (Polytron) in 20 mL of 0.7 M NaAc, pH 5.5, and then centrifuged for 1 hour at 50,000 x g. The pellet was washed once with the same buffer, and then CPE activity in either the supernatant fraction or the membrane homogenate was determined in triplicate with dansyl-Phe-Ala-Arg in 0.7 M Tris acetate buffers at the indicated pH.*

levels of CPE are present in rat pituitary, with moderately high levels in brain. Moderate levels of CPE are present in adrenal and intestine, and low levels are detectable in heart and eye. In most of the tissues examined, approximately two-thirds of the total CPE activity is soluble (table 1). Less of the CPE activity in brain and heart is soluble, with values of 35 percent and 45 percent, respectively. The tissue-specific variations in the amount of CPE that is soluble is presumably due to differences in the posttranslational processing of CPE.

The level of CPE activity in many, but not all, fetal (E17) rat tissues is similar to the level in the adult (table 1). A large difference is found in the adrenal, where CPE activity is approximately 10-fold higher in adult tissue. There is also a difference in the relative amounts of the soluble and membrane-bound forms of CPE for the two age groups (table 1). In all tissues except brain, the fraction of CPE that is soluble increases substantially between E17 and adult rats. In

**TABLE 1.** *Comparison of levels of CPE in adult and* fetal *rat tissues*

| Tissue | Adult CPE Activity nmoles/min/mg | Adult % Soluble | Fetal (El 7) CPE Activity nmoles/min/mg | Fetal (El 7) % Soluble |
|---|---|---|---|---|
| Pituitary | 62 ±27 | 66±13 | | |
| Brain | 10 ± 2 | 35± 7 | 6.4 ± 2.3 | 56±9 |
| Adrenal | 5.3± 1.2 | 62± 8 | 0.55 | 20 |
| Intestine | 4.0± 1.6 | 64± 2 | 6.2 ± 3.3 | 39± 6 |
| Heart | 1.2± 0.4 | 45±10 | 1.2 ± 0.6 | 28±10 |
| Eye | 1.1± 0.2 | 61 ±10 | 1.5 ± 0.5 | 35± 9 |

NOTE: Data are the average of three rats±standard error (except fetal adrenal: tissue from three animals was pooled). Carboxypeptidase activity was determined with the substrate dansyl-Phe-Ala-Arg (Fricker and Snyder 1983); CPE, expressed in nmole/min per mg protein, is defined as the difference in carboxypeptidase activity measured in the presence of 1 mM $CoCl_2$ compared with the activity in the presence of 1 µM GEMSA (Stack et al. 1984). E17=embryonic day 17.

brain, a larger fraction of CPE activity is soluble in the fetal rats than in the adult animals. These findings suggest that both the total amount of CPE and the relative amounts of the soluble and membrane-bound forms are developmentally regulated in a tissue-specific manner.

The tissue distribution of CPE activity generally is consistent with previous studies examining the localization of this enzyme in various tissues using a radiolabeled active site-directed inhibitor, [$^3$H]-GEMSA (Strittmatter et al. 1984, 1986), and with the levels of CPE mRNA (Fricker et al. 1989; Rodriguez et al. 1989). The major difference in the results of the various studies is in the levels of CPE in the intestine. This tissue has moderate amounts of CPE activity and [$^3$H]-GEMSA binding (Strittmatter et al. 1984; Lynch et al. 1987) but extremely low levels of CPE mRNA (Fricker et al. 1989). The reason for this discrepancy is not known.

## REGULATION OF CPE SECRETION

CPE activity is secreted from a variety of peptide hormone-producing cells, including a mouse pituitary corticotrophic cell line (AtT-20 cells), a rat anterior pituitary-derived cell line ($GH_4C_1$ cells), and primary cultures of bovine adrenal

chromaffin cells. The secretion of both CPE and prooplomelanocottin (POMC)-derived peptides from the AtT-20 cell line can be stimulated by corticotrophic-releasing hormone (CRH) and inhibited by dexamethasone (Mains and Eipper 1984). The secretion of CPE from primary cultures of bovine adrenal chromaffin cells is stimulated by nicotine and inhibited by nicotinic antagonists (Hook and Eiden 1985). CPE secretion from $GH_4C_,$ cells is stimulated by either KCI or thyrotropin releasing hormone (Fricker et al. 1990b). These observations are consistent with the localization of CPE to the secretory granules of rat pituitary and bovine adrenal medulla (Fricker and Snyder 1982; Docherty and Hutton 1983). The release of CPE is presumably a consequence of the secretion of the peptide, although it is possible that secreted CPE performs some unknown function.

Recently, CPE has been found to be present in type I astrocytes that were cultured from E17 rat hypothalami (Vilijn et al. 1989). The level of CPE activity in the cultures of astrocytes is approximately 10-fold lower than the level of CPE activity in rat hypothalamic neuronal cultures (table 2). However, the amount of CPE activity secreted from the neurons is only fourfold to fivefold higher, indicating that the basal rate of secretion is different for these two cell types. The regulation of the secretion also differs: KCI stimulates the secretion of CPE from the neuronal cultures but does not alter the amount of CPE secreted from the cultured astrocytes (table 2). The level of CPE mRNA is fourfold higher in astrocytes than in neurons, suggesting that the production of CPE protein in astrocytes is higher than predicted from the low cellular levels of enzymatic activity (table 2).

**TABLE 2.** *CPE activity and mRNA levels in primary cultures of hypothalamic neurons (neonatal) and type I* astrocytes *(E 17)*

| Cell | Specific Activity Cellular CPE. | Secreted CPE[b] Basal | Secreted CPE[b] KCI Stimulation | CPE mRNA[C] |
|---|---|---|---|---|
| Neurons | 120±20 | 35 | 103 | 1.0 |
| Astrocytes | 11± 2 | 8 | 8.8 | 4.4 |

[a]CPE was measured with the substrate dansyl-Phe-Ala-Arg (Fricker and Snyder 1983) and is expressed in pmoles/min-$10^6$ cells±standard error of the mean for triplicate determinations (Vilijn et al. 1989).
[b]pmoles/min secreted from $10^6$ cells per hour.
[C]Relative densitometric units/µg total RNA (Vilijn et al. 1989).

## PHARMACOLOGICAL REGULATION OF CPE IN TISSUES AND CELL CULTURE

The regulation of CPE has been examined in a variety of systems. Initial experiments focused on the levels of CPE activity in rat and mouse brain upon addiction to morphine. The results of these studies were inconclusive: In some experiments, high doses of morphine caused a 50-percent decrease in the level of S-CPE activity but had no effect on the level of membrane-associated CPE activity (Fricker, Supattapone, and Snyder, unpublished observation). However, this effect was not consistently reproducible. Since CPE is present in many cell types within the brain, it is possible that changes in a small number of cells are not detected by measuring CPE activity in whole brain.

Recent studies on the *in vivo* regulation of CPE have focused on the rat adrenal medulla since there is only a single type of peptide-producing cell in this tissue. In the rat adrenal medulla, reflex splanchnic stimulation caused by insulin-induced hypoglycemic shock increased the level of enkephalin mRNA approximately 16-fold and increased the level of enkephalin peptides nearly 150-fold (Viveros et al. 1987). This treatment also caused a onefold increase in the levels of both CPE mRNA and enzymatic activity within several days of treatment (Fricker et al. 1990c). Although this degree of change was substantially smaller than the changes in enkephalin mRNA and peptide levels, the direction of the change was the same (table 3).

The regulation of CPE also has been examined in a variety of cell culture systems. Primary cultures of bovine adrenal chromaffin cells produced both enkephalin-containing peptides of high molecular weight and enkephalin peptides of lower molecular weight. Reserpine treatment increased the levels of the low molecular weight species and decreased the amount of the larger peptides, suggesting that the processing rate is stimulated (Wilson et al. 1980; Eiden et al. 1984; Lindberg 1986; Viveros et al. 1987). The effect of reserpine on the level of enkephalin mRNA is not consistent between different studies: Eiden and colleagues (1984) found a decrease in enkephalin mRNA levels upon reserpine treatment, but Viveros and colleagues (1987) found no change. The effect of reserpine treatments on CPE activity also differs between studies. Hook and colleagues (1985) found that CPE activity was stimulated by treatment of the cells with reserpine and that this stimulation was due to a change in the $K_m$ for substrate hydrolysis. We have also found that reserpine stimulates CPE activity in chromaffin cells, but this treatment does not change the $K_m$ for substrate hydrolysis (Fricker, Das, and Lindberg, unpublished observation). Instead, we found that the apparent $V_{max}$ was increased twofold to threefold by the treatment, which could be due either to a change in the turnover rate of the enzyme or to an increase in the number of CPE molecules.

**TABLE 3. Regulation of CPE activity in cell culture and in tissue**

| Cells/Tissue | Hormone | Treatment | Effect on Hormone | | Effect on CPE | | Source |
|---|---|---|---|---|---|---|---|
| | | | mRNA | Peptide | mRNA | Activity | |
| Rat adrenal medulla | Enkephalin | Insulin shock | ↑↑ | ↑↑↑ | ↑ | ↑ | Fricker et al. 1990c |
| Bovine adrenal chromaffin cells | Enkephalin | Reserpine | ↑↓ | ↑ | ? | ↑ ($K_m$) | Hook et al. 1985 |
| | | Reserpine | | | ? | ↑ ($V_{max}$) | Fricker, Das, and Lindberg, unpublished data |
| GH4C1 (rat anterior pituitary) | Prolactin | $E_2$/Insulin/EGF | ↑↑ | ↑↑↑ | 0 | ↑ ($V_{max}$) | Fricker et al. 1990b |
| AtT-20/D16v (mouse anterior pituitary) | ACTH | Dexamethasone | ↓ | ↓ | 0 | 0 | Thiele and Fricker 1988 |
| | | Dexamethasone | | | ↓ | ? | Rodriguez et al. 1989 |
| | | CRH | ↑ | ↑ | 0 | 0 | Thiele and Fricker 1988 |
| | | CRH | | | ↑ | ? | Rodriguez et al. 1989 |

KEY: ↑=1- to 2-fold increase; ↑↑=5- to 15-fold increase; ↑↑↑=50- to 150-fold increase; ↓=decrease; ↑↓=conflicting results; 0=no change; ?=not determined

This kinetic change does not appear to be the result of the redistribution of CPE from the membrane-associated form to the soluble form: The amount of CPE that is soluble is not affected by reserpine treatment (Fricker, Das, and Lindberg, unpublished observation).

The $GH_4C_1$ cell line, derived from rat anterior pituitary, produces both prolactin and growth hormone (Tashjian et al. 1968; Tashjian 1979). The treatment of these cells with 1 nM estradiol, 300 nM insulin, and 10 nM epidermal growth factor increases both the number of secretory granules and the intracellular level of prolactin approximately 30- to 50-fold (Scammell et al. 1986), Prolactin mRNA is elevated approximately 10-fold by the hormonal treatment, but CPE mRNA is not altered by more than 10 percent (Fricker et al. 1990b). The level of CPE activity is increased approximately onefold by the treatment, which is due to an increase in the apparent $V_{max}$, with no effect on the $K_m$.

The regulation of CPE activity and mRNA levels also has been examined in the AtT-20/D16v cell line. This mouse anterior pituitary-derived cell line produces high levels of POMC-derived peptides, such as adrenocorticotropic hormone (ACTH) (Eipper and Mains 1980). Treatment of these cells with

dexamethasone causes a decrease in POMC mRNA levels and ACTH levels (Eipper and Mains 1980). CRH increases both POMC mRNA and ACTH levels in this cell line. in addition, the level of peptidyl-glycine-α-amidating monooxygenase, the enzyme responsible for the amidation of the C-terminus of many peptide hormones, also is lowered by the dexamethasone treatment and is elevated by the CRH treatment (Eipper et al. 1987). These treatments do not appear to alter the level of CPE activity (Mains and Eipper 1984; Thiele and Fricker 1988) or the level of CPE mRNA (Thiele and Fricker 1988), although another study reported changes in CPE mRNA that are comparable to the changes in POMC mRNA (Rodriguez et al. 1989). The treatment paradigm and culturing conditions differed slightly between the two studies, and it is possible that these differences influenced the results.

These studies indicate that CPE can be regulated *in* vivo and in cell culture. In general, the changes in CPE activity and/or CPE mRNA levels are in the same direction but of a smaller magnitude than the changes in bioactive peptide and/or mRNA levels. This is consistent with the proposal that CPE is not a rate-limiting enzyme in the production of most peptide hormones. However, purified CPE has recently been found to slowly convert α-neoendorphin to ß-neoendorphin, and porcine ß-endorphin 1-27 to ß-endorphin 1-26, *in vitro* (Smyth et al. 1989). Since tissue levels of α-neoendorphin and ß-endorphin 1-27 are often comparable to the levels of the C-terminally shortened peptides, it is likely that CPE is the rate-limiting enzyme in the production of ß-neoendorphin and ß-endorphin 1-26. Small changes in CPE activity presumably would have a significant effect on the production of these peptides.

## SUMMARY

CPE, EC 3.4.17.10, is involved in the production of numerous peptide hormones and neurotransmitters. To investigate whether the regulation of this enzyme may play a role in the control of peptide hormone biosynthesis, we have examined potential mechanisms by which CPE activity can be regulated. These studies have involved kinetic analysis of purified enzyme and of tissue extracts, as well as the determination of enzyme activity and mRNA levels in a variety of tissues and cell lines. The major findings are that (1) pH has a significant influence on the activity of purified CPE; (2) pH also has an effect on the binding of CPE to membranes, and the membrane binding influences CPE activity; (3) the levels of CPE activity and the degree of membrane binding vary between tissues and developmental state; and (4) in several cell lines and tissues, CPE activity is increased by treatments that increase the levels of peptide hormones, although the changes in CPE levels are typically much smaller than the corresponding change in peptide levels.

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## ACKNOWLEDGMENTS

This work was supported by National Institute on Drug Abuse grant DA-04494 (LDF), by an Alfred P. Sloan research fellowship (LDF), and by National Institute of Arthritis, Diabetes, and Digestive Kidney Diseases (now called National Institute of Diabetes and Digestive and Kidney Diseases) training grant T32-AM07513 (RSK).

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# Use of Site-Directed Mutagenesis To Elucidate the Active Site of Neutral Endopeptidase 24.11 (Enkephalinase)

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It is a reasonably well-accepted hypothesis that the physiological actions of the enkephalins and probably other opioid peptides are terminated by peptidase action. Thus, these peptidases play a key role in regulating the action of opioid peptides. Neutral endopeptidase 24.11 (NEP) is one such peptidase. The enzyme is a 750-amino acid integral membrane protein composed of a 23-amino acid cytoplasmic domain; a 24-amino acid hydrophobic segment that functions as a transmembrane region and a signal peptide; and a 699-amino acid extracellular domain, which contains the active site (Malfroy et al. 1987; Devault et al. 1987; Shipp et al. 1988). Hydrolysis by NEP appears to represent the major pathway by which enkephalins are inactivated at enkephalinergic synapses. Based on this function, NEP has been given the trivial name "enkephalinase" (Malfroy et al. 1978). In addition to the enkephalins, there are other opioid peptides that have been shown to be substrates for NEP *in vitro.* These include met-enkephalin-Arg-Phe, gamma endorphin, and beta lipotropin 61-68 (Hersh 1984). However, the relative importance of the enzyme in inactivating these opioid peptides *in vivo* has not been investigated thoroughly. NEP also has been implicated as being involved in the regulation of blood pressure. It is the major degradative enzyme for the potent vasso active peptides: atrial natriuretic peptide (Koehn et al. 1987; Sonnenberg et al. 1988) and endothelin (Vijayaraghavan et al. 1990). Altogether, there are more than 20 physiologically active peptides that are reported to be cleaved by the enzyme (table 1).

It has been demonstrated recently that NEP is identical to the tumor antigen CALLA (common acute lymphoblastic leukemia antigen) (Shipp et al. 1989; LeTarte et al. 1988) and that CALLA represents active NEP on these cells (table 2). CALLA is expressed by early lymphoid progenitors that are as yet uncommitted to B- or T-cell lineage or are at the earliest stages of B-cell differentiation. CALLA is barely detectable on mature B cells, suggesting that it may play a role in the early stages of lymphoid differentiation. In addition,

**TABLE 1.** *Physiologically active peptides cleaved by NEP*

| | | |
|---|---|---|
| Met-enkephalin | Bradykinin | Neurokinin A |
| Met-enkephalin-Arg-Phe | Gamma-endorphin | Neurokinin B |
| Angiotensin I | Chemotactic peptide | Interleukin I |
| Angiotensin II | Beta lipotropin 61-69 | Somatostatin |
| Angiotensin III | Leu-enkephalin | CCK-8 |
| Dynorphin 1-9 | Substance P | ANF |
| Dynorphin 1-13 | Oxytocin | Endothelin |
| Physalaemin | Neurotensin | |

**TABLE 2.** *Identification of active NEP on CALLA+ leukemic and* glioma cell *lines*

| | Specific Activity (pmols/min/$10^6$ cells) | |
|---|---|---|
| Cell Line | - Phosphoramidon | + Phosphoramidon |
| Leukemic cells | | |
| Nalm-6 (CALLA+) | 80 | 2.8 |
| Nall-1 (CALLA+) | 42 | 2.6 |
| SMS (CALLA-) | 3.6 | 3.5 |
| Gliomas | | |
| #71 | 32 | 2.1 |
| #229 | 14 | 1.6 |
| #427 | 10 | 1.1 |

NOTE: Enzyme assays were conducted on whole cell suspensions in 25 mM HEPES buffer, pH 7.4, using 0.1 mM glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide as substrate.

CALLA/NEP has been detected on several gliomas (Carrel et al. 1982). As shown in table 2, this activity is comparable to that found on leukemic cells.

It has been demonstrated that NEP produces a naloxone reversible analgesia in experimental animals (Roques et al. 1980; Chipkin et al. 1982; Greenberg and O'Keefe 1982; Lecomte et al. 1986), and several NEP inhibitors currently are being evaluated clinically for their analgesic properties. A more detailed knowledge of the active site of NEP would be useful for the design and synthesis of new inhibitors of the enzyme of potential clinical use.

The specificity of NEP is directed toward cleavage of substrates on the amino side of hydrophobic amino acids (Hersh and Morihara 1986; Pozsgay et al. 1986; Hersh 1982). In this respect, NEP resembles the well-characterized bacterial neutral endopeptidases of which thermolysin is the best characterized. Comparison of the specificity of thermolysin and NEP with synthetic substrates indicates that the enzymes are similar although not identical (Hersh and Morihara 1986; Pozsgay et al. 1986). This is illustrated in table 3. As can be seen in the table, the relative order of reactivity between the two enzymes is similar with the three methoxynaphthylamide (4MeO2NA) substrates; however, with a nitrophenylalanine-containing substrate, activity can be detected only with NEP. It is believed that the substrate binding site in NEP is larger and less hydrophobic than in the bacterial endopeptidases, but these differences are relatively modest when one considers the similarities in the overall specificity of NEP and the bacterial endopeptidases.

TABLE 3. Comparison of NEP and thermolysin with synthetic substrates

| | NEP | | Thermolysin | |
|---|---|---|---|---|
| Substrate | Km (mM) | Kcat ($s^{-1}$) | Km (mM) | Kcat ($s^{-1}$) |
| glut-Ala-Ala-Phe-4MeO-2NA | 0.07 | 23.00 | 0.55 | 101.00 |
| glut-Gly-Gly-Phe-4MeO-2NA | 0.09 | 0.14 | 0.55 | 0.70 |
| glut-Ala-Ala-Pro-Phe-4MeO-2NA | 0.27 | 6.40 | 0.95 | 1.40 |
| dansyl-D-Ala-Gly-(NO,)Phe-Gly | 0.08 | 3.00 | (0.10)[a] | <)0.005 |

[a]No reactivity was detected at a substrate concentration of 0.1 mM.

NOTE: glut=glutaryl

Both NEP and the bacterial endopeptidases are inhibited specifically by the dipeptide analog phosphoramidon, a property that has been used to distinguish these enzymes from other peptidases. The similarity between NEP and the bacterial endopeptidases extends beyond substrate and inhibitor specificity. There are two regions of amino acid sequence that are conserved between the bacterial and mammalian enzymes (table 4). One of these is a helical segment containing (1) two histidines that, in thermolysin, serve as zinc ligands and (2) a glutamic acid that, in thermolysin, is believed to act as an acid-base catalyst. The other region contains a histidine that, in thermolysin, is proposed to stabilize a tetrahedral reaction intermediate but appears nonfunctional in NEP (Erdos and Skidgel 1989).

**TABLE 4.** *Sequence homology between NEP and bacterial endopeptidases*

| | | |
|---|---|---|
| NEP | G G I G M V I G H E I T H G F | 582 |
| Thermolysin | G G I - D V V A H E L T H A V | 146 |
| B. cereus | G G I - D V I G H E L T H A V | 146 |
| B. subtilis | G S L - D V T A H E M T H G V | 146 |
| NEP | G G Q H L N - G I - N | 639 |
| Thermolysin | G G V H I N S G I I N | 238 |
| B. cereus | G G V H T N S G I I N | 238 |
| B. subtilis | - G V H T N S G I P N | 238 |

NEP does differ, however, from the bacterial endopeptidases in that NEP contains an active site arginine that is proposed to interact with the C-terminal carboxylate of its substrates (Jackson and Hersh 1986; Malfroy and Schwartz 1982; Beaumont and Roques 1986). The presence of this active site arginine results in the preferential binding of substrates containing a free C-terminal carboxylate as compared to their corresponding amide (table 5), and it leads to the preferential cleavage of C-terminal carboxylate containing substrates near their C-terminus. For example, angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) contains four potential cleavage sites, which are located on the amino side of valine[3], tyrosine[4], isoleucine[5], and phenylalanine[8] (shown in bold); yet, NEP cleavage of this peptide occurs preferentially at the Pro-Phe bond (Gafford et al. 1983). Based on this observation and the sequence homology between the carboxy-terminal region of NEP and the N-terminal domain of carboxypeptidase A, it has been suggested that NEP exhibits properties similar to those of the carboxypeptidases (Malfroy et al. 1987).

**TABLE 5.** *Comparison of binding of enkephalin free acid and amide to NEP and thermolysin*

| | Ki (μM) | | Ratio Ki(amide)/Ki(acid) | |
|---|---|---|---|---|
| Peptide | NEP | Thermolysin | NEP | Thermolysin |
| Met-enkephalin | 13 | 186 | 9.1 | 1.5 |
| Met-enkephalinamide | 119 | 278 | | |
| Leu-enkephalin | 47 | 313 | 7.9 | 2.9 |
| Leu-enkephalinamide | 373 | 900 | | |

Studies in this laboratory have been initiated to elucidate the active site of NEP using a combined approach of chemical modification of the enzyme to identify potential active site residues and site-directed mutagenesis to evaluate the role of these residues in catalysis. To conduct studies involving site-directed mutagenesis, a full-length cDNA for the enzyme was required. This was accomplished by the isolation of two partial NEP cDNA clones that, when joined together, encompassed the entire coding sequence for the enzyme (Bateman et al. 1969). The first clone, designated NE-1, was isolated from a bacteriophage lambda gtl 1 cDNA library constructed from rat kidney mRNA by screening with an antibody generated against rat kidney NEP. The insert in this clone starts at the codon for $Lys^{360}$ and extends into the 3' untranslated region. This clone was used to rescreen the library from which a second overlapping clone, NE-2, was isolated. NE-2 starts 78 nucleotides into the 5' untranslated region and overlaps NE-1 by 363 nucleotides. The two clones were linked together through an overlapping Bgl II site, yielding a full-length cDNA. Subcloning of this cDNA into the vector pCMV permitted transient expression of NEP activity in COS cells. The recombinant enzyme, like the native enzyme, is glycosylated and integrated into the plasma membrane with its catalytic domain extracellular. It exhibits the same kinetic properties as the enzyme derived from rat tissue.

The arginine-specific reagents butanedione and phenylglyoxal were used to demonstrate that NEP contains a reactive active site arginine (Jackson and Hersh 1986) (figure 1). The modification of this arginine results in an increase in the Km of the substrate with a smaller effect on Vmax (table 6). The reason for the apparent loss in enzyme activity seen in figure 1 was that the substrate concentration used in the enzyme assays was below its Km. Since these reagents incorporate relatively bulky groups into the enzyme, their effect could result from steric interactions rather than direct modification of an active site residue. Therefore, it was crucial to identify the reactive arginine and to examine its function through site-directed mutagenesis.

14C-Phenylglyoxal was used to radiolabel the enzyme and was found to incorporate approximately 2 mols of reagent/mol enzyme (Bateman et al. 1989). However, incorporation of only one of the two phenylglyoxal molecules was blocked by substrates and inhibitors, indicating only one active site arginine. To identify this active site arginine, NEP was radiolabeled with phenylglyoxal in the presence and absence of inhibitors. The radiolabeled enzyme was separated from free phenylglyoxal by molecular sieve chromatography and then digested with trypsin. The resultant peptides were separated by reverse-phase HPLC and, after further digestion with pepsin, the 14C-labeled active site peptide was isolated (figure 2). The isolated peptide was subjected to automated Edman

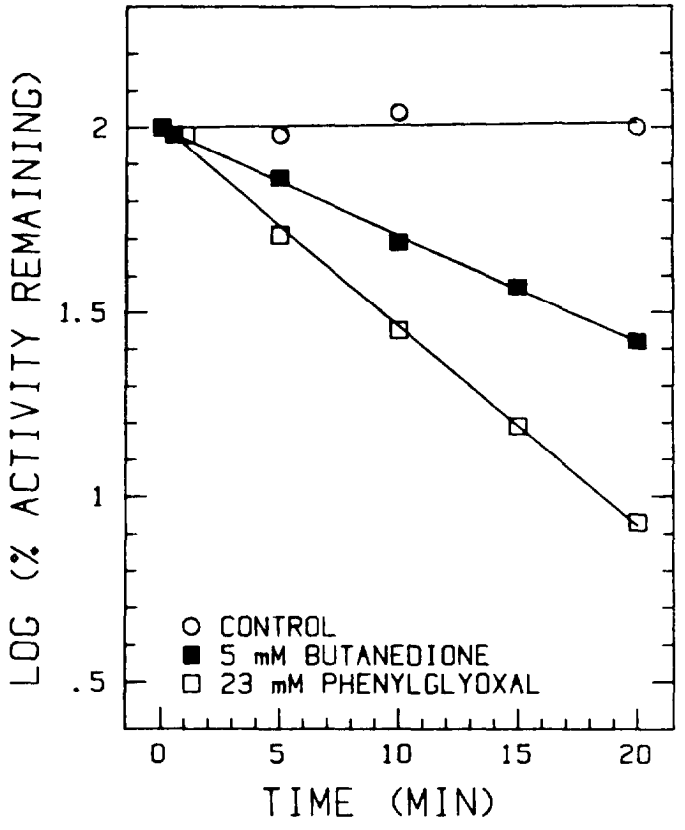


**FIGURE 1.** *Inactivation of NEP by arginine reagents*

NOTE: Purified NEP was incubated with phenylglyoxal; with butanedione; or, in the absence of any additions (control), in 50 mM triethanolamine/50 mM sodium borate buffer, pH 7.8. At the periods indicated, aliquots were removed and assayed for residual enzyme activity with 0.1 mM dansyl-D-Ala-Gly-($NO_2$)Phe-Gly as substrate. Data are plotted as pseudo first order reactions.

degradation, yielding the following amino acid sequence: Asn-Val-lle-Pro-Glu-Thr-Ser-Ser-Arg-Tyr-Ser-Asn-Phe 106.

This sequence tentatively identified arginine 102 as the active site arginine. To confirm the functionality of arginine 102, site-directed mutagenesis was used to

TABLE 6. *Kinetic properties of NEP modified with arginine reagents*

| Treatment | Km (mM) | Kcat ($s^{-1}$) |
|---|---|---|
| None | 0.25 | 46.4 |
| Phenylglyoxal | 1.05 | 37.1 |
| Butanedionone | 1.30 | 49.6 |

NOTE: Purified NEP was pretreated with the arginine reagents until no further decrease in activity was detected using 0.1 mM dansyl-D-Ala-Gly-($NO_2$)Phe-Gly as a substrate. Due to the insolubility of this substrate, the kinetic properties of the treated enzyme then were determined with Cbz-Ala-Gly-Leu-Ala as substrate.

generate an enzyme form in which this arginine was converted to glutamine. This was accomplished using the mutagenesis procedure of Zoller and Smith (1982) as modified by Craig (1985). The codon for arginine was changed to the codon for glutamine by a single base change. Mutants were identified by differential hybridization and confirmed by nucleotide sequencing. The wild-type and mutant enzymes were transiently expressed in COS cells. The amount of NEP recovered from COS cells was determined by quantitative Western blot analysis, as illustrated in figure 3.

The R102Q mutant enzyme reacted equally well as the wild-type enzyme with a substrate amide but exhibited low activity with a substrate containing a free C-terminal carboxyl group (figure 4). The wild-type enzyme exhibits a 10-fold greater affinity for free acids compared to the corresponding amide, and this ability to discriminate between an acid and an amide is essentially lost in the mutant enzyme (table 7). This results from a greater than 15-fold increase in the Km for the free acid substrate in the mutant enzyme, with less than a 3-fold increase in the Km for the amide substrate, These changes in the binding properties of the mutant enzyme provide strong evidence for the involvement of arginine 102 in the binding of the C-terminal carboxylate of substrates.

Modeling studies based on the x-ray crystallographic structure of thermolysin-inhibitor complexes indicates that the enzyme contains an active site valine residue (valine 137 in table 4) within the conserved helical segment common between NEP and the bacterial endopeptidases. This valine forms a part of the substrate binding pocket of thermolysin and makes a hydrophobic contact with the amino acid side chain in the S'1 subsite. In NEP, a valine residue occupies this same relative position (valine 573 of NEP in table 4). It was therefore of interest to determine whether valine 573 in NEP has a similar binding function.

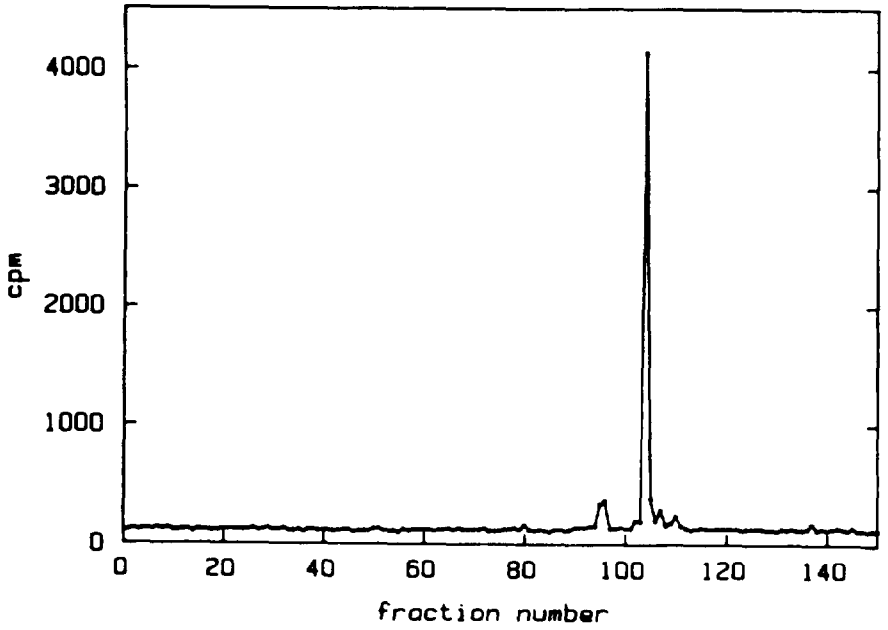


**FIGURE 2.** *Isolation of phenylglyoxal modified peptide*

NOTE: NEP radiolabeled with 14C-phenylglyoxal initially was digested with trypsin and the resultant peptides separated by HPLC. A radiolabeled peptide was digested further with pepsin and chromatographed on a C-8 reverse-phase column using a 0.02 percent trifluoroacetic acid/ acetonitrile gradient system. Shown is the radioactive content of the various fractions eluted from the HPLC column.

To examine this possibility, a mutant enzyme was prepared in which this valine residue was changed to a leucine. By adding an additional methylene group to the amino acid side chain in this position, it was anticipated that the size of the binding pocket might be decreased and result in a lowering of the affinity of the enzyme for bulky side chains in the P'1 position.

A series of peptides of the general structure N-[1-(RS)-carboxy-2-phenylethyl-X-pAB, where X represents various amino acids and pAB is para-aminobenzoic acid (Pozsgay et al. 1988), were used to probe the binding characteristics of the S'1 site of the wild type and valine to leucine mutant. As shown in table 8, when the P'1 residue is the small alanine side chain, the wild-type and mutant

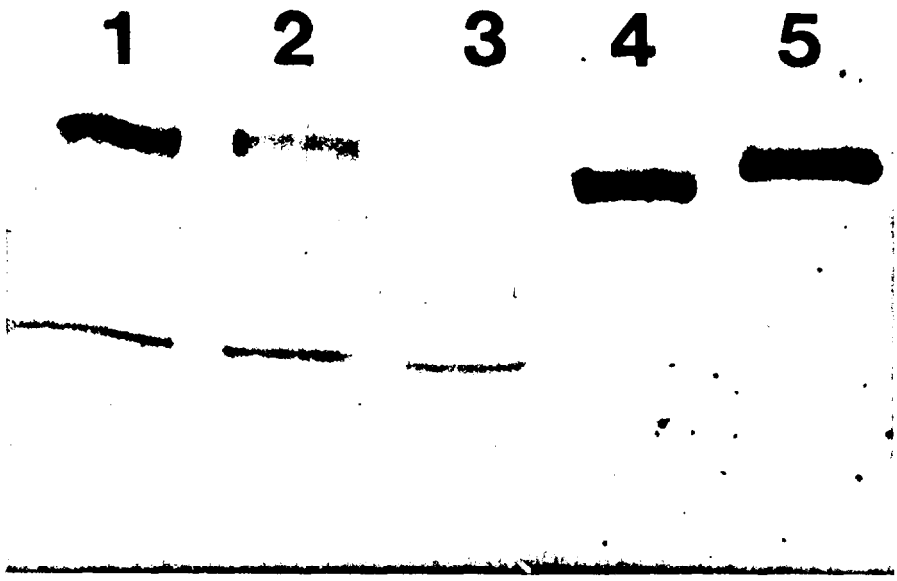


**FIGURE 3.** *Western blot of NEP* expressed *in COS cells and comparison to enzyme from rat kidney*

NOTE: Samples: lane 1, wild-type NEP expressed in COS cells, 50 µg of protein; lane 2, R102Q mutant expressed in COS cells, 50 µg of protein; lane 3, COS cell extract from cells transfected with NEP cDNA in the reverse orientation, 54 µg of protein; lane 4, purified NEP papain solubilized from rat kidneys, 2 µg of protein; lane 5, rat kidney membranes solubilized with Triton X-100, 40 µg of protein.

enzymes exhibit the same binding characteristics. However, when the size of the P'1 residue is increased, there is decreased binding of the inhibitor to the mutant enzyme as compared to the wild type. This decrease in the affinity of bulky P'1 residues to the mutant enzyme is consistent with the hypothesis that valine 573 forms a part of the S'1 subsite of NEP.

Although the expression of NEP in COS cells provides a useful system for evaluating mutant forms of the enzyme, it has the drawback that the enzyme is integrated into the plasma membrane and thus must be solubilized with detergents prior to study. It is known that NEP can be converted to a soluble

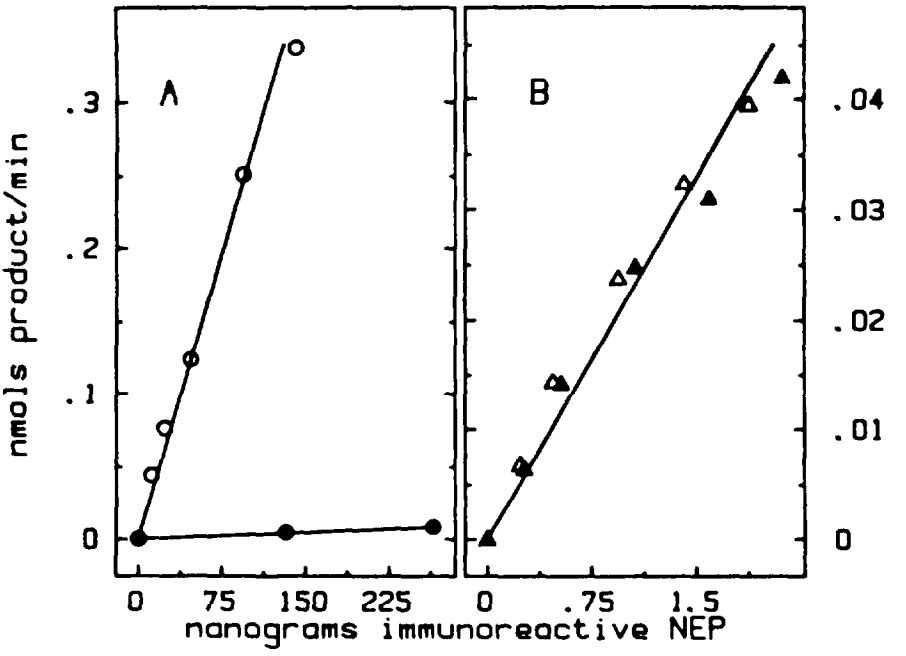


**FIGURE 4.** *Comparison of the activities of wild-type and R102Q forms of NEP*

NOTE: The activity of the wild-type and R102Q mutant forms of the enzyme was determined with either 0.1 mM dansyl-D-Ala-Gly-($NO_2$)Phe-Gly as substrate (A) or 0.1 mM glutaryl-Ala-Ala-Phe-4MeO-ZNA (B) as substrate in 100 mM MES buffer at pH 8.5. The enzyme concentration was determined by its reactions with an anti-NEP antisera and thus is expressed in terms of immunoreactive material. Open symbols represent the wild-type enzyme; closed symbols represent the R102Q mutant.

TABLE 7. *Binding properties of wild-type and R102Q* mutant *with a substrate free acid and its amide*

| Substrate | Km (μM) Wild Type | Mutant |
|---|---|---|
| D-Ala$^2$-Met$^5$-enkephalin | 22 | 385 |
| D-Ala$^2$-Met$^5$-enkephalinamide | 370 | 1,260 |
| Ratio $\frac{\text{Km enkephalinamide}}{\text{Km enkephalin}}$ | 16.8 | 3.3 |

**TABLE 8.** *Binding of inhibitors with variable P'1 residues to wild-type and V573L* mutant forms *of NEP*

| N-[1-(RS)-carboxy-2-phenylethyl-X-pAB | Ki (μM) | |
|---|---|---|
| X | Wild Type | Mutant |
| Ala | 34 | 34 |
| Leu | 0.6 | >50 |
| Phe | 0.05 | 9 |
| Trp | 5 | 51 |
| Tyr | 0.7 | 11 |

form by digestion with papain; however, this requires initial extraction of the membrane proteins into deoxycholate, and the yield of NEP is relatively low. Therefore, site-directed mutagenesis was used to delete the first 52 amino acids from the enzyme, which includes both the cytoplasmic and the hydrophobic membrane-spanning domains. Both domains were replaced with a cleavable hemagglutinin leader sequence. Expression of this NEP fusion protein in COS cells led to the secretion of soluble active enzyme into the media (table 9). This secreted enzyme form retained the catalytic properties of the native enzyme; however, analysis by SDS-PAGE indicated a molecular weight slightly larger than the papain-solubilized enzyme, indicating that it is glycosylated differently. This system represents one in which mutant enzyme forms can be prepared in a secreted form and recovered in a highly purified state in the culture media. It should prove very useful for the expression and analysis of other membrane proteins,

**TABLE 9.** *Secretion of NEP* activity *from transfected COS cells*

| Sample | Activity in Media (munits/mL) Hours After Transfection | | |
|---|---|---|---|
| | 0-24 | 24-48 | 49-72 |
| Fusion protein | 0.02 | 0.12 | 0.17 |
| Wild type | ND | ND | >0.01 |
| Cultured cells | ND | ND | ND |

NOTE: ND=not detectable

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## ACKNOWLEDGMENTS

This research was supported in part by grant DA-02243 from the National Institute on Drug Abuse and by grant l-391 from the Robert A. Welch Foundation, Houston, TX. Glioma cell lines cited in table 2 were provided by Dr. N. De Tribolet.

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# Human Cytochrome P450: Possible Roles of Drug-Metabolizing Enzymes and Polymorphic Drug Oxidation in Addiction

***Frank J. Gonzalez***

## INTRODUCTION

When a drug or other foreign chemical enters the body, it usually is metabolized by one or more of a group of proteins collectively known as the drug-metabolizing enzymes. In general, these enzymes convert nonpolar compounds into water soluble derivatives that can be easily eliminated from the body via either the urine or the bile. A chemical, however, does not have to be hydrophobic to be metabolized. Practically, drug metabolism usually results in the therapeutic inactivation of drugs, although, as discussed below, a drug also can be converted from an inactive to an active form.

Metabolism of many drugs occurs in two steps termed “phase I” and “phase II” reactions. The principal enzymes involved in the first step are the cytochrome P450s (P450s). These enzymes or monooxygenases serve to functionalize the chemical through the introduction of a hydroxyl group or an epoxide. The hydroxyl group can be added to carbon, nitrogen, or sulfur atoms. In many cases, unstable intermediates can rearrange themselves in the enzyme-active site resulting in other biotransformations, including dealkylation, dearylation, dehalogenation, deamination, and reduction (Ortiz de Montellano 1989). Another enzyme, the flavin-containing monooxygenase, can perform oxidations at sulfur and nitrogen groups. Subsequent to addition of a functional group into a substrate by phase I enzymes, the phase II enzymes can use this group for conjugation of such compounds as glucuronic acid, glutathione, cysteine, glycine, and sulfate. The phase II enzymes include glutathione transferases, uridine diphosphate (UDP) glucuronosyltransferases, sulfotransferases, and others. The end product is a rather hydrophilic compound that can be excreted easily. Other drug metabolism enzymes, which are not necessarily classified as phase I or II, also exist; these include epoxide hydratatases, numerous exterases, *N*-acetyltransferase, dihydrodiol dehydrogenases, DT-diaphorase,

aldehyde dehydrogenases, glycosidases, and glucosidases. A large number of different enzymes, therefore, exist to metabolize drugs and other foreign compounds (table 1). It is believed that several of these enzymes are present in multiple forms, thus accounting for their large substrate specificities. However, a single form sometimes is capable of metabolizing numerous structurally diverse chemicals. In addition, several drug-metabolizing enzymes are expressed polymorphically in both rodents and humans: based on these findings and other considerations, it is believed that many of them evolved to metabolize the numerous toxins found in plant life (see below).

Most of the drug-metabolizing enzymes are expressed in liver, which serves as the main "clearinghouse" for foreign chemicals. Once blood passes through the liver, a large percentage of a particular drug can be metabolized. Other tissues also contain drug-metabolizing enzymes-the most notable being the lung and intestine, organs that are in direct contact with the environment.

This chapter focuses on the cytochrome P450s, the major class of drug-metabolizing enzymes, and discusses polymorphisms in these enzymes and the possible role of genetic differences in P450 in drug addiction.

## CYTOCHROME P450s

These enzymes exist as a gene superfamily composed of 10 families in vertebrates (Gonzalez 1988; Nebert et al. 1991). P450s within six of these families are involved in steroidogenic pathways and in cholesterol-bile acid synthesis. One family, the P450 4 family, encodes fatty acid hydroxylases, while P450s within families 1, 2, and 3 encode the drug-metabolizing enzymes.

The cytochrome P450s are the most common and well-studied enzymes of drug metabolism. These enzymes contain a noncovalently bound heme, in the form of protoporphyrin IX, and receive electrons from the flavoprotein NADPH-P450 oxidoreductase. Those P450s involved in drug metabolism are embedded in the intracellular endoplasmic reticulum network, a membrane location that is ideally suited for interaction of P450s with hydrophobic substrates. It is generally believed that these enzymes are bound to the lipid bilayer via a hydrophobic amino terminus, with the bulk of the enzyme facing the cytoplasmic face of the endoplasmic reticulum (Nelson and Strobel 1988).

### Evolution of Cytochrome P450s

One of the most fascinating aspects of P450s is the large degree of species differences in their regulation and their substrate specificities (Gonzalez 1988). For example, rats, mice, rabbits, and humans can differ widely in their

**TABLE 1.** *List of drug-metabolizing enzymes and* their *reactions and multiplicities*

| | Multiplicity | Polymorphisms | Reactions |
|---|---|---|---|
| Phase I Enzymes | | | |
| Cytochrome P450s | 9 gene families | Yes | *C*-hydroxyiatlon<br>*N*-hydroxylatlon<br>Deamination<br>Dehalogenation<br>Sulfoxidatlon<br>*N*-dealkylation<br>*S*-dealkylatlon<br>(others) |
| Flavin-containing monooxygenase | Yes | Yes | *S*-oxldation<br>*N-oxldation* |
| Phase II Enzymes | | | |
| UDP-glucuronosyl-transferase | Yes | Yes | Glucuronate conjugatlon |
| Sulfotransferase | Unknown | Unknown | Sulfate conjugatlon |
| Glutathione transferase | 4 gene families | Yes | Glutathione conjugation |
| *N-Acetyltransferaee* | Yes | Yes | Acetate conjugatlon |
| Other Drug Metabolizing | | | |
| Carboxyleaterase | Yes | Unknown | Carboxyester hydrolysis |
| Aldehyde dehydrogenase | Yes | Yes | Aldehyde oxidation |
| Epoxide hydrolase (microeomal) | No | Unknown | Epoxlde hydrolysis |
| Epoxide hydrolase (cytosolic) | Unknown | Unknown | Epoxyde hydrolysis |
| Dihydrodlol dehydrogenase | Unknown | Unknown | |
| NAD(P)H-quinone oxidoreductase | N o | Unknown | Quinone reduction |
| Drug Metabolism Support Enzymes | | | |
| NADPH-P450 oxidoreductase | No | Unknown | P450 reduction |
| Cytochrome b6 | No | Unknown | P450 reduction |

metabolism of drugs. This property, which has been recognized for over 40 years (Williams 1974), Is of utmost importance when attempting to extrapolate rodent pharmacological data to humans.

The molecular basis for species differences has become more evident in recent years, with a large amount of data from studies on purified P450s, cDNA, and genes. It has become evident that P450s have evolved quite differently in rodents and humans. Even between rats and mice, which split from a common ancestor about 17 million years ago, many P450s and their genes have evolved species-unique properties (Gonzalez and Nebert 1990). For example, certain mouse genes are expressed in males but not females. Genes within the same subfamily in rats, on the other hand, are expressed in both sexes. Likewise, certain rat genes may be expressed in females, but genes in the same subfamily of mice are under different regulation. Interestingly, no sex-specific P450s have been detected in humans. P450s within a single subfamily of mice also may have the capacity to metabolize androgens, whereas P450s within the same subfamily of rats may not be able to catalyze androgen hydroxylation (Gonzalez 1988; Gonzalez and Nebert 1990). Again, these findings illustrate the fact that P450s have evolved in a species-specific manner.

It is generally believed that the basis for the species-specific evolution of mammalian P450s is the environment-more specifically, the consumption of plants (Nebert and Gonzalez 1985; Nelson and Strobel 1987; Gonzalez and Nebert 1990). As a defense against animal consumption, plants have developed a large arsenal of toxins and stress metabolites or phytoalexins. As each animal species developed among a population of plants producing toxins and phytoalexins, specific P450s were required for detoxification and survival. The appearance of a new species or subspecies of plant within an animal's environment would necessitate the involvement of P450-catalyzed detoxification. Therefore, two species evolving in different environments of vegetation would have their own group of P450 enzymes. The role of plants in P450 evolution also is supported by the fact that a large explosion and diversification of P450 genes occurred about 400 million years ago, soon after animals began to emerge from an aquatic environment to a terrestrial habitat that had been populated for several million years by plants having unique toxins previously not seen by animals (Nelson and Strobel 1987). In any case, it is likely that P450s evolved to metabolize plant chemicals, many of which are similar to, and were used as the basis for, drugs. Illicit drugs, such as opiates, cannabidiol, and cocaine, are probably plant toxins developed as a defense against animals.

Another important feature of the P450s is the high degree of polymorphisms found in rodents and humans. In the context of drug metabolism, a

polymorphism can be defined as a common genetic defect in the ability to carry out a certain drug oxidation reaction, By definition, the deficiency giving rise to a polymorphic trait Is so common (generally >1 percent) that it is not maintained by recurrent mutation. Among the most well-studied polymorphisms in rodents are the mouse “Ah receptor” polymorphism in which certain strains of mice have a receptor with weak affinity for the inducing ligand (Nebert 1986). Therefore, the P450 responsible for oxidizing a compound such as benzo(a)pyrene is never induced because benzo(a)pyrene is incapable of interacting with the receptor that causes an increase in transcription of the P450 gene. Another well-studied polymorphism is the Dark Agouti rat deficiency in debrisoquine metabolism (Al-Dabbagh et al. 1980). This deficiency is due to a defective P450 gene (Matsunaga et al. 1989).

Polymorphisms in drug metabolism also have been described in humans. Two polymorphisms involving P450s were found and were termed the ‘mephenytoin polymorphism” and ‘debrisoquine polymorphism.” These were uncovered by screening groups of people for their abilities to metabolize drugs. From 1 to 5 percent of all Caucasians are unable to carry out *S*-mephenytoin 4’-hydroxylation (Kupfer and Preisig 1984; lnaba et al. 1986). The frequencies of this polymorphism in Japanese and Chinese subjects reaches 23 percent and 17 percent, respectively (Horai et al. 1989), indicating a large degree of ethnic differences in polymorphic drug metabolism. The P450 gene responsible for the mephenytoin polymorphism has not been clearly identified as yet, although it is believed to reside within the 2C subfamily (Gonzalez 1988).

The most well-characterized P450 polymorphism is that associated with debrisoquine metabolism. The earliest clue to the existence of this deficiency came from clinical studies in which the antihypertensive agent debrisoquine (Declinax) was found to produce exaggerated effects in a large number of patients. This toxicity was determined to be due to a genetic deficiency that is inherited as an autosomal recessive trait (Idle and Smith 1979). The P450 involved in debrisoquine oxidation, designated 2D6, also is capable of metabolizing numerous other drugs (figure 1). This defect was found to be the result of mutant alleles for a single gene, designated CYP2D6 (encoding P450llD6), that is located on chromosome 22 (Gonzalez et al. 1988a, 1988b). In some cases, defective CYP2D6 alleles can be detected in lymphocyte DNA by Southern blotting analysis (Skoda et al. 1988). However, a noninvasive test in which all mutant P450 gene alleles can be diagnosed has not been developed.

It should be emphasized that individuals who lack a particular P450, such as the debrisoquine 4-hydroxylase and Smephenytoin hydroxylase, are otherwise normal. Based on this fact, it appears that some P450s are not required for

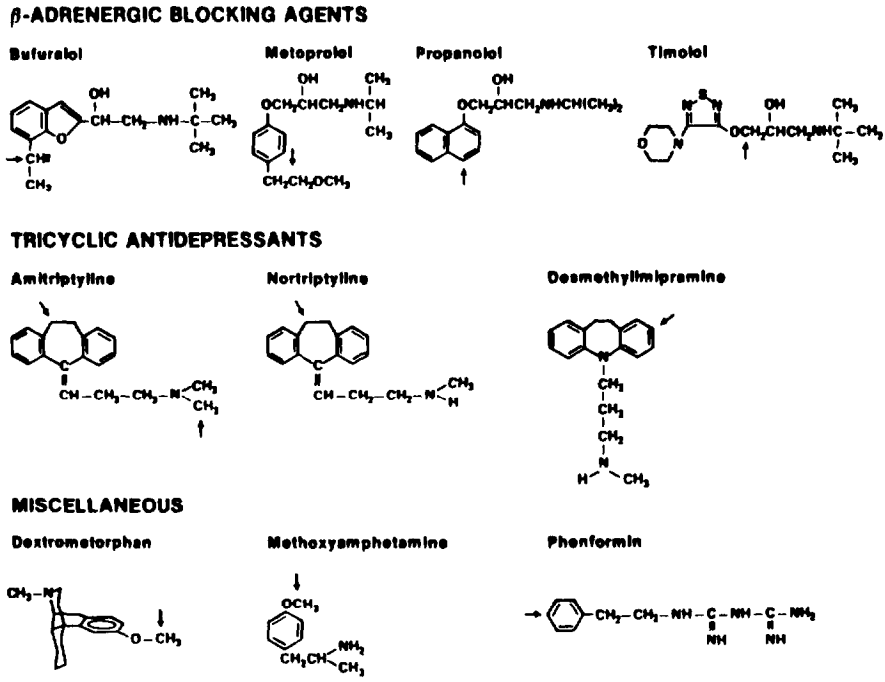



**FIGURE 1.** *Substrates for CYP2D6. The arrows indicate the position of hydroxyation of each substrate.*

SOURCE: Meyer et al. 1990, copyright, 1990, Academic Press

normal physiological function. Indeed, this is compatible with an exclusive role of P450s in metabolism and elimination of foreign compounds. It is likely, therefore, that humans may not need particular P450s for survival and the P450 no longer renders a selective advantage. This is probably because humans are now capable of freely choosing what they eat, and a poisonous substance simply can be avoided and a substitute found-a luxury that was not afforded our ancestors. This might be the reason for P450 loss and for the high frequency of drug metabolism polymorphisms in humans. Rodents, on the other hand, are more generally victims of their environment; in fact, there does seem to be a trend that mice, rats, and rabbits have more active P450s than do humans. Finally, it should be noted that polymorphisms in other drug-metabolizing enzymes exist in humans. Among the most well-studied are the *N*-acetyltransferase polymorphism (Weber 1987) and a glutathione transferase polymorphism (Seidegard et al. 1988).

## CONSEQUENCE OF P450 POLYMORPHISMS

The most serious consequence of P450 polymorphism is in drug therapy. As indicated above, the ß-blocking agent debrisoquine was found to be toxic in about 10 percent of all patients and, hence, is no longer prescribed. The debrisoquine 4-hydroxylase P450 is also capable of metabolizing numerous drugs (figure 1). In this connection, other drugs, such as perhexiline, are capable of causing toxicities and even death due to this polymorphism (Shah et al. 1982). Recently, certain cardiac antiarythmic drugs have caused problems during clinical trials because of a P450 deficiency (Ruskin 1989). The ability to diagnose mutant P450s prior to treatment with drugs should greatly aid in avoiding toxic consequences.

The unknown consequence of P450 polymorphism is the association of the presence or absence of a P450 with cancer or chronic toxicity. For example, smokers who develop lung cancer are much more likely to actively metabolize debrisoquine, indicating that individuals who have a normal P450 gene are at higher risk than those who do not (Ayesh et al. 1984; Caporaso et al. 1989). Many P450s are capable of activating carcinogens to DNA-binding and mutagenic metabolites; however, convenient assays for expression of these forms in humans have not been developed. When assays are available, it may be possible to conduct molecular epidemiology studies, both retrospective and prospective, to determine if P450 phenotypes are risk factors in human cancer.

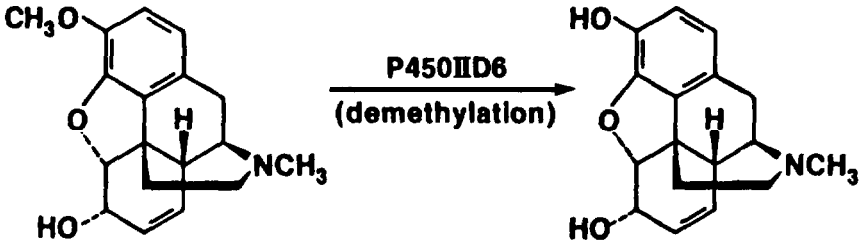
## ROLE OF P450 POLYMORPHISMS IN ILLICIT DRUG USE

Rodent P450s are capable of metabolizing cannabidiol and $\Delta^9$-tetrahydrocannabinol (Watanabe et al. 1987); these compounds have dramatic effects on total hepatic P450 content, which decreases following acute administration (Bornheim and Correia 1989a). However, when cannabidiol is administered chronically, a specific P450 is highly induced in mouse that is capable of metabolizing $\Delta^9$-tetrahydrocannabinol (Bornheim and Correia 1989b). The effects of metabolites generated by P450s in humans is unknown, although it has been established that they have pharmacologic activity (Christensen et al. 1971). P450s are also able to metabolize and inactivate cocaine, and this metabolism can produce hepatotoxic metabolites in rodents (Shuster et al. 1988).

Recently, it was discovered that the debrisoquine 4-hydroxylase CYP2D6 enzyme can convert codeine to morphine by a demethylation reaction (figure 2) (Dayer et al. 1988). This has been confirmed by studying the metabolism of debrisoquine and codeine in a population of subjects in which lack of codeine O-demethylation was found to cosegregate with the debrisoquine hydroxylation

deficiency (Yue et al. 1989). In this case, codeine is converted by a P450 to its analgesically active form, morphine. Individuals who do not have this P450 are unable to respond to codeine treatment.

It is tempting to speculate that oxidation polymorphisms might play a role in abuse of certain illicit drugs. For example, certain individuals having or lacking a P450 may not respond favorably to drug ingestion in a way similar to that of early patients who received debrisoquine. Either an individual cannot metabolize a drug to its active component or, conversely, the drug is metabolized too rapidly and, hence, is ineffective; or a drug may be toxic. Any of these possibilities might play roles in who does or does not become addicted.




CODEINE MORPHINE

**FIGURE 2.** *Conversion of codeine to morphine by human hepatic P450*

## FUTURE EXPERIMENTATION

Future areas of drug abuse research could focus on the metabolism of illicit drugs by human P450s and other drug-metabolizing enzymes. These studies can be performed on liver tissue samples *in vitro.* Human liver can be obtained from organ donor transplant units and various agencies, such as the National Disease Research Interchange. Although it is difficult to purify P450s from limited sources of human tissues, it is possible to isolate cDNAs and genes encoding a large number of these enzymes using rodent antibodies and probes. These cDNAs can be expressed into active enzymes using various cDNA expression systems, such as vaccinia virus (Aoyama et al. 1989); the expressed enzymes can be used to screen for metabolism of illicit drugs. A list of known human P450s in which cDNAs are available and some of their substrates is shown in table 2. Knowledge of which of these P450 forms inactivate or activate specific drugs, and whether these forms are polymorphic, might aid in prediction of individuals

who are susceptible to drug abuse. In addition, these data might also prove useful in strategies for effective therapies for addicts.

**TABLE 2.** *Human P450s and some of their substrates*[a]

| | |
|---|---|
| 1A1 | Benzo(a)pyrene, ethoxyresorufin, ethoxycoumarin |
| 1A2 | Acetaminophen, acetylaminofluorine, aflatoxin, ethoxyresorufin, heterocyclic arylamines, phenacetin, theophyline |
| 2A3 | Coumarin, nitrosamines |
| 2B7 | Etoxycoumarin |
| 2C8 | *R*-Mephenytoin, tolbutamide |
| 2C9 | *R*-Mephenytoin, tienilic acid, tolbutamide, warfarin |
| 2D6 | Bufuralol, debrisoquine, dextrometorphan, metoprolol, nortryptiline, propranolol, sparteine |
| 2E1 | Acetoacetate, acetol, acetaminophen, acetone, benzene, ethanol, halothane, nitrosamines, pyridine |
| 2F1 | 7-Ethoxycoumarin, testosterone |
| 3A4 | Cortisol, cyclosporine, erythromycin, lidocaine mephenytoin, midazolam, nifedipine, progesterone, testosterone, quinidine, warfarin |
| 4B1 | Testosterone |

[a]These lists do not represent every known substrate and every known human P450; for example, at least four P450s are related to 3A4.

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GPO out of stock NCADI out of stock
NTIS PB #89-134936/AS $31

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GPO out of stock NTIS PB #89-158422/AS $39

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GPO out of stock NTIS PB #89-158430/AS $45

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GPO out of stock NTIS PB #89-151955/AS $31

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GPO Stock #017-024-01302-1 $6 NTIS PB #69-151963/AS $31

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GPO Stock #017-024-01313-7 $3.75 NTIS PB #89-151971/AS $23

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GPO out of stock NCADI out of stock
Not available from NTIS

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GPO out of stock NCADI out of stock
NTIS PB #88-206111/AS $53

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GPO Stock #017-024-01348-0 $4 NCADI out of stock
NTIS PB #89-125488/AS $23

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GPO out of stock NTIS PB #88-245683/AS $31

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GPO Stock #017-024-01354-4 $17 NTIS PB #89-109227/AS Contact NTIS for price

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GPO out of stock NTIS PB #89-125496/AS $23

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GPO Stock #017-024-01360-g $5.50 NTIS PB #89-123178/AS $31

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GPO Stock #017-024-01362-5 $17

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**DHHS Publication No. (ADM) 92-1798**
**Alcohol, Drug Abuse, end Mental Health Administration**
**Printed 1991**