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Contents

	Page
Introduction *Theresa N.H. Lee*	1
Neuronal Nicotinic Acetylcholine Receptor Diversity *James W. Patrick*	4
Neurotransmitter and Drug Receptor Genes *George R. Uhl*	14
The Diversity of the Dopamine Receptors *Olivier Civelli, James R. Bunzow, Qun-Yong Zhou, and David K. Grandy*	23
Cloned Dopamine Receptors: Targets in Therapy of Drug Abuse *Philip Seeman*	34
Cannabinoid Receptors: Which Cells, Where, How, and Why? *Lisa A. Matsuda, Tom I. Bonner, and Stephen J. Lolait*	48
Studies on μ-Opioid-Binding Sites With Peptide Antibodies *Eric J. Simon, Theresa L. Gioannini, Yi-He Yao, and Jacob M. Hiller*	57
Molecular Cloning and Characterization of Neurotransmitter Transporters *Randy D. Blakely*	66

Regulation of Gene Expression by Dopamine: Implications in Drug Addiction 84
Orla M. Conneely, Ronan F. Power, and Bert W. O'Malley

Regulation of Opioid Gene Expression: A Model To Understand Neural Plasticity 98
Michael J. Comb, Linda Kobierski, Hung Ming Chu, Yi Tan, David Borsook, Karl Herrup, and Steven E. Hyman

Cellular and Molecular Analysis of Opioid Peptide Gene Expression 113
Cynthia T. McMurray, Karen M. Pollock, and James Douglass

The Prohormone and Proprotein Processing Enzymes PC1 and PC2: Structure, Selective Cleavage of Mouse POMC and Human Renin at Pairs of Basic Residues, Cellular Expression, Tissue Distribution, and mRNA Regulation 132
Nabil G. Seidah, Robert Day, Suzanne Benjannet, Normand Rondeau, Alain Boudreault, Timothy Reudelhuber, Martin K.-H. Schafer, Stanley J. Watson, and Michel Chrétien

List of NIDA Research Monographs 151

Introduction

Theresa N.H. Lee

At the first National Institute on Drug Abuse (NIDA) technical review meeting on "Molecular Approaches to Drug Abuse Research" held in August 1989, one member of each of the three families of receptors and channels was chosen to illustrate various strategies of gene cloning due to the limited number of drug receptor genes cloned. These are the dopamine D2 receptor of the G protein-coupled receptor family, the nicotinic acetylcholine receptor of the ligand-gated channel family, and the potassium channel of the voltage-gated channel family. Because of the extensive and innovative studies on the nicotinic acetylcholine receptor, it was selected as an example to cover postcloning research endeavors such as regulation of expression using molecular genetics and transgenic mice as well as studies of structure and function relationship using site-directed mutagenesis and other techniques.

The second NIDA technical review meeting on "Molecular Approaches to Drug Abuse Research" was held on July 30 and 31, 1991, at the National Institutes of Health. This technical review encompassed almost all the pivotal research developments of the past 2 years in the field. What extraordinary progress scientists in this field have made since the 1989 meeting! The proceedings of this conference are presented in the following chapters of this monograph.

As a logical extension of the agenda of the 1989 conference, the 1991 technical review began with Dr. James W. Patrick's update on the diversity of ligand/receptor interactions of neuronal nicotinic acetylcholine receptors based on studies of functional oligomeric receptors. Presentations followed on the successful cloning of genes of two other members of the ligand-gated channel family—the glutamate (AMPA/Kainate) receptor and the γ-aminobutyric acid (GABA) p-1 receptor by Dr. Stephen Heinemann and Dr. George R. Uhl, respectively.

Perhaps as a reflection of the intense interest in the molecular cloning of the opioid receptor genes, there have been an unprecedented number of genes cloned in the G protein-coupled receptor family in these 2 years. These scientists have made international news recently with their breakthroughs in

receptor gene cloning and subsequent remarkable studies. Some of the examples presented in the 1991 conference were Dr. Olivier Civelli and Dr. Philip Seeman on the molecular biology and pharmacology of dopamine receptors D1, D2, D3, D4, and D5 and Dr. Lisa A. Matsuda on the cannabinoid receptor. Dr. Eric J. Simon gave an update on the μ-opioid receptor. Even though the cloning of the κ-opioid receptor gene was not conclusive at the time of the conference, the recent cloning and expression of the long-awaited prohormone and proprotein convertases PC1 and PC2 genes opened up numerous avenues in the field (Dr. Nabil G. Seidah).

This technical review also coincided with another breakthrough in the field, namely, the successful cloning of the gene for the dopamine transporter with 12 transmembrane domains. This work will undoubtedly help elucidate the molecular mechanism of cocaine action. The leader of one of the groups that accomplished this important task, Dr. Susan Amara, has also joined the NIDA scientific community recently as a grantee. Although Dr. Amara was unable to attend the conference, Dr. Randy D. Blakely spoke instead on the molecular cloning and characterization of GABA and norepinephrine transporter genes.

This technical review would not have been complete without coverage of the enormous contributions of NIDA scientists to our understanding of the regulation of expression of opioid peptides. Dr. Michael J. Comb and Dr. James Douglass provided two superb examples with their presentations on this subject. From a different perspective, Dr. Orla M. Conneely discussed the recent evidence from their laboratory that members of the steroid/thyroid receptor family of transcription factors could be activated by dopamine and dopamine D1 receptor agonists.

The 1991 technical review also commemorated the establishment of the Molecular Biology and Genetics Program initiated 3 years earlier in the Division of Preclinical Research of NIDA. This program now encompasses nearly every important area employing molecular approaches to drug abuse research; this is clearly illustrated in the technical review and this monograph. I am extremely proud to point out that, of the 11 people contributing to this monograph, 8 are members of the NIDA scientific community, and another 2 are in the process of applying for support.

Drug abuse researchers interested in molecular approaches will find this coming decade challenging and fruitful, with ground-breaking results expediting understanding of the underlying basis of addiction to generate better strategies for effective treatment, education, and prevention.

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Neuronal Nicotinic Acetylcholine Receptor Diversity

James W. Patrick

INTRODUCTION

Society must deal with the dangerous dichotomy of drugs, a dichotomy that arises from the fact that the drugs that provide the best treatment for many illnesses also present tremendous opportunity for abuse. Furthermore, both naturally occurring and synthetic drugs provide extremely powerful tools for the study of biological systems. The use of these drugs as research tools has resulted in a refinement of understanding of both the drugs and their biological sites of action. This has, in turn, led to the development of ever more specific and powerful drugs with subsequent new opportunities for abuse. This trend is expected to continue because recent studies have revealed an unexpected and, for the moment, bewildering diversity of drug receptors. This diversity suggests that eventually one will be able to engineer exquisitely specific drugs targeted to specific areas of the brain with minimal side effects. On the one hand, one expects that this work will reveal new sites and mechanisms of action of drugs and, unfortunately, will offer more opportunity for abuse through the development of new classes of drugs. On the other hand, one hopes that the insights gained in this work will result in the production of drugs with minimal abuse potential or perhaps generate therapies to deal with abuse. There is every reason to believe that new insights into receptors, new drugs, and rapidly expanding access to the nervous system will lead to a new level of appreciation and understanding of both the function of the brain and the uses and abuses of drugs.

The rationale that underlies much of the research in this field is based on the recent observation that there is a large family of genes that encode neurotransmitter receptors and that almost all the ligand-gated ion channels are members of this superfamily. Because these receptors are all members of the same family they share structural and functional features. However, they differ in many important ways, and these differences provide powerful tools with which to probe their important common features. The family of genes that compose the nicotinic receptors is studied to understand the structure, function,

and regulation of the nicotinic receptors. The information thus obtained has provided the foundation for all other studies of ligand-gated ion channels and will very likely continue to be important for understanding other receptors such as the members of the glutamate, GABA, glycine, or serotonin receptor gene families. Therefore, insights provided into the structure and function of the neuronal nicotinic receptors will help one's understanding of the ligand-gated ion channels in general.

NICOTINIC ACETYLCHOLINE RECEPTORS

The nicotinic acetylcholine receptor has long been the prototypical ligand-gated ion channel, and most of what is known about the structure and function of these ion channels derives from studies of the muscle-type nicotinic receptor isolated from the electric rays and fishes. Significant progress in this area has come in the last 5 to 10 years following the application of the techniques of molecular biology to the study of the muscle nicotinic receptor. These studies led to the elucidation of the primary structure of the subunits of the receptor (Ballivet et al. 1981; Noda et al. 1982, 1983a, 1983b; Sumikawa et al. 1982; Claudio et al. 1983), its synthesis from RNA derived from cDNA clones (Mishina et al. 1984), the determination of functional domains by mutagenesis (Mishina et al. 1985), mapping of antibody binding sites (Tzartos et al. 1988, 1990), the discovery of a new receptor subunit (Takai et al. 1985) and its role in synaptogenesis at the neuromuscular junction (Methfessel and Sakmann 1986; Sakmann et al. 1985; Gu and Hall 1988), and the identification of genomic sequences that control synthesis of receptor subunits (Gardner et al. 1987). Understanding of the nicotinic receptor and the neuromuscular junction in normal and diseased states has advanced dramatically as a result of these studies.

The acetylcholine receptor at the neuromuscular junction was chosen for these studies because good ligands were available and because the junction was well studied and amenable to the types of experiments the author and his colleagues thought were necessary. However, it seemed likely that a great deal could be learned from the nicotinic receptors found at other nicotinic cholinergic synapses. It also seemed likely that this information would be important for understanding the receptor at the neuromuscular junction and for understanding the role these receptors play in neurons. It has long been known that cholinergic transmission at sympathetic ganglia is nicotinic, that the nicotinic acetylcholine receptors on neurons differ from those found at the neuromuscular junction, and that nicotinic receptors might play a very important role in neurotransmitter release. The potential for understanding the nicotinic receptors and ligand-gated ion channels in general, as well as the potential for appreciating new roles for cholinergic function on neurons, led many laboratories to the neuronal nicotinic acetylcholine receptors.

One approach was based on the idea that sequences encoding the muscle-type nicotinic acetylcholine receptors would hybridize to, and thus identify, the sequences encoding the neuronal nicotinic acetylcholine receptors. This general approach led to the identification in the rat and chicken of a family of genes encoding subunits of nicotinic acetylcholine receptors that are expressed in the central nervous system (CNS) (for reviews, see Luetje et al. 1990a; Nordberg et al. 1989). Currently, the products of six different genes (alpha2, alpha3, alpha4, alpha7, beta2, and beta4) are known to generate at least seven different functional receptors (Nef et al. 1988; Deneris et al. 1988; Wada et al. 1988; Boulter et al. 1986, 1987; Goldman et al. 1986; Duvoisin et al. 1989; Couturier et al. 1990; Schoepfer et al. 1990). There are additional members of this gene family (alpha5, alpha8, and beta3) whose gene products have not yet been shown to be associated with a function (Schoepfer et al. 1990; Boulter et al. 1990; Deneris et al. 1989). The proteins encoded by these nine genes have homologous extracellular, transmembrane, and cytoplasmic domains and, in general, are classified as alpha or beta subunits. The alpha subunits are identified by contiguous cysteines in the extracellular domain. The beta (or nonalpha) subunits are identified by the lack of these cysteines and by the ability of either beta2 or beta4 to substitute for the beta1 subunit in the formation of a functional muscle-type nicotinic receptor.

The proteins derived from six of these genes associate in various combinations to form functional receptors in the *Xenopus* oocyte. The alpha2, alpha3, and alpha4 subunits each form functional receptors in combination with either beta2 or beta4. Receptors thus formed vary with respect to their single channel properties (Papke et al. 1989) and pharmacology (Luetje and Patrick 1991; Luetje et al. 1990b) depending on which of the three different alpha subunits are present. Likewise, the substitution of a beta4 subunit for a alpha2 subunit alters the response of the receptor to various agonists and antagonists (Duvoisin et al. 1989; Luetje and Patrick 1991). There is also good evidence that the complement of receptor subtypes present on a neuron changes during development (Moss et al. 1989).

The receptor formed in the oocyte from alpha4 and beta2 subunits is probably a pentamer comprising three beta subunits and two alpha subunits (Cooper et al. 1991). Although it is clearly possible to form receptors containing more than one kind of alpha subunit (S. Helekar and J. Patrick, unpublished observations), the diversity of oligomeric receptor structures formed in the CNS is not known.

Two additional members of the gene family have been identified in the chick (Couturier et al. 1990; Schoepfer et al. 1990). These new members are alpha7 and alpha8, and the proteins encoded by these clones differ from the other alpha subunits in several regards. The alpha7 subunit forms an acetylcholine-

gated ion channel in the absence of other added subunits (Couturier et al. 1990). Therefore, alpha7 appears to form homooligomeric receptors. The functional receptors thus formed are blocked by alpha-bungarotoxin, unlike any of the other neuronal receptors studied to date. Finally, they are derived from genes with an intron/exon structure that is different from that of the other neuronal receptor subunits (Couturier et al. 1990).

There are additional members of the gene family for which a function has not yet been identified. Alpha5 is homologous to the other alpha subunits in its overall architecture and in the presence of the two contiguous cysteines in the extracellular domain (Boulter et al. 1990). However, this protein does not form a functional receptor when RNA encoding it is injected into oocytes in combination with any other receptor encoding RNA. It seems unlikely that these clones fail to encode functional receptors as a consequence of some cloning artifact that generated an incorrect sequence because the sequence has been confirmed by analysis of genomic clones. The gene encoding alpha5 is expressed in the CNS in a precise set of neuronal structures (Wada et al. 1990). There has not yet been a systematic analysis of the contribution this subunit might make as a third component of an oligomeric receptor. Beta3 likewise fails to form functional receptors when injected in pairwise combinations with the known functional receptors and, like alpha5 is expressed in a well-defined set of neuronal structures.

In situ hybridization has shown that the members of this gene family are expressed throughout the CNS (Wada et al. 1989, 1990). The beta2-encoding RNA is found in almost all brain nuclei examined, and the other alpha- and beta-encoding RNAs are expressed in specific but generally overlapping subsets of nuclei. The beta4 subunit was first described as localized to the medial habenula but is now known to be widely expressed in the central and peripheral nervous systems (Moss et al. 1989). The alpha subunits are expressed in discrete but overlapping sets of loci. However, these data do not show the location of the expressed protein, and it is not yet known which particular subunit combinations are located on dendrites, cell bodies, or axons.

In summary, molecular biological approaches to neuronal nicotinic receptors have defined a gene family, documented the expression of this gene family in the CNS, and demonstrated that various combinations of the proteins derived from these genes form functionally different receptors. It is not yet known if all the members of the gene family have been identified. Nor does one know the full spectrum of combinations of subunits that exist in vivo or the roles that these different subunit combinations play in the function and/or modification of synapses.

It is the case, however, that neuronal nicotinic receptors differ in their functional properties depending on which beta subunits are included in the oligomer. Receptors containing a beta2 and an alpha3 subunit are sensitive to the neuronal bungarotoxin (Boulter et al. 1987), whereas those in which an alpha2 replaces the alpha3 subunit are 100-fold less sensitive (Wada et al. 1988). Receptors substituting an alpha4 are intermediate in sensitivity (Boulter et al. 1987; Luetje et al. 1990b). This is consistent with the view that the ligand binding site is on the alpha subunit. However, substitution of a beta4 for beta2 renders the alpha3-containing receptor insensitive to this toxin, suggesting that the beta subunits either contribute to the toxin binding site or modify the conformation of the alpha subunits (Duvoisin et al. 1989; Luetje et al. 1990b).

Different combinations of alpha and beta subunits are also distinguishable in their responses to agonists. The relative sensitivities for acetylcholine, nicotine, dimethylphenylpiperainium, and cytisine were determined for all six receptor combinations that can be made from alpha2, alpha3, or alpha4 in combination with either beta2 or beta4. These experiments were done in the *Xenopus* oocyte where it was also established that the expressed muscle-type receptor had a pharmacological profile indistinguishable from the cell line from which the clones were derived. The results of these studies demonstrate that both the alpha and the beta subunits contribute to agonist specificity. Receptors containing a beta2 subunit differ in their response to acetylcholine and nicotine depending on which alpha subunit is present. Nicotine is about thirtyfold more effective on receptors containing alpha2 than on receptors containing alpha3, suggesting that the alpha subunits determine agonist specificity. Cytisine is the least effective agonist in all receptors containing a beta2 subunit. However, substitution of the beta4 for the beta2 renders cytisine the best of the four agonists. Although cytisine is the best agonist on beta4-containing receptors, competition experiments suggest that cytisine is an antagonist on beta2-containing receptors. These results demonstrate that both the alpha and beta subunits contribute to both agonist and antagonist recognition and suggest that one possible consequence of the expression of different combinations of receptor subunits in the CNS is altered ligand affinity and specificity (Luetje and Patrick 1991).

The neuronal nicotinic receptors differ from the muscle nicotinic receptors in two additional interesting ways. The neuronal nicotinic acetylcholine receptors are more permeable to calcium and in this property more closely resemble the N-methyl-D-aspartate type of glutamate receptor than a muscle nicotinic acetylcholine receptor (Vernino et al. 1992). The neuronal nicotinic receptors are also modulated by external calcium ions. The author of this chapter and his colleagues observed that increasing the external calcium ion concentration resulted in large increases in the current produced by a given concentration of

receptors expressed in bovine chromaffin cells. The effect is not seen with either barium or calcium, is not a consequence of activation of the calcium-activated chloride channel, and is not a consequence of an increased contribution of calcium to the current but rather a modulation by calcium of the response of the receptor to agonist. This result distinguishes the neuronal nicotinic receptors from the muscle nicotinic receptors, which, in contrast, show a decrease in current in the presence of elevated extracellular calcium and suggest a different mechanism for regulation of receptor function at synapses in the CNS. Both the extent of regulation by external calcium and the magnitude of the calcium permeability are determined by the particular combination of subunits that make up the receptor. Both of these phenomena could contribute to cholinergic neurotoxicity and could be particularly relevant if the receptors were located presynaptically.

DISCUSSION

One consequence of the diversity of receptor subunits and of the receptors they form is a diversity of responses to different ligands. This observation suggests that efforts to more carefully define the properties of the ligands that activate or block different receptors could have important results. It might prove possible to define classes of agonists or antagonists that are exquisitely specific for particular combinations of subunits. These ligands might have several important uses. They might be valuable for dissecting the function of particular cholinergic systems in the brain by providing the investigator with sharp tools. They might also be powerful drugs to help overcome addiction to nicotine. Alternatively, they might prove to be drugs that provide some of the beneficial aspects of nicotine but with fewer of the harmful results and prove useful in treating diseases in which misfunction of nicotinic function is suspected, such as Alzheimer's disease. This diversity of ligand binding specificity is clearly a double-edged sword because, although it may allow the creation of powerful tools, it may (as pointed out above) provide yet additional drugs with abuse potential.

However, it seems reasonable that there is a window of opportunity in the pharmacological diversity of the ligand-gated ion channels. There is the potential for the design of drugs that would provide important access to function in the CNS. It also seems likely that the magnitude of the window is currently underestimated. There may be many more aspects of receptor function that vary with subunit combination, and access to the diversity related to these aspects may be important. For example, different receptor subunit combinations might be differentially regulated by peptides or external ions, allow passage of different combinations of ions, be subject to regulation by different cytoplasmic mechanisms, or be found in different portions of the

neuron. An interesting result in this regard might be the presence of nicotinic receptors in the presynaptic membrane where they might regulate release of such neurotransmitters as dopamine or serotonin. The discovery of the diversity of ligand-gated ion channels is recent, but the idea has been rapidly assimilated. However, there remains the exploitation of this diversity to better understand the brain and to design drugs to better deal with the various diseases that affect the brain.

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Neurotransmitter and Drug Receptor Genes

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INTRODUCTION

Understanding neurotransmitter and drug receptor genes has potential for enhancing the molecular neurobiology of substance abuse in at least two fashions. First, understanding the molecular mechanisms whereby drugs and neurotransmitters affected by drugs interact with their initial biological targets, their receptors, can enhance understanding of acute drug action. Structure-function relationships can be approached by modifying both the ligands and the receptors, enhancing possibilities for development of, for example, antiabuse medications. Second, since receptor genes likely are key to the mechanisms underlying substance abuse, they are promising candidates for the population variants that could explain some of the individual-to-individual differences in susceptibility to drug abuse in human populations. Recent studies of genes encoding a novel γ-aminobutyric acid (GABA) receptor and of variant dopamine D2 receptor genes in drug-using and drug-free human populations clearly demonstrate these points.

NOVEL GABA RECEPTORS: GABA C?

Classical pharmacological, binding. and electrophysiological studies suggest that GABA produces most of its activities through interactions with two major receptor classes: (1) GABA A receptors, composed of subunits that form GABA-gated chloride channels and bind bicuculline, muscimol, barbiturates, and benrodiazepines and interact with ethanol, and (2) GABA B receptors that are responsive to baclofen and can alter calcium fluxes. Thus, GABA A receptors are principal sites for the action of three classes of abused substances: ethanol, barbiturates, and benzodiazepines.

Studies of cloned GABA A receptor subunits reveal that mixtures of α-, β-, γ-, and δ-subunits may form functional brain receptors. Variation in subunit composition can alter the activities of the resultant receptors; the specific

receptor subunit profile expressed by a neuron thus determines its differential cellular responsiveness to GABA or related drugs (Pritchett and Seeburg 1990; Luddens et al. 1990). With the diversity of the possible GABA receptors that could be formed in vivo, the exact criteria for defining GABA A receptors have become debatable: (1) Should classical pharmacologic criteria, such as inhibition by the competitive GABA antagonist bicuculline, define these receptors, or (2) should structural membership in the family of multimeric ligand-gated chloride channels defined by cloning studies constitute the defining feature?

Some GABA responses in visual pathways are mimicked by muscimol and inhibited by picrotoxin but are insensitive to the competitive GABA A antagonist bicuculline and to GABA B antagonists (Sivilotti and Nistri 1988, 1989, 1991; Nistri and Sivilotti 1985). These receptors are also insensitive to barbiturates and benzodiazepines. Miledi and coworkers have recently shown that GABA responses conferred by retinal mRNA in the *Xenopus* oocyte system display the same bicuculline and baclofen resistance (Polenzani et al. 1991). Based on the pharmacologic criteria noted above, GABA C responses thus would be neither GABA A nor GABA B. GABA responses that are insensitive to both bicuculline and baclofen have been called GABA C by Johnston (1986). However, electrophysiological features of GABA C responses are consistent with their mediation by ligand-gated ion channels such as those characteristic of GABA A receptors (Olsen and Venter 1986): Conceivably, bicuculline-insensitive GABA responses could be conferred by a receptor highly homologous to other known GABA A receptors.

We have recently cloned a cDNA for a receptor subunit, GABA p-1, whose mRNA is highly expressed in retina. When expressed as a single subunit in the *Xenopus* oocyte system, p-1 mRNA consistently and robustly confers picrotoxin-sensitive GABA responses whose reversal potential indicates changed chloride conductance. Although several GABA A receptor subunits can form functional ligand-gated channels when expressed in various combinations, such responses are typically variable and inconsistent unless several subunits are coexpressed (Blair et al. 1988; Khrestchatisky et al. 1989; Shivers et al. 1990; Malherbe et al. 1990; Verdoorn et al. 1990).

GABA p-1 responses are strikingly insensitive to inhibition by bicuculline (Shimada et al., in press). The GABA binding site of this receptor thus shows substantial differences from the GABA binding sites on α- and ß-homo-oligomeric receptors (Blair et al. 1988; Khrestchatisky et al. 1989; Shivers et al. 1990; Malherbe et al. 1990; Verdoorn et al. 1990). This site's properties fit with those of the "unusual" bicuculline-resistant GABA receptor in visual pathways, with GABA responses in *Xenopus* oocytes injected with

retina mRNA, and with the GABA C receptor defined by Johnston (Sivilotti and Nistri 1989; Johnston 1986). Expression of the GABA p-1 cDNA as a homo-oligomer thus creates a unique GABA binding site. When the primary structure of this receptor cDNA is compared with other known ligand-gated channels, its closest homology is with GABA A receptor subunits. Nevertheless, its sequence is more divergent from the other classes of GABA receptor subunits than they are from each other. If a GABA A receptor is defined based on its membership in this family and based on its ability to form a ligand-gated channel, then the GABA p-1 receptor belongs in the GABA A family. To the extent that GABA A receptors are defined based on a pharmacologic feature, bicuculline sensitivity, this receptor falls into the class of "unusual GABA receptors" or GABA C receptors (Sivilotti and Nistri 1991; Johnston 1986).

Could this receptor be expressed as a p-1 homo-oligomer in the retina or brain? The unique properties of the p-1 receptor are maintained when it is coexpressed with either an α- or a ß-GABA A subunit (Shimada et al., in press). Although studies defining which subunits are coexpressed by neurons are necessary before the significance of such observations for in vivo receptor function can be known, these results—and observations that the major component of GABA responses obtained in *Xenopus* expression studies of mRNA isolated from the retina is bicuculline insensitive—are consistent with the p-1 subunit's self-association in vivo.

The pharmacologic profile obtained from the expressed p-1 subunit suggests that the resultant receptors could be termed GABA C. If further evidence supports a self-associating role for this subunit, this role may be sufficiently unique to demand this designation. In any case, these receptors' properties make them ideal molecular tools with which to investigate the features necessary to confer ethanol, benzodiazepine, and barbiturate sensitivity on ligand-gated chloride channel GABA receptors. By making chimeric receptors containing specific regions of GABA A α-subunits spliced onto the p-1 backbone, researchers have the opportunity to produce single subunits that should express at high levels by themselves and gain sensitivity to barbiturates and benzodiazepines with the addition of specific α-sequences. Such constructions will allow testing of hypotheses such as that advanced by Pritchett and Seeburg (1990) that specific N-terminal amino acids are important molecular features for benzodiazepine actions.

DOPAMINE D2 RECEPTOR ALLELES IN SUBSTANCE ABUSERS

Several substances that share the potential for abuse by humans also share the ability to enhance dopamine activity in mesolimbic/mesocortical circuits thought

to be important for behavioral reward and reinforcement (Lippa et al. 1973; Di Chiara and Imperato 1988; Wise and Rompre 1989). For example, cocaine's ability to inhibit reuptake of dopamine indicates a possible direct action for this highly reinforcing drug in these dopaminergic circuits (Ritz et al. 1987; Grigoriadis et al. 1989).

Blum and coworkers (1990) suggested that the "A1" allele of the dopamine D2 receptor gene may display an association with alcoholism. This allele, identified by a *Taq* I restriction fragment length polymorphism (RFLP) of the human dopamine D2 receptor (Grandy et al. 1989), was present in 69 percent of alcoholics but only 20 percent of nonalcoholics. However, Bolos and colleagues (1990) found that the A1 allele frequency was not significantly higher in 40 alcoholics than in 127 individuals from two other samples not characterized with respect to alcohol use. A substantial genetic contribution to susceptibility to alcoholism is supported by family, twin, and adoption studies (Goodwin 1979; Cloninger et al. 1981; Cloninger 1987). A genetic component of vulnerability to drug abuse is less clearly documented but has been suggested in both twin and adoption studies (Cadoret et al. 1987; Pickens et al. 1991). These considerations led to the examination of whether subjects with substantial self-reported alcohol, other drug, or nicotine use also display elevated A1 allelic frequencies.

Examining such an allelic association in drug abusers raises methodological concerns relating to polysubstance abuse, reliability of subjects, and means used to categorize factors such as extent of drug use and dependence. Recognizing these difficulties and the importance of a possible association between drug use and specific receptor gene alleles, O'Hara and colleagues (submitted for publication) studied D2 receptor alleles in almost 400 individuals volunteering for research protocols at the National Institute on Drug Abuse Addiction Research Center or presenting to the Johns Hopkins hemodialysis and genetics clinics to provide population controls.

The A1 allele was present at a higher frequency in blacks than in whites ($z=4.37$, $P<.00001$) for all groups combined. The A3 allele was present in 15 of 206 black subjects but absent in the 225 white subjects ($z=3.64$, $P<.001$), providing further evidence for a racial difference in D2 allelic status. No association between ethnicity and presence of the A1 allele was found in an analysis of Greek (7 of 25=28 percent A1 present) and Italian (8 of 25=32 percent A1 present) individuals.

Associations between A1 allele presence and drug use were examined separately in blacks and whites. In whites, a trend toward an association was found in comparisons of subjects with substantial (++ and +++) vs.

minimal (0 and +) total drug use (z=1.57, P<.06). Bartholomew's test also showed a trend for an increasing gradient in proportion of subjects with the A1 allele across the four levels of substance use (P<.10). Black subjects displayed no apparent association for either comparison.

Allelic association was also examined for individual drugs. A1 allele presence was higher for white heavy users (++ and +++) of most substances compared with whites with the lowest overall substance use (0 on total use); none of these differences reached statistical significance. However, since many of the subjects used multiple substances, the comparisons are not between independent groups. Black subjects displayed no association between A1 presence and use of any substance class.

The hypothesis that individual differences in substance abuse may be due, in part, to different alleles of the dopamine D2 receptor gene arose from initial work in alcoholics (Blum et al. 1990) and was strengthened by a compelling biological rationale for interactions between abused drugs and brain dopamine systems (Di Chiara and Imperato 1988; Wise and Rompre 1989).

The lack of strong association between D2 receptor gene alleles and substance use evident in this study is consistent with estimates of the heritable components of alcoholism and drug abuse (Devor and Cloninger 1989; Cadoret et al. 1987). One recent study of concordance rates for alcoholism in twin populations suggests that between 20 and 30 percent of the vulnerability to abuse or depend on these substances may be genetic in origin (Pickens et al. 1991). Attempts to link familial alcohol susceptibility to specific chromosomal markers and patterns of inheritance in families have not been consistent with a single genetic locus (Gilligan et al. 1987; Aston and Hill 1990). The strong association between a single gene allele and alcoholism found by Blum and coworkers (1990) would thus fit poorly with this extent of heritability. The large environmental influences on expression of alcoholism and Blum and colleagues' study of unrelated individuals rather than defined pedigrees also make the strength of their findings surprising.

The associations that Uhl and colleagues (1992) found reach only the margins of statistical significance and are evident in only one race. Thus, they should be approached with both interest and caution, although an association of this magnitude would fit better with previous results concerning heritability of drug abuse. Striking results (P<0.001) can be obtained when Uhl and colleagues' (1992) data are combined with other published studies: 31 percent (78 of 253) of white individuals lacking documented substance abuse and 46 percent (88 of 193) of whites with alcohol abuse/dependence or heavy substance use display the A1 allele (Blum et al. 1990; Grandy et al. 1989; Bolos et al. 1990; Parsian et

al. 1991). Although alternative explanations must be considered (see below), these results cannot rule out an association between a D2 receptor genotype and substance use.

In contrast to the finding of a modest possible association between receptor gene alleles and level of drug use, there was a highly significant effect of race on allelic frequencies. The 20-percent A1 allele frequency in whites contrasts with 37 percent for blacks. No white individual had an A3 allele, whereas the frequency in blacks was 3 percent. These data agree with findings of higher A1 allele presence in blacks than in whites (Blum et al. 1990) and no A3 alleles in 167 whites (Bolos et al. 1990).

Gene allelic frequency differences among distinct white populations are well documented for disease-related genes such as those causing thalassemia (southern European predominance) and cystic fibrosis (northern European predominance) (Cystic Fibrosis Genetic Analysis Consortium 1990; Orkin et al. 1982). Population-to-population differences within Caucasian groups presumably exist for many anonymous RFLP markers as well, although analysis comparing Greek and Italian individuals fails to provide support for such differences in D2 allelic distribution (O'Hara et al., submitted for publication). The modest possible allelic association with substance use in whites could result from disproportionate membership of heavy substance users in ethnic groups with high A1 frequencies, but the broad populations from which the different groups were drawn and O'Hara and colleagues' (submitted for publication) failure to find substantial differences in two Caucasian populations make this hypothesis less likely.

The results of this study could be extended in several ways. Sequencing genes cloned from A1 and A2 homozygotes could pinpoint base pair changes at the polymorphic *Taq* I sites and address the possibility that other base pair changes linked to these alleles might produce functional differences. Individuals with A1, A2, and A3 genotypes could also be tested to identify differing physiological and psychological responses to administered drugs. Finally, study of the genotypes of additional white individuals with carefully ascertained ethnicity and drug use, members of more kindreds displaying striking familial patterns of substance use, and individuals meeting criteria for drug abuse/dependence diagnoses could enhance the data presented here. Positive results of such investigations would strengthen confidence that a D2 receptor allele confers vulnerability to substance abuse and provides a biological marker allowing targeted interventions for vulnerable individuals.

SUMMARY

Studies of the two neurotransmitter receptor genes described here illustrate the rich possibilities that this area of molecular neurobiology holds for drug abuse research. Since many of the 100 to 150 different neurotransmitters could have multiple receptor genes, as many as 1,000 different genes or 1 percent of the human genome might encode neurotransmitter receptors. Clearly, as the other chapters in this monograph also indicate, the field is ripe with possibilities of discovery with implications for drug action.

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The Diversity of the Dopamine Receptors

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INTRODUCTION

Among all the neuronal pathways, the dopamine system is thought to have the leading role in modulating drug addiction. This system relies on the interaction of one neurotransmitter, dopamine, with several receptors.

Classically, dopamine was thought to exert its effects by binding to only two G protein-coupled receptors, known as the D1 and D2 receptors (Kebabian and Calne 1979). These receptors have been differentiated pharmacologically, biologically, and physiologically and also by their anatomical distribution. These two receptors exert their biological actions by coupling to and activating different G protein complexes. The D1 receptor interacts with the Gs complex to activate adenylyl cyclase, whereas the D2 interacts with Gi to inhibit cyclic adenosine monophosphate (cAMP) production.

CLONING OF THE D2 DOPAMINE RECEPTOR

The cloning of the D2 dopamine receptor resulted from the use of a strategy based on the sequence homology expected to exist among G protein-coupled receptors. The ß2-adrenoreceptor coding sequence was used as a hybridization probe to screen a rat genomic library under low-stringent hybridization conditions. By screening the equivalent of three genomes, 90 positive clones were identified, from which 20 were characterized and partially sequenced. This allowed the characterization of the clones encoding the rat ß1-adreno (Machida et al. 1990), the serotonin 1a (Albert et al. 1989), the muscarinic 4 (J.R. Bunzow and O. Civelli, unpublished observation), and another clone, RGB-2, which, as described below, encodes the dopamine D2 receptor (Bunzow et al. 1988).

The RGB-2 clone was used to screen a rat brain cDNA library. One positive 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, 21 amino acid residues that are 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.

The full-length RGB-2 cDNA 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. Stable transfectants were prepared and analyzed for their ability to bind dopamine ligands (Bunzow et al. 1988). L-RGB2Zem-1 membranes bound D2 dopamine agonists and antagonists with the same pharmacological profile as do rat striatal membranes. These studies used the antagonist [3H] spiperone, whose binding was shown to be saturable and of high affinity. [3H] spiperone binding to L-RGB2Zem-1 membranes was displaced by several antagonists with the stereospecificity expected of a D2 receptor and with the same Kis as determined in rat striatal membranes.

D2 receptors are present on lactotroph cells of the anterior pituitary where they regulate prolactin (PRL) secretion. The somatomammotroph cell line GH_4C_1 is derived from a rat pituitary tumor, is devoid of endogenous dopamine receptors, and is known to secrete PRL. GH_4C_1 cells were transfected with the RGB-2 cDNA metallothionein construction. One clone, GH_4ZR_7, was found to express high levels of RGB-2 mRNA (Albert et al. 1989). Since the D2 receptor is expected to inhibit cAMP levels, vasointestinal peptide (VIP) was used to stimulate endogenous cAMP production. Dopamine inhibited both basal and VIP-stimulated cAMP levels in media from GH4ZR7 cells. Intracellular cAMP levels were also inhibited. The stereospecificity of these inhibitions was demonstrated using isomers of sulpiride: The active enantiomer (-)sulpiride blocked the inhibition, whereas (+)sulpiride had no effect. To demonstrate that the changes in cAMP levels were the result of an inhibition of adenylate cyclase, dopamine was added to membranes of VIP- or forskolin-stimulated GH4ZR7 cells, and adenylate cyclase activity was measured. Dopamine inhibited adenylate cyclase activity in a stereoselective manner. Finally, the inhibition of PRL secretion by dopamine was assayed in GH4ZR7 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 by (+)sulpiride. Therefore, the RGB-2 cDNA encodes a D2 dopamine receptor that is functional.

CLONING OF THE DOPAMINE D1 RECEPTOR

The success of the homology approach in D2 receptor cloning opened the door for the cloning of other dopamine receptors, in particular the D1 receptor. The authors and colleagues took advantage of the polymerase chain reaction (PCR)-based approach, which had been developed to clone several thyroid G protein-coupled receptors (Libert et al. 1989). This approach consists of synthesizing two sets of synthetic oligonucleotides that correspond to two highly conserved regions among all the G protein-coupled receptors (found generally in transmembrane domains III and VI). These oligonucleotides are used as primers in a PCR reaction for specific amplification of cDNAs containing complementary sequences. The cDNAs used for the D1 receptor cloning were synthesized from rat striatum. To direct the PCR approach toward the specific cloning of the D1 receptor, we added another technical step. Because it is known that Gs-coupled catecholamine receptors have a putative third cytoplasmic loop of 52 to 78 amino acids (Zhou et al. 1990), our total population of PCR products was size fractionated, and products in the expected range were sequenced. Of 24 PCR products, 7 encoded potential G protein-coupled receptors, one of which showed structural features expected to belong to Gs-coupled catecholamine receptors. This clone was used to screen a rat cDNA and human and rat genomic libraries since most catecholamine receptor genes lack introns in their coding regions (Lefkowitz et al. 1988). The isolated clone was sequenced and shown to contain all the characteristics expected of G protein-coupled receptors (i.e., share the highest degree of similarity and prototypical structural features of the catecholamine receptors). The absence of a glutamic residue found in the third transmembrane domains of all the ß-adrenoreceptors and the size of the third cytoplasmic loop suggested that the cloned receptor could be a Gs-coupled dopamine receptor, namely the D1 receptor.

The demonstration that the cloned receptor was the D1 receptor was accomplished by expressing the corresponding gene. First, the putative D1 receptor human gene was expressed by transient expression in COS-7 cells. Membrane proteins from the transfected cells were tested for their ability to bind D1 receptor ligands. The specific antagonist SCH 23390 was found to have the highest affinity for the cloned receptor, and the overall pharmacological profile was that of a D1 receptor binding site. The biological activity of the cloned receptor was studied upon transient transfection in human kidney 293 cells and analysis of dopamine stimulation of adenylyl cyclase activity. The cloned receptor was shown to stimulate adenylyl cyclase activity according to a pharmacological profile expected for the D1 receptor. Therefore, we concluded that we had cloned the D1 dopamine receptors.

DIVERSITY OF THE DOPAMINE RECEPTORS

As discussed above, pharmacological analyses had agreed on the existence of only two dopamine receptors (Hess and Creese 1987; Creese 1986; Leff and Creese 1985). With the cloning of these two receptors, new tools were at hand to further understanding of the dopamine system. In 2 years, several studies using molecular biological approaches have shown that the classical view of the dopamine system was incomplete.

Two Forms of the D2 Receptor

The discovery that not one but two dopamine D2 receptor forms exist was reported in 1989 (Selbie et al. 1989; Grandy et al. 1989; Dal Toso et al. 1989; Giros et al. 1989; Monsma et al. 1989; Chio et al. 1990; Miller et al. 1990; O'Malley et al. 1990) and showed that the two D2 receptor forms exist in human, rat, and bovine cells. These two forms differ in 29 amino acid residues located in the putative third cytoplasmic loop of the receptor. The short form is the one originally cloned (as described above); the long form is new and was discovered either by screening cDNA libraries or by PCR analyses.

Several data were obtained about the 29 amino acid addition. First, the additional residues do not modify the affinity or the profile of the D2 receptor for antagonists (Grandy et al. 1989; Giros et al. 1989). Second, they do not affect significantly the ability of the receptor to inhibit cAMP production (Dal Toso et al. 1989) as could have been expected from their location in the third cytoplasmic loop. Third, it was found that the 29-amino acid addition contains two potential glycosylation sites, but thus far, nothing is known about their importance (Grandy et al. 1989). Fourth, it was found that although the two forms of the D2 receptor coexist in all tissues analyzed, their ratio varies. The short form is the least abundant; its concentration is very low in the pituitary but represents about half of the D2 receptor mRNA in the pons or medulla (Giros et al. 1989; O'Malley et al. 1990). Fifth, the generation of the two forms of D2 receptor was shown to be the result of an alternative splicing event occurring during the maturation of the D2 receptor pre-mRNA (Grandy et al. 1989; Dal Toso et al. 1989; O'Malley et al. 1990), which was demonstrated by the discovery of an 87-bp exon encoding the additional amino acid residues. These studies also led to the description of the organization of the D2 receptor gene, with the coding part of the D2 receptor encoded by seven exons, one of which (exon 5 in Grandy et al. 1989) is alternatively spliced.

In summary, the two D2 receptor forms have not been shown to differ in their pharmacological or biological activities. They are generated by alternative splicing and coexist in a tissue-specific ratio. Any differences in their biological significance have yet to be demonstrated.

New Dopamine Receptors

Probably the most striking discovery to emerge from the cloning of G protein-coupled receptors is their diversity. Every class of G protein-coupled receptor studied by recombinant DNA techniques has been proven more complex than had been characterized pharmacologically (Bonner et al. 1987; Schwinn et al. 1990; Emorine et al. 1989). In view of the diversity of physiological responses modulated by dopamine receptors, there was ample reason to believe that the dopamine receptor class would also follow this trend. The success of the homology approach in cloning the D2 receptor and the availability of dopamine receptor probes led to the search for new, undescribed dopamine receptors.

D3 Receptor. Through the combination of cDNA library screening, PCR extension, and genomic library screening, a cDNA was isolated that encodes for a novel receptor related to the D2 receptor, thereafter coined the D3 receptor (Sokoloff et al. 1990). This receptor shares 75 and 41 percent of its putative transmembrane sequences with the D2 and D1 receptors, respectively. Moreover, it is encoded by a gene that contains five introns in its coding region and whose organization is similar to that of the D2 receptor. The D3 receptor structure is also highly similar of that of the D2 receptor, in that it contains a large third cytoplasmic loop of similar size to that of the long form of the D2 receptor. However, the organization of the gene in this loop does not allow for alternatively spliced forms. The D3 receptor also contains the residues found to be important for catechol and amine groups recognition in the catecholamine receptors. Altogether, the structure of the D3 receptor suggested a close relationship to the D2 receptor, which was confirmed by pharmacological analyses.

When expressed in eucaryotic cells (COS-7 or CHO cells), the D3 receptor was shown to have a pharmacological profile reminiscent of that of the D2 receptor. It binds D2 ligands (not D1 or other catecholamine ligands), although its affinity to most neuroleptics was 10- to 100-fold less than that of the D2 receptor. However, the D3 receptor was found to bind two particular antagonists, (+)AJ76 and (+)UH232, with three to five times more affinity than the D2 receptor. These antagonists are thought to have a higher specificity for the dopamine presynaptic receptors or autoreceptors. Interestingly, the binding of dopamine to the D3 receptor was not affected by the addition of guanylnucleotides, which block G protein-coupled receptors in their high affinity state and are used to measure G protein coupling. This result might be explained by the absence of suitable G proteins in the transfected cells (COS-7 and CHO cells) or by a low modulation of dopamine binding by guanylnucleotides at the D3 receptor. The latter was not shown to modulate cAMP formation in CHO cells, in contrast to the other dopamine receptors.

The tissue distribution of the D3 receptor was determined by Northern blot and in situ hybridization analyses. It not only was found to overlap but also to differ from that of the D2 receptor. The D3 receptor is absent in the pituitary and is expressed at low levels in the neostriatum, whereas high densities of the D2 receptor are present in both. The distribution of the D3 receptor overlaps with the D2 receptor in the olfactory tubercules and the hypothalamus. Moreover, the D3 receptor is expressed at high levels in the islands of Calleja and in the nucleus accumbens, regions that are part of the limbic system and where the concentrations of D2 receptor are relatively lower. In addition, the D3 receptor was found to colocalize with the D2 receptor in presynaptic cells that produce dopamine in the substantia nigra and in the ventrotegmental areas.

D4 Receptor. By analyzing the mRNA population of the neuroepithelioma SK-N-MC cells with D2 receptor cDNA probes under conditions of low stringency, the existence of a D2-related mRNA was detected (Van Tol et al. 1991). The corresponding cDNA and gene were sequenced and found to encode another novel dopamine receptor, the D4 receptor. In its putative transmembrane domains, the D4 receptor is 41, 52, and 51 percent identical to the D1, D2, and D3 receptors, respectively, and it contains the residues necessary for catecholamine recognition. Its putative third cytoplasmic loop is shorter than that of the D2 receptor. At the genomic level, the D4 coding sequence is separated by four introns that are positioned similarly to those of the D2 receptor. In addition, a 52-bp repeat borders both sides of the third intron. This unusual intron-exon junction does not contain conventional splice sites and allows for potentially variable alternative splicing events without changes in protein sequence.

The D4 receptor was expressed in COS-7 cells, and its pharmacological profile was determined. Most of the tested agonists and antagonists displayed affinities for the D4 receptor similar to or lower than that for the D2 receptor. However, the D4 receptor binds one particular antipsychotic, clozapine, with an affinity tenfold higher than either the D2 or D3 receptors.

Clozapine is a particularly interesting antipsychotic agent whose action is not associated with the motor control side effects that plague other neuroleptics. However, clozapine has its own side effects. Most deleterious is that it causes agranulocytosis in a few cases, which has prevented its general use. In any case, the discovery of a dopamine receptor specific for a neuroleptic that might not affect the centers for motor control in the central nervous system is important. A preliminary analysis of the D4 receptor tissue location indicated that the D4 receptor is expressed in the mesocorticolimbic system rather than in the nigrostriatal systems (Van Tol et al. 1991), adding credit to the hypothesis that stimulation of this receptor has little impact on control of movement. These

observations suggest that the D4 receptor is most likely the primary target mediating the antipsychotic action of clozapine.

Other Dopamine Receptors. There are indications that the diversity in dopamine receptors does not stop at the D4 receptor. The existence of another D2-like receptor has been reported (Todd et al. 1989). This receptor was expressed upon transfection of rat genomic DNA into mouse fibroblasts and was identified by its ability to bind iodinated spiperone. This receptor has a pharmacological profile closely resembling that of the cloned D2 receptor, yet it has a sequence different from that of the cloned D2 receptor as shown by the inability of its mRNA to hybridize to three sets of oligonucleotides specific to the D2 sequence. Stimulation of this receptor by dopamine leads to an increase in intracellular calcium concentrations that appears not to be mediated through G protein coupling.

Other D1-like receptors have also been described. D1-like receptors have been detected in renal tissue (Felder et al. 1989). These are linked to the activation of phosphatidylinositol-specific phospholipase C. These D1-like receptors are different from the cloned D1 receptor because the latter is not expressed in renal tissue (Zhou et al. 1990; Dearry et al. 1990). A D1-like receptor that couples to inositol phosphate production has also been detected by expression in *Xenopus* oocytes (Mahan et al. 1990). This receptor is encoded by a mRNA found in the rat striatum but is of different size than the one encoding the cloned D1 receptor.

There are also indications that yet other dopamine receptors might exist. Pharmacological and biological data discussing the existence of putative receptors have been discussed (Andersen et al. 1990). Moreover, several laboratories have recently cloned a new D5 or D1b receptor (D.G. Grandy, personal communication, 1991; H.H.M. Van Tol, personal communication, 1991; M.G. Caron, personal communication, 1991).

CONCLUSIONS AND PERSPECTIVES

Based on pharmacological studies, the dopamine receptors have been classified into two subtypes, the D1 and D2 receptors. The cloning of the dopamine receptors has revealed that, although the number of dopamine receptors is larger than expected, they can still be classified under the broad categories of D1- and D2-like receptors. The D1-like receptors are encoded by genes that have no intron in their coding sequence, share more than 50 percent identity with the D1 receptor in the sequences part of the transmembrane domains, have a pharmacological profile that resembles that of the D1 receptor and that binds efficiently the antagonist SCH 23390, and

stimulate adenylyl cyclase activity. The D2-like receptors have similar characteristics when compared with the D2 receptor—they efficiently bind spiperone and inhibit adenylyl cyclase activity.

The complexity of the dopamine receptor family is not surprising in view of the complexity of the other receptor families that are part of the G protein-receptor supergene family (Bonner et al. 1987; Schwinn et al. 1990; Emorine et al. 1989). How many dopamine receptors will be found is unknown. However, as presently understood, there are two widespread and quantitatively predominant dopamine receptors, the classical D1 and D2 receptors. The other dopamine receptors are present in significantly lower amounts in restricted localizations, and they can be related to the two major dopamine receptors through their pharmacological profile. Therefore, most of what has been known about dopamine agonists' and antagonists' actions has to be reevaluated in view of the existence of the different dopamine receptors. This underscores the impact that the cloning of the D2 receptor will have on the field of dopamine receptors. Finally, the discovery of new dopamine receptors is also interesting from an evolutionary point of view. One neurotransmitter interacts with a variety of receptors, suggesting that nature has spent a large effort in developing the targets in mechanism of synaptic transmission, a fact that could increase understanding of higher brain function.

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Cloned Dopamine Receptors: Targets in Therapy of Drug Abuse

Philip Seeman

INTRODUCTION

Most self-rewarding behaviors operate through the brain dopamine system. This generalization also holds for drug abuse. For example, many common drugs of abuse, including amphetamine, cocaine, LSD, nicotine, ethanol, and opiates, promote the release of dopamine in vivo (Di Chiara and Imperato 1988). Moreover, the acute overdoses of some of these drugs of abuse (e.g., amphetamine, cocaine, or LSD, but not ethanol or opiates) often are treated by dopamine receptor-blocking medications such as haloperidol or other neuroleptics.

EFFECTIVE THERAPY TARGETS: TRANSPORTERS OR RECEPTORS?

In clinical psychiatry and neurology, most medications that are used to alleviate various illnesses block neurotransmitter transporter sites or receptor sites. In general, the more effective medications are those that block receptors directly. Medications that block the transmitter uptake sites (or transporter sites), although useful, generally have been less effective clinically than those that block or stimulate receptors directly.

For example, imipramine or chlorimipramine are used in the treatment of clinical depression. These compounds inhibit the uptake or transport of norepinephrine into nerve terminals. These medications generally require about 2 or 3 weeks before clinical improvement occurs. Moreover, the magnitude of the overall efficacy of these medications is unclear, because many studies indicate that admitting a depressed patient into a hospital alleviates depression in 60 percent of patients. This compares with about 70 percent improvement when using both medication and hospitalization of depressed patients.

The mechanism thought to explain the action of these antidepressant drugs is that, by inhibiting the uptake of norepinephrine, the drugs increase or prolong

the release of the transmitter, which in turn leads to a reduction of ß-adrenoceptors in the postsynaptic neurones. Thus, the ß-adrenoceptors are indirectly and slowly affected by the transporter-inhibiting drugs.

A more rapid and effective antidepressant action may be obtained by stimulating the ß-adrenoceptors directly by means of ß-adrenoceptor agonists. Although this agonist-type strategy has been found experimentally to be rapid and effective in alleviating depression, the cardiovascular side effects currently preclude routine treatment by agonist therapy.

However, fluoxetine is an important example of a successful transporter blocker that alleviates depression relatively rapidly in about one-third of depressed patients. The effect becomes apparent within 1 week of treatment.

A second example of presynaptic therapy affecting the transporter site is in Parkinson's disease. Dopamine uptake inhibitors, including benztropine, are no longer used in treating this illness. However, agonists that stimulate dopamine D1 or D2 receptors are effective in Parkinson's disease.

Presynaptic receptor therapy is also less effective clinically. For example, compounds such as BHT 920 and 3-PPP act on presynaptic dopamine receptors to inhibit the release of dopamine. Although such inhibition does confer neuroleptic-like effects clinically (e.g., against psychosis or schizophrenia), the clinical action alleviates only about 40 percent of psychotic patients, compared with an improvement of 35 percent of such patients without neuroleptics, and approximately 70- to 90-percent improvement with dopamine receptor-blocking neuroleptics.

GOAL: TARGETING DIFFERENT DOPAMINE RECEPTORS IN BRAIN PSYCHOMOTOR REGIONS

Although receptors are effective clinical targets, recent medications are not sufficiently receptor selective to yield clinical actions free of unwanted side effects. The recent discoveries of multiple receptors for each transmitter now permit the design and development of medications selective for a specific subtype of receptor.

Thus, designing a neuroleptic that selectively targets D2-like receptors in nonmotor brain regions will obviate motor side effects of such a neuroleptic in psychotic patients. This appears to be the case with clozapine, a neuroleptic that is selective for the D4 dopamine receptor (Van Tol et al. 1991).

TYPES OF DOPAMINE RECEPTORS

The first type of dopamine receptor reported, now termed D1, was identified by its ability to respond to dopamine and to stimulate adenylate cyclase (Kebabian et al. 1972). The DNA for this receptor has been cloned (Sunahara et al. 1990; Zhou et al. 1990; Dearry et al. 1990); the amino acid sequence of D1 is shown in figure 1.

The second type of dopamine receptor, now termed D2, was identified by its affinity for nanomolar concentrations of antipsychotic drugs (Seeman et al. 1974, 1975a, 1975b, 1976, 1984, 1987; Titeler et al. 1978; Seeman and Niznik 1990). The DNA for D2 has been cloned (Bunzow et al. 1988; Grandy et al. 1989; Martens et al. 1991); the amino acid sequence for D2 is shown in figure 1.

Additional dopamine receptors have been found recently by homology probing, that is, by probing genomic DNA or cDNA with oligonucleotides containing bases similar or identical (homologous) to D1, D2, and related catecholamine receptors.

This approach yielded short and long forms of D2 (Giros et al. 1989; O'Dowd et al. 1990), a D3 dopamine receptor (Sokoloff et al. 1990; Giros et al. 1990), a D4 dopamine receptor (Van Tol et al. 1991), and a D5 dopamine receptor (Sunahara et al. 1991).

In addition, this approach yielded truncated forms of these receptors, such as a D3 receptor with its third transmembrane portion deleted and a D3 receptor with its second outer (or extracellular) portion deleted (Giros et al. 1991). The sequences of these truncated D3 receptors, named D3 (TM-del) and D3 (O2-del), respectively, are shown in figure 1.

Moreover, the method of homology probing has revealed at least two human pseudogenes of D5 (Nguyen et al. 1991).

Of the amino acid sequences shown in figure 1, 13 amino acids are homologous in at least 75 various membrane-located receptors (for various amines, peptides, and hormones) that are all GTP sensitive. These 13 amino acids, which are common to all the G-linked receptors, are illustrated by arrows in figure 2 and by solid black triangles in figure 1.

FIGURE 1. *Amino acid sequences of dopamine receptors. D1 human is from Sunahara et al. 1990; D1 rat from Zhou et al. 1990; D2 human from Grandy et al. 1989 and Dal Toso et al. 1989; D2 rat from Bunzow et al. 1988; D2 frog from Martens et al. 1991; D3 human from Giros et al. 1990; D3 rat from Sokoloff et al. 1990; D3 rat (with transmembrane segment 3 deleted) and D3 rat (with extracellular segment 2 deleted) from Giros et al. 1991; D4 human from Van Tol et al. 1991; and D5 human from Sunahara et al. 1991. The solid black triangles indicate the 13 amino acids that are found to be identical (homologous) in more than 75 G-linked receptors for various amines, peptides, and hormones.*

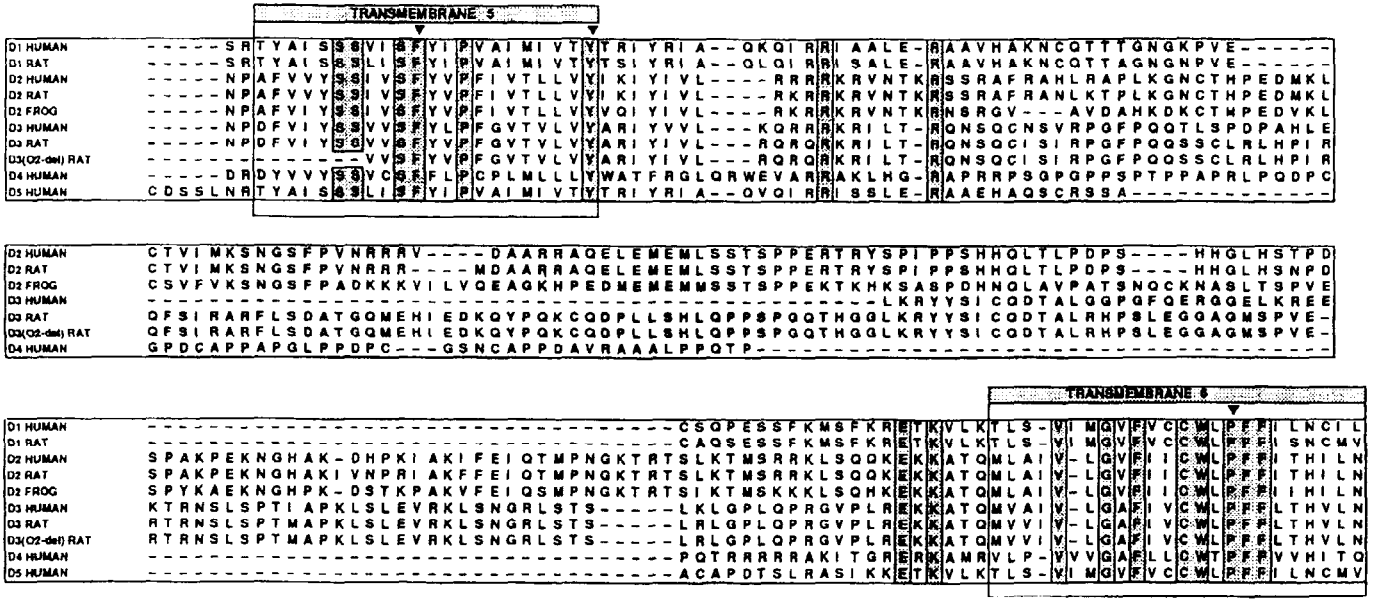

FIGURE 1. *continued*

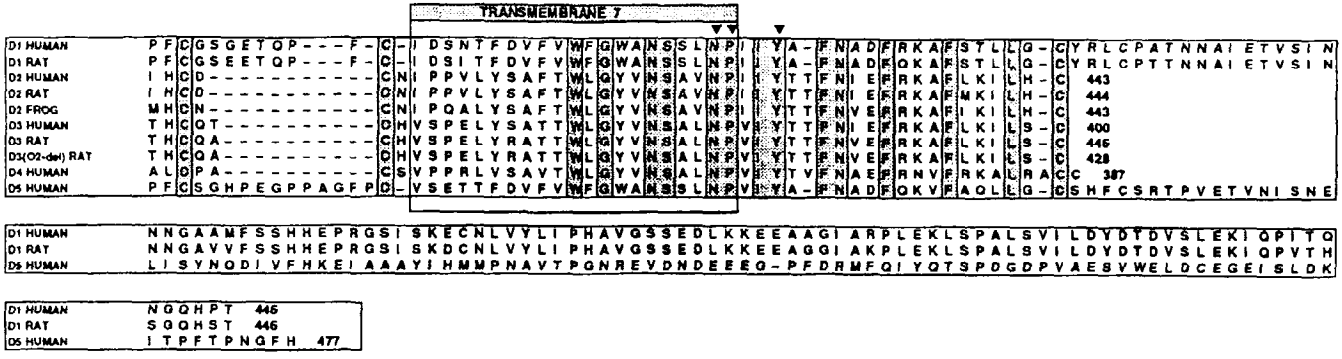

FIGURE 1. *continued*

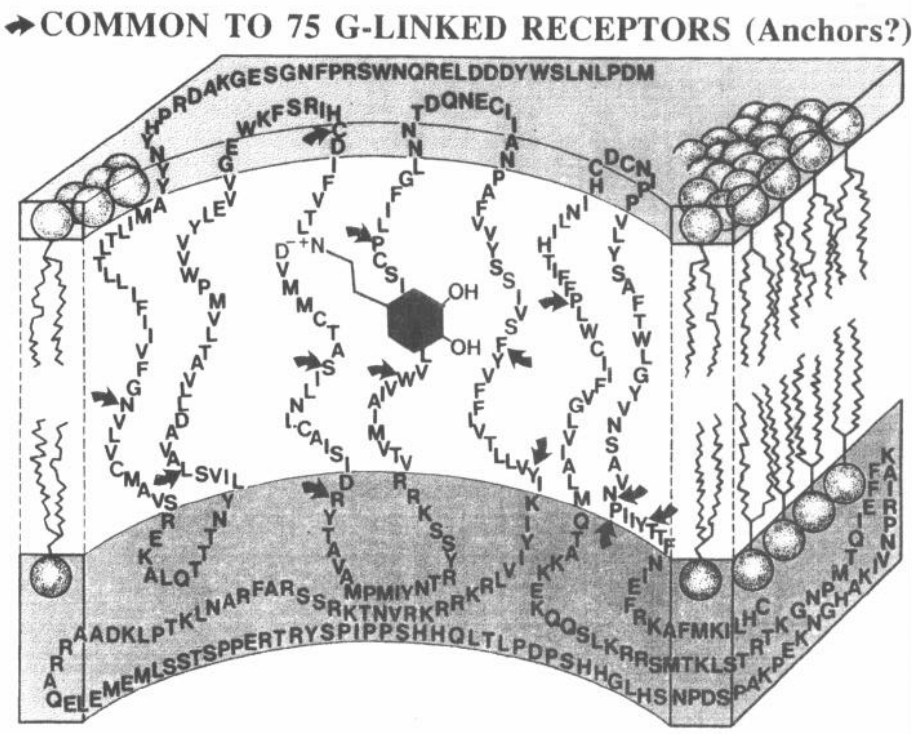

FIGURE 2. *Illustrating the rat dopamine D2 (short) receptor within the membrane. The arrows indicate the 13 amino acids that are homologous in more than 75 G-linked receptors for amines, peptides, and hormones. Dopamine is assumed to attach its hydroxyl groups to the serine residues in the fifth transmembrane segment.*

CLONED DOPAMINE RECEPTOR PHARMACOLOGY

The sensitivities of the various dopamine receptors to agonists and antagonists are listed in table 1. For each of these sites to be termed a dopamine receptor, it is essential that dopamine be the most potent endogenous transmitter to inhibit binding; otherwise, the site is not a dopamine receptor, by definition. All the binding sites shown in the table meet this criterion, because epinephrine, norepinephrine, and serotonin were all weaker than dopamine in inhibiting the binding of each radioligand.

TABLE 1. *Sensitivities of dopamine receptors to agonists and antagonists*

	Human	Various Species	Rat		Human		Rat	Human	
Site or Clone	D1	Tissue D2	D2 short	D2 long	D2 short	D2 long	D3	D4 hybrid	D5
Tissue/cell	Cos-7[7,13,14]	Striatum ant. pit.[6]	Ltk-[1,8] GH4ZR7[2.5] Cos-7[11]	CHO	293 cell	Ltk-LT[8,12] Cos-7[9] 293[10]	CHO	Cos-7	Cos-7[14]
Ligand	*Sch23982[7] *Sch23390[13,14]	*Spip	*Spip[1.2] *Spip[5] *Dom[8] †I-Sul[11]	†I-Sul	*Spip	*Dom[8] *Spip[12] *Rac[9] *Spip[10]	†I-Sul	*Spip[12]	*Sch23390[14]
	K, nM	K, nM	K, nM	K, nM	K, nM	K, nM	K, nM	K, nM	K, nM
Agonists									
ADTN-(+)	4,600[14]	Hi:1.7[6]						Hi:24	909
Apomorphine	210[7]	Hi:~2[6]		24[3]		250[9]	20[3]	Hi:4.1	363
Bromocriptine		1.9[6]	14[5]	5.3[3]		12.6[12]	7.4[3]	340	454
Dopamine	2,340[14]	Hi:7.5[6]	Hi:73[11]	Hi:2.8[11]		24,800[9]	25[3]	Hi:28	228
Dopamine+GN			17,000[1]	1,705[3]			27[3]	Hi:49-450	
Epinephrine	>55,000[7]								
Fenoldopam	20;60	Hi:2.8[6]						321	15
(-)Norepinephrine	50,000[13]	~6,000[6]						1,760	12,000
NPA	1,816[14]	Hi:0.4[6]						6.5	1,136
Pergolide	1,363[14]			21[3]			0.62[3]		918
PHNO-(+)		Hi:1.2[6]						79	
Quinpirole-(-)	14,000[7]	Hi:4.8[6]		576[3]			5.1[3]	46	>20,000
Serotonin	9,690[14]	~10,000[6]						4,180	3,000
SKF 38393	87[7]	Hi:157[6]		9,560[3]			5,000[3]	1,800	100
SKF 76783	645[14]								530

TABLE 1. *continued*

	Human	Various Species	Rat		Human		Rat	Human	
Site or Clone	D1	Tissue D2	D2 short	D2 long	D2 short	D2 long	D3	D4 hybrid	D5
Antagonists									
Butaclamol-(+)	0.9;3	0.9[6]	0.9[5]			0.8[12]		40	27
Chlorpromazine	73[14]	3[6]		2.8[3]		1.5[9]	6.1[3]	37	133
Clozapine	141[14]	100±20[4]	168[5]	56[3]		138[12]	180[3]	9	250
Eticlopride	18,000[14]	0.09[6]						2.1	19,000
Fluphenazine	21[14]	1±0.5[5]						46	14
Haloperidol	27[14]	1.5±0.5[4]	0.8-3	0.5-0.8		1[8]	9.8[3]	5.1	48
*Iodosulpnde				0.61[3]			1.2[3]		
Ketanserin	190[7]	208[6]	>1,000[1]					148	2,500
Pimozide		4[6]		2.4[3]			3.7[3]	43	
Prochlorperazine				4.7[3]			35[3]		
Radopride	>72,000[7]	2[4]	1.6[5]	1.8[3]		10.5[12]	3.5[3]	237	
*Radopride		1.9[4]				3.2[9]			
Remoxipride		300±90[4]						3,690	
SCH 23390	0.1;0.4	1,690[6]	714[8]			913[8]		3,560	0.3
SKF 83566	0.3[14]								0.4
Spiperone	220[7]	0.07[6]	0.096[2]	0.069[3]		0.053[8]	0.61[3]	0.06	4,500
*Spiperone		0.05[6]	0.09[5]		055[10]	.05[10]		0.08	
Sulpiride-S	36,000[14]	18[6]	5.5[11]	9.2[3]		46[9]	25[3]	52	77,270
Do.			15.9[5]	4.8[11]		31[12]			
Sulpiride-R	20,454[14]		115[11]	103[11]			422[3]		28,636
Thioridazine	100[14]	15±5[4]		3.3[3]			7.8[3]	12	300
YM-09151-2		0.06[6]						0.09	
*YM-09151-2			0.09[5]			0.09[12]			

TABLE 1. *continued*

KEY: *=tritium; †I-Sul=[^{125}1]iodosulpiride; ant. pit.=anterior pituitary; GN=guanine nudeotide; Dom=domperidone; Halo=haloperidol; Hi=high-affinity state of D_2; hybrid=hybrid of gene and cDNA from SKNMC neuroblastoma; NPA=N-propylnorapomorphine; Rac=raclopride; Spip=spiperone

[1]Bunzow et al. 1988
[2]Albert et al. 1990
[3]Sokoloff et al. 1990
[4]Seeman 1990
[5]P. Seeman, unpublished data
[6]Seeman and Niznik 1988
[7]Dearry et al. 1990
[8]Grandy et al. 1989
[9]Stormann et al. 1990
[10]Dal Toso et al. 1989
[11]Giros et al. 1989
[12]Van Tol et al. 1991
[13]Sunahara et al. 1990
[14]Sunahara et al. 1991

The D1-like receptors are D1 and D5. They are approximately equally sensitive to all agonists except dopamine. The dopamine dissociation constant (K value) shown in the table for D1 is 2,340 nM and for D5 is 228 nM. Both these values refer to the low-affinity state of D1 and D5, because under these experimental conditions the Cos-7 cells did not reveal the high-affinity state. Because D5 is approximately 10 times more sensitive to dopamine, this suggests that D5 is more readily activated than D1 in vivo. Thus, D5 may provide the major background tone in the nervous system, or it may be recruited during massive discharges of dopamine from nerve terminals, as might occur during episodes of drug abuse.

Although the expressed D1 and D5 receptors did not reveal the high-affinity state in tissue culture under these conditions, it is known that the high-affinity state of D1 in brain tissue has a K of about 0.7 nM for dopamine, compared with a value of about 10 nM for the high-affinity state of D2 for dopamine. Thus, the D1/D5 receptors are about one order more sensitive to dopamine than the D2-like receptors (that is, D2, D3, and D4) (see also Gehlert et al. 1992). The reason for emphasizing this point is that the high-affinity state of these receptors is the functional state (George et al. 1985).

As exemplified for clozapine in the table, this drug has about a tenfold higher affinity for D4 than for D2 or D3. This supports the principle of developing receptor-selective medications in the future.

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Cannabinoid Receptors: Which Cells, Where, How, and Why?

Lisa A. Matsuda, Tom I. Bonner, and Stephen J. Lolait

INTRODUCTION

In both laboratory animals and humans exposed to marijuana or cannabimimetic compounds, the majority of effects appear to be mediated by the central nervous system. Although Δ^9-tetrahydrocannabinol (Δ^9-THC) has been known to be the primary psychoactive component of marijuana since the mid-1960s (Gaoni and Mechoulam 1964), a receptor-mediated mechanism for marijuana's actions has been recognized only within the past several years (Howlett et al. 1990). The existence of a cannabinoid receptor implies that an endogenous "cannabinoid" ligand also must exist. However, the identity of this ligand is unknown. In addition, a lack of readily available cannabinoid receptor antagonists has further hindered the elucidation of the physiological and pathological significance of cannabinoid receptors. Recent cloning and identification of a cDNA that encodes a G protein-coupled cannabinoid receptor not only provides solid evidence for a cannabinoid receptor protein but also provides nucleic acid sequence information that can be used to localize the mRNA for this receptor (Matsuda et al. 1990). By using the technique of in situ hybridization histochemistry (ISHH), the neuroanatomical localization of cannabinoid receptor mRNA identifies populations of brain cells that potentially could mediate cannabinoid-induced effects. Furthermore, comparing the localization of the mRNA for the receptor with the localization of cannabinoid receptor proteins (Herkenham et al. 1991a) reveals clues regarding the circuitry of the cells expressing these receptors.

LOCALIZATION OF RECEPTOR mRNA IN RAT BRAIN

The expression of the cannabinoid receptor gene was visualized in coronal tissue sections of adult rat brain using ISHH with a ^{35}S-tailed 48-base oligonucleotide probe, SKR6-1. This probe complements sequence from the rat cDNA, SKR6 bases 349-396 (Matsuda et al. 1990). In transfected cells, this cDNA encodes a receptor that mimics the cannabinoid receptor previously characterized in both neural cell lines and brain tissues in vitro (Howlett et al.

1986; Bidaut-Russell et al. 1990). In response to Δ^9-THC and CP 55940, a potent synthetic nonclassical cannabinoid analog, cyclic AMP (cAMP) production in SKR6-transfected cells decreases in a dose-dependent, stereoselective, and pertussis toxin-sensitive manner (figure 1) (Matsuda et al. 1990). Moreover, the gene for the human homolog of this receptor encodes a protein that binds ^{3}H-CP 55940, a potent, synthetic cannabinoid analog, in a saturable and specific manner (Felder et al. 1991).

In sections from adult rat brain, a regionally specific and unique localization pattern for the cannabinoid receptor mRNA was found (Matsuda et al., submitted for publication). In general, labeling intensities were highest in forebrain regions (olfactory areas, caudate nucleus, hippocampus) and in

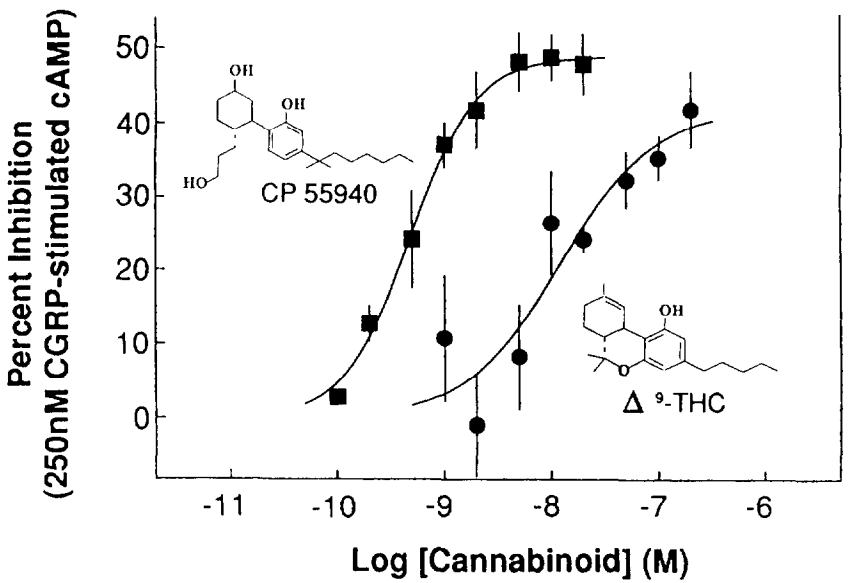

FIGURE 1. *Cannabinoid-induced inhibition of calcitonin gene-related peptide (CGRP, 250 nM)-stimulated cAMP production. Intact Chinese hamster ovary cells stably expressing the SKR6 cDNA were exposed to various concentrations of CP 55940 or Δ^9-THC for 5 minutes. Data points represent the average percent inhibition of cAMP (±SEM) from three experiments, each performed in triplicate. Chemical structures of CP 55940 and Δ^9-THC are shown. Δ^9-THC was obtained from the National Institute on Drug Abuse; CP 55940 was donated by the Pfizer Research Corp.*

the cerebellar cortex (figure 2). Although the localization of cannabinoid receptor mRNA was quite similar to that of the cannabinoid receptor protein (Herkenham et al. 1991a), numerous discrepancies were evident. In most cases, mismatches between cannabinoid receptors and receptor mRNA likely resulted from the expression of receptor proteins in the axons and/or terminals of projection neurons. Although receptor and mRNA discrepancies could result from comparing ISHH labeling with binding data obtained with a ligand that recognizes multiple cannabinoid receptor subtypes, the ligand used to localize cannabinoid receptors (^{3}H-CP 55940) appears to recognize a single cannabinoid receptor subtype (Devane et al. 1988; Herkenham et al. 1990). Indeed, Herkenham and colleagues (1991b, 1991c) have reported experimental evidence for the localization of cannabinoid receptors to projection target areas of both striatal and cerebellar neurons.

In the midbrain and hindbrain, ISHH labeling generally was uniform (of similar intensity) among all labeled cells within a given region or anatomical nucleus, and intensities varied from moderate to very low for different regions (data not shown). In the forebrain, the intensity of ISHH labeling varied from very low to very high. Several forebrain areas displayed a nonuniform labeling pattern in which heavily labeled cells were present with cells labeled to a more moderate extent. This nonuniform labeling was clearly evident in the hippocampus, where cells displaying very high intensity labeling were scattered throughout Ammon's horn and in a monolayer subjacent to the granular layer of the dentate gyrus; moderately labeled cells included those in the pyramidal cell layer and those in the hilar region of the dentate gyrus (figure 3). Other forebrain regions—anterior olfactory nuclei (figure 2, panel a), nucleus of the lateral olfactory tract, basolateral amygdaloid nucleus (data not shown), horizontal limb of the nucleus of the diagonal band (figure 3, panel b), cerebral cortex (figure 2, panels b and c)—also displayed cells that were labeled to a high intensity. Although the physiological significance of high levels of mRNA likely will depend on the density of cannabinoid receptors on these cells, the mechanism(s) involved in this range of gene expression would be of interest.

Possible explanations for high amounts of receptor mRNA include an increased rate of transcription of the receptor gene and/or a decreased rate of transcript degradation. If transcription rate varies (degradation rates remaining constant), high amounts of mRNA could result from different promoters and cell-specific induction mechanisms. Although splicing variants in 5'-untranslated sequences have been identified by comparisons of the human cannabinoid receptor gene (T.I. Bonner, M.J. Brownstein, C.C. Felder, C. Chen, and L.A. Matsuda, unpublished manuscript), the SKR6 cDNA, and a human cannabinoid receptor cDNA (Gerard et al. 1990), more detailed information concerning the sites that regulate cannabinoid receptor gene expression is not yet available. In the

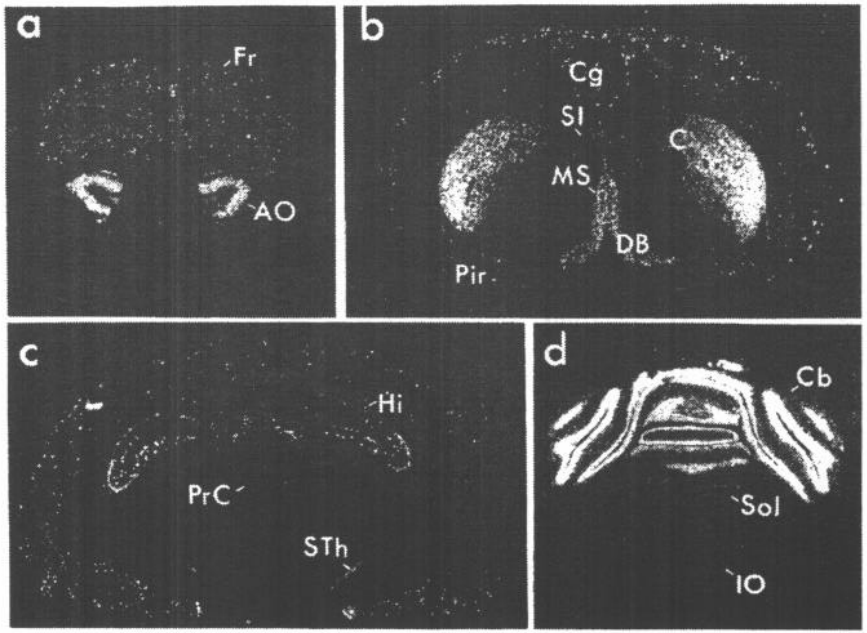

FIGURE 2. *ISHH labeling of cannabinoid receptor mRNA in adult rat brain. Panels a through dare negative film images of coronal sections taken at various levels through the brain; labeling by the SKR6-1 probe appears as white on a dark background. Abbreviations: Fr, frontal cortex; AO, anterior olfactory nucleus; Cg, cingulate cortex; SI, intermediate septal nucleus; C, caudate nucleus; MS, medial septal complex; DB, nucleus of the diagonal band; Pir, piriform cortex; Hi, hippocampus; PrC, precommissioned nucleus; Sth, subthalamic nucleus; Cb, cerebellum; Sol, nucleus of the solitary tract; IO, inferior olive.*

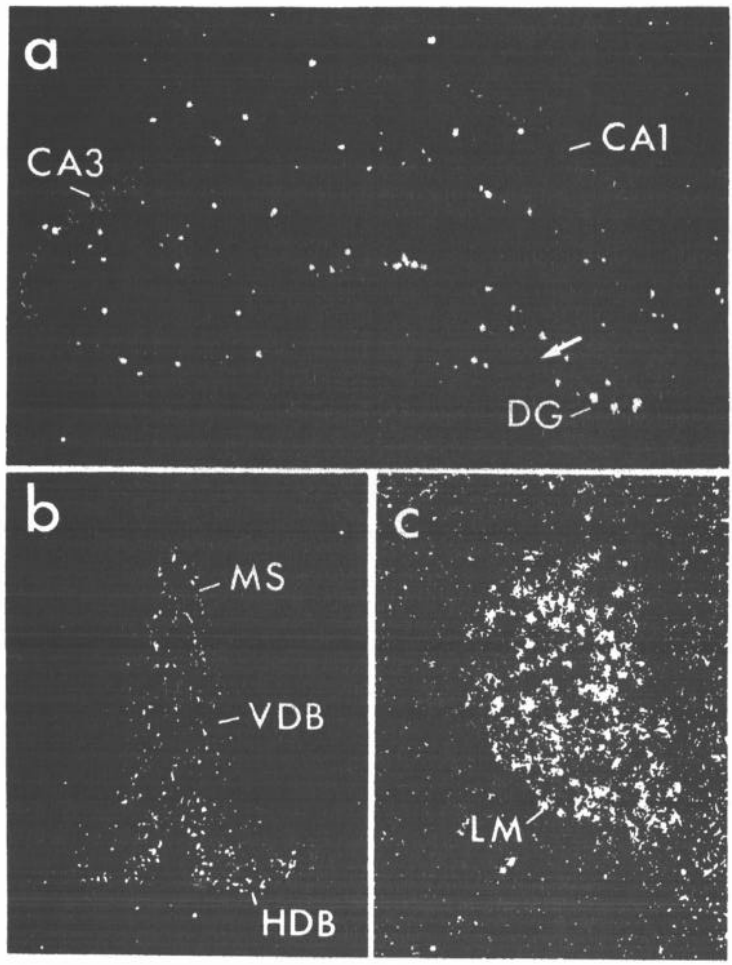

FIGURE 3. *Expression of the cannabinoid receptor gene in areas associated with memory. Images were produced in a manner similar to those pictured in figure 2 but were taken at higher magnifications. Panel a: hippocampus, CA1, pyramidal cell layer of field CA1 of Ammon's horn; CA3, pyramidal cell layer of field CA3 of Ammon's horn: DG, dentate gyrus (arrow features the hilar region of the dentate gyrus). Panel b: medial septal complex, MS, medial septum; VDB, vertical limb of the nucleus of the diagonal band; HDB, horizontal limb of the nucleus of the diagonal band. Panel c: mammillary body, LM, lateral nucleus.*

situation where mRNA stablity may vary (constant rates of transcription), less mRNA would accumulate in cells in which the cannabinoid receptor mRNA is more readily degraded. Conclusive information regarding this possibility is also not available; however, cDNA clones with different lengths of 3'-untranslated sequence have been described and appear to be the result of two distinct polyadenylation sites (Matsuda et al. 1990). This finding may be relevant since motifs in sequences 3' of the coding region appear to influence message stability in many eukaryotic genes (Shaw and Kamen 1986; Brawerman 1987). Clearly, additional research is needed to determine the mechanism(s) by which certain subpopulations of cells express high levels of cannabinoid receptor mRNA.

In animals, cannabimimetic activity is routinely assessed by drug discrimination, specific motor responses (dog ataxia, rodent catalepsy) (Razdan 1986), or an increase in pain thresholds (Johnson and Melvin 1986). High levels of receptor mRNA in the caudate nucleus and cerebellum (figure 2) are consistent with the marked effects that cannabinoids exert on the motor behavior of laboratory animals. Potential sources for cannabinoid-induced analgesia include numerous regions that displayed low-to-moderate amounts of receptor mRNA, including the cingulate cortex (moderate), intralaminar thalamic nuclei (moderate), central grey (low), nucleus raphe mangus (low), pontine reticular formation (low), and paragigantocellular reticular nucleus (moderate) (data not shown).

In humans, memory deficits are some of the most consistently reported effects of marijuana (Miller and Branconnier 1983). Clear labeling observed in the rat forebrain suggests several potential sites in the human brain that could mediate this effect, including the hippocampus, medial septal complex, lateral nucleus of the mammillary body (figure 3), and the amygdaloid complex (data not shown)—regions that traditionally have been associated with various aspects of memory functions. Similarly, labeling was detected clearly in rat forebrain regions that correspond to those that could mediate marijuana-induced effects on human appetite and mood (hypothalamus, amygdaloid complex, anterior cingulate cortex [data not shown]).

In contrast to the effects on mood and cognition, marijuana-induced autonomic effects in humans are mild (Hollister 1986). However, in animals, cannabimimetics decrease respiration, heart rate, and blood pressure (Dewey 1986). These cardiovascular and respiratory responses are likely due to the moderate levels of cannabinoid receptor mRNA that were found in numerous midbrain and hindbrain regions (the pontine nucleus, the paragigantocellular reticular nucleus, the lateral reticular nucleus, the area postrema [data not shown], nucleus of the solitary tract [figure 2]). Although

the cannabinoid-induced effects on autonomic functions are rarely lethal in humans, further study of the cells that express the cannabinoid receptor gene in the midbrain and hindbrain may reveal an important physiological role(s) for the cannabinoid receptor and its endogenous ligand.

SUMMARY

Localization of the mRNA for this receptor has identified many regions of the rat brain in which the gene for this receptor is active. Several of these regions are consistent with the cannabinoid- or marijuana-induced effects that occur in both laboratory animals and humans. However, other labeled regions are not easily associated with well-known effects of marijuana (Matsuda et al., submitted for publication). Although great progress has been achieved in elucidating the mechanism of action of cannabis in recent years (Howlett et al. 1990), much remains to be discovered about the expression of cannabinoid receptors in the brain and exactly how this receptor influences numerous brain functions.

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Studies on μ-Opioid-Binding Sites With Peptide Antibodies

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PURIFICATION OF A μ-OPIOID-BINDING PROTEIN AND ITS CHARACTERIZATION

The purification to homogeneity of a μ-opioid-binding protein (OBP) from bovine striatal membranes and its characterization by its ability to bind opioid antagonists saturably, reversibly, and with high affinity was reported previously by Gioannini and colleagues (1985). Briefly, the purification involved two major steps: (1) affinity chromatography on a then-novel derivative of naltrexone, 6-desoxy-aminoethylaminonaltrexone, coupled to the carboxyl side chain of CH-Sepharose, followed by (2) lectin chromatography on wheat germ agglutinin agarose. A single band of apparent molecular weight (mol wt) of 65 kD was obtained on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The purification achieved was 60,000- to 70,000-fold, close to the theoretical value for a protein of this size with a single binding site. The purified OBP binds opioid antagonists with high affinity, but it binds against agonists with low affinity, presumably because it is uncoupled from the Gi protein to which it is normally coupled. The evidence that OBP is a μ-binding opioid protein is as follows:

1. OBP is isolated on an affinity matrix that contains a ligand that binds μ-receptors preferentially.

2. Binding of opioid antagonists is displaced by the μ-selective ligand D Ala2Gly-I5 enkephalin (DAGO), albeit at high concentrations, but not by selective δ- or κ-ligands.

3. OBP binds the μ-specific antagonist 3H-cyprodime (Schmidhammer et al. 1989). At saturating concentrations of ligand, OBP binds as much cyprodime as bremazocine does, suggesting that all the binding to OBP of bremazocine, a universal ligand, is to μ-sites.

4. Finally, the molecular weight of OBP (65 kD) is the same as that observed when 125I-ß-endorphin is cross-linked to opioid-binding sites in tissues and a cell line, which were shown to be μ-sites by specific displacement with μ-ligands (Howard et al. 1985, 1986).

The presence of disulfide bridges (-S-S-) that contribute to the secondary structure of OBP is indicated by the difference in mobility of OBP in SDS-PAGE under nonreducing vs. reducing conditions (Gioannini et al. 1985). As shown in figure 1, treatment of OBP with increasing concentrations of reducing reagent dithiothreitol (DTT) produced a stepwise shift from an apparent molecular weight of 53 to 65 kD (Gioannini et al. 1989). The importance of disulfide bonds, not only for secondary structure but also for function, is reflected in the sensitivity of opioid ligand binding to inhibition by DTT. The major opioid receptor types differed in their sensitivity to DTT as follows: μ>δ>>κ with κ-sites being virtually resistant to even very high concentrations of DTT (Gioannini et. al. 1989). The inhibition produced by DTT is reversible, was observed to a much lesser degree with antagonist ligands than with agonists, and was due to a reduction in affinity (increase in kD) rather than in the number of receptor sites (Bmax). The possibility that these disulfide bridges may have a role in receptor activation by agonist ligands is being explored.

MICROSEQUENCING OF PURIFIED OBP AND GENERATION OF ANTISERA AGAINST PEPTIDE SEQUENCES

The availability of pure OBP encouraged the authors to attempt to determine a portion of its primary sequence. Direct sequencing of OBP proved unsuccessful, indicating that OBP is an N-terminally blocked protein and must be fragmented to obtain peptides for amino acid sequencing, which greatly increases the quantities of purified protein required. Two peptide fragments, 21 and 13 amino acids in length, respectively, were generated by chemical cleavage of OBP with CNBr followed by isolation of the peptides on reverse phase high-performance liquid chromatography. The amino acid sequences obtained were not found in databases of known protein sequences. Polyclonal antibodies have been generated against portions of the peptides by injection of rabbits with the synthetic peptides coupled to thyroglobulin, followed by appropriate booster injections. The interaction of these antibodies with purified OBP, bovine brain regions, and several cell lines are discussed below.

INTERACTION OF PEPTIDE ANTIBODIES WITH PURIFIED OBP

The peptide fragments used for antibody production and the antibodies generated against each are indicated in table 1.

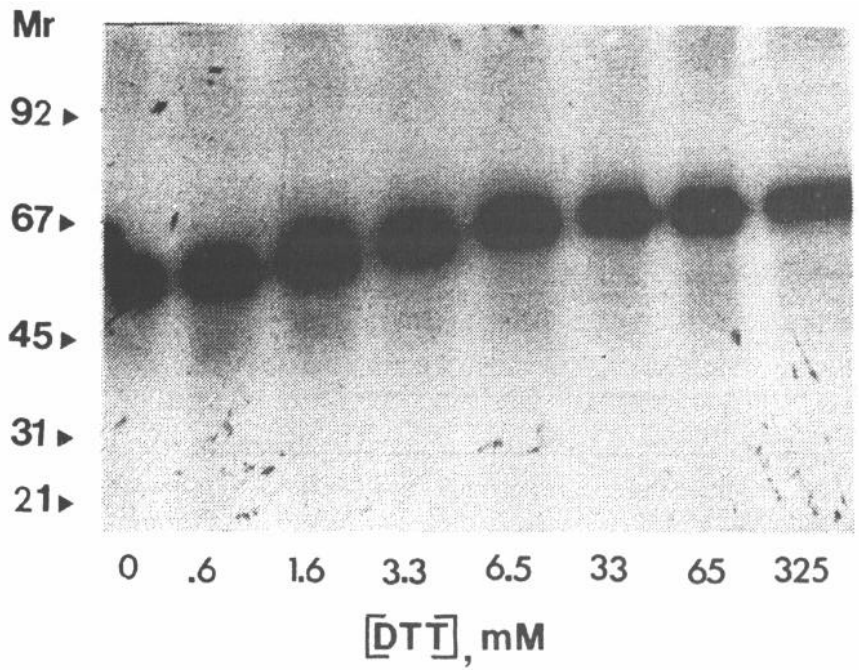

FIGURE 1. *The effect of increasing DTT concentrations on the apparent molecular weight of purified μ-OBP. Purified μ-OBP was radioiodinated and separated from free iodine. Samples were treated with the indicated concentrations of DTT and subjected to SDS- PAGE.*

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All the antibodies were able to immunoprecipitate a major portion of 125I-labeled purified OBP after incubation with the antibody followed by incubation with protein A. The amount precipitated after correction for background (radioactivity precipitated by normal rabbit serum [NRS] or irrelevant immune sera) ranged from 40 percent for the weakest antibody (Ab 161) to more than 60 percent for the strongest, Ab 162 and Ab 165, at dilutions of 1:200. Background of the assay accounted for 5 to 7 percent of added radioactivity. Ab 162 and Ab 165 can immunoprecipitate 30 percent of 125I-OBP even at a 1:1,000 dilution. The protein precipitated by these antibodies was dissociated from the complex and subjected to SDS-PAGE. Autoradiography of the gel,

TABLE 1. *Antibodies generated against peptide sequences derived from purified OBP*

Antibody	Fragment
165, 166, 6639	N-terminal 12 amino acids of peptide 1
163	C-terminal 7 amino acids of peptide 1
161, 162	N-terminal 10 amino acids of peptide 2

carried out under reducing conditions, showed a radioactive band with an apparent molecular weight of 65 kD (i.e., the molecular weight of OBP).

Sequential treatment of OBP with antibodies, derived from the two different peptides, indicated that initial treatment with either antibody removed all immunoprecipitable antigen (i.e., no further immunoprecipitation occurred with the second antibody). These results confirm that the two peptides isolated and sequenced from fragmented OBP are derived from the same protein.

The interaction of OBP with the antipeptide antibodies also was examined in immunoblots. An immunoreactive protein corresponding to a 65 kD mol wt was detected with each antibody examined. The strongest signal was produced with antibodies against the N-terminal portion of peptide 1 (Ab 165, 166, 6639) at a 1:100 dilution. The 65-kD band obtained in immunoblots with OBP can be blocked by preincubation of the antisera with the appropriate peptide (100 μM), but not by other peptides. No signal was detected when OBP was immunoblotted against nonimmune serum. It is noteworthy that no signal was detected unless OBP was reduced with DTT. Apparently, the presence of the disulfide bridges and the resulting secondary structure of the protein prevent access of the antibodies to the epitopes.

IMMUNOBLOTS OF TISSUES AND CELL LINES WITH ANTISERA AGAINST OBP PEPTIDES

The authors decided to determine whether the peptide antibodies were capable of producing positive immunoblots with extracts of tissues containing high concentrations of μ-opioid receptors. The first tissue examined was an extract of bovine striatal membranes, the source of purified OBP. Immunoblotting with peptide antisera gave a band at the appropriate molecular weight of 65 kD.

The positive signal at 65 kD mol wt seen in immunoblots with both digitonin and CHAPS extracts of bovine striatal membranes prompted us to examine cell lines and other bovine brain tissues for positive signals in immunoblots

with the antipeptide antibodies. Immunoblots of SDS-solubilized bovine tissues with the various antibodies indicated the presence of immunoreactive protein (65 kD mol wt). The signal could be blocked by preincubation of antisera with the appropriate peptide (50 to 100 μM). The tissues that reacted with antibody, in addition to striatum, were frontal cortex, hippocampus, and thalamus, all regions known to have moderate-to-high levels of μ-opioid receptors. Pons and white matter produced no or a barely detectable response, which correlates with their low levels of opioid receptors. The sensitivity of Ab 165, which produces the strongest signal, is evidenced by its ability at a dilution of 1:100 to detect μ-opioid-binding material equivalent to 0.001 percent of the 30 to 50 μg of protein loaded per sample (300 to 500 pg of OBP) in an immunoblot.

Immunoblots with the cell line SK-N-SH, which contains predominantly μ-binding sites, produced a strong positive reaction at a position comparable to that seen with purified OBP and brain tissue extracts (65 kD). The immunoreactive protein detected in NG1O8-15 cells, a cell line that is reported to contain only δ-receptors, migrated to a position slightly lower than that seen in the SK-N-SH cells or with purified OBP (apparent molecular weight of approximately 58 kD). In both cell lines, the response could be blocked by preincubation of the serum with 100 μM concentration of peptide. Two negative control cell lines, HELA cells and C6 glioma cells, produced no detectable response. The detection of a response with the NG108-15 cells suggests cross-reactivity of the antibodies with δ--receptors, although the presence of small amounts of μ-receptors, hitherto not detected by binding assays, could not be ruled out.

The ability of the antipeptide antibodies to react with native receptors was investigated by examining the effect on opioid ligand binding and by evaluating the extent to which active receptors can be removed from solution by immunoprecipitation. None of the antibodies inhibited binding of opioid ligands to either membrane-bound or soluble receptors. No depletion of receptors was detected in the supernatants after immunoprecipitation with any of the antibodies. It was concluded that the antibodies recognize only denatured receptors. Not unexpectedly, the short amino acid sequences to which the antibodies were made may not be accessible to the antibody in the native receptor or may assume a secondary structure not recognized by the antibody.

All the above results, with antisera generated against microsequence from the purified OBP, support the authors' contention that we have purified an opioid-binding site—in particular, the immunoblots of tissues and cell lines that give bands of the appropriate molecular weight. Moreover, there is good correlation between positive signals and levels of opioid receptors in the tissues or cultures.

CROSS-REACTIVITY OF PURIFIED OBP WITH ANTISERA GENERATED AGAINST BOVINE RHODOPSIN

Biochemical and physiological evidence indicates that all three major types of opioid receptors, μ, δ, and κ, negatively modulate adenylate cyclase and, therefore, are coupled to guanine nucleotide regulatory proteins (G proteins). This suggests that opioid receptors belong to the large family of receptors for hormones, neurotransmitters, and peptides that effect signal transmission by activating a G protein. Analysis of the amino acid sequences of many proteins of this class has revealed some significant structural features common to all members of this family, the most striking of which is the presence of seven hydrophobic domains thought to span the cell membrane. The following results further support the hypothesis that OBP belongs to this class of G-coupled proteins.

Antibodies generated against membrane-associated rhodopsin and against five specific amino acid sequences in rhodopsin were used. In immunoblots against OBP, two antibodies, one against membrane-associated rhodopsin and one against a sequence in the carboxyl terminal tail (CT1), reacted strongly, whereas an antibody against the 1-2 loop (first cytoplasmic loop between transmembrane domains 1 and 2) reacted weakly. Weiss and colleagues (1987) had previously studied the interaction of purified ß-adrenergic receptor from S49 lymphoma cells with this same series of antibodies under identical reaction conditions. The pattern of reactivity of these antibodies with OBP was the same as that previously reported by Weiss and coworkers (1987) for purified ß-adrenergic receptor. These researchers had shown that preincubation of antiserum CT1, an antiserum against a sequence in the C-terminal tail of rhodopsin, with the peptide (rhodopsin 325-343) used to generate it, was able to diminish greatly the signal obtained in immunoblots of the ß-adrenergic receptor. This supports the specificity of the immune cross-reaction. A rhodopsin peptide (324-348), which contains the sequence (325-343) used to generate the CT1 antiserum, was made available to the authors' laboratory. This peptide, when preincubated with antiserum CT1 at 100 μM concentration, completely abolished the positive signal in immunoblots with OBP. A rhodopsin peptide (rhodopsin sequence 331-348), which lacked the first six amino acids of the CT1 sequence, was ineffective in reducing the intensity of the 65-kD signal.

To verify that rhodopsin antibodies recognize the same protein as the peptide antibodies, OBP was immunoprecipitated by Ab 165. An immunoblot of the proteins remaining in the supernatant after immunoprecipitation showed a strong diminution of the signal (65-kD band) obtained with the rhodopsin antibody CT1, relative to a control supernatant from "immunoprecipitation" with

NRS. The protein immunoprecipitated with Ab 165 was eluted from the antigen-antibody complex and examined in an immunoblot against CT1 . A positive signal was observed at 65 kD mol wt, whereas no signal was detected with the NRS control. This experiment indicates that the protein recognized by the rhodopsin antibodies is the same as that recognized by OBP-derived peptide antibodies.

The results reported here, in conjunction with those of Weiss and colleagues (1987), indicate that the three proteins—bovine rhodopsin, S49 lymphoma ß-adrenergic receptor, and OBP purified to homogeneity from bovine striatal membranes—share common epitopes. Since there seems to be relatively little amino acid sequence homology in the areas used for antibody production (at least between rhodopsin and the ß-adrenergic receptor), structural features, perhaps at the level of secondary and/or tertiary structure, along with limited amino acid homology, may be responsible for the immunological cross-reactivity. Weiss and coworkers (1987) have reported evidence that three amino acids, K, N, and P in positions 325-327, may be important for recognition of antibody CT1 by rhodopsin and by ß-adrenergic receptor. Current data suggest that this also may be true for OBP.

The results presented constitute evidence, beyond that previously obtained, that μ-opioid-binding sites are members of the family of G protein-coupled receptors and are likely to show the typical seven membrane-spanning domain structure of these proteins, when their complete amino acid sequence becomes known. It should be noted that similar results have been obtained by W.A. Klee and coworkers (personal communication, October 1991). These researchers found that δ-receptors labeled with the affinity label 3H-FIT and purified from NG108-15 cells in culture were immunoprecipitated by an antiserum generated against a peptide sequence from the C-terminal tail of rhodopsin. This finding lends credence to ours and suggests that μ- and δ-opioid receptors both share antigenic sites with rhodopsin.

UPDATE ON ATTEMPTS TO CLONE THE μ-OBP

Oligonucleotides have been prepared based on the amino acid sequence of the isolated peptides. Bovine brain and striatal cDNA libraries have been screened with the labeled oligonucleotide probes, and clones have been obtained. Partial sequencing revealed that the clones did not contain the full probe used to detect them; that is, they were false-positive clones. This is undoubtedly due to the considerable degeneracy of the authors' sequences, which contain many amino acids coded by four or six codons. Screening is currently in progress using different oligonucleotides. Clones are being sequenced to see if they contain the sequence present in the probe. If so, sequencing of the complete

cDNA will be carried out. The resulting structure should give an idea whether it is the opioid receptor, although proof must await transfection of suitable cells and expression of an active binding site with the appropriate ligand specificity.

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Molecular Cloning and Characterization of Neurotransmitter Transporters

Randy D. Blakely

INTRODUCTION

For precise chemical signaling between neurons and target cells, the magnitude and duration of neurotransmitters in synaptic spaces must be tightly regulated. Two principal and distinct mechanisms are classically recognized as being responsible for rapid transmitter inactivation. Transmitter can be either enzymatically metabolized, as with acetylcholinesterase hydrolysis of acetylcholine (Taylor 1991), or actively accumulated into presynaptic terminals and/or surrounding glia (Iversen 1967; Schousboe 1981; Hendley 1984; Cooper et al. 1986; Horn 1990; Nicholls and Atwell 1990). First demonstrated in the periphery by Axelrod and colleagues while studying the fate of intravenous radiolabeled catecholamine (Axelrod 1971), rapid transport across neuronal and glial membranes has been extensively characterized for many central neurotransmitters. In addition to norepinephrine (NE), the neurotransmitters dopamine (DA), serotonin (5-HT), L-glutamic acid (Glu), γ-aminobutyric acid (GABA), and glycine (Gly) each can be actively transported across brain membranes in a region-dependent manner consistent with the localization of neuronal terminals releasing these compounds. Choline availability is the rate-limiting process in acetylcholine biosynthesis. Therefore, it is no surprise that even cholinergic neurons utilize an active transport process to actively recapture synaptic choline following acetylcholine hydrolysis (Kuhar and Murrin 1978). The physiologic importance of transmitter inactivation by reuptake carriers is particularly apparent when examining the clinical and societal impact of agents with a high selectivity for blocking monoamine transport, including cocaine, amphetamines, and many antidepressants (Ritz et al. 1987; Galloway 1988; Richelson 1990). Furthermore, the ability of synaptic transporters to accumulate neurotransmitter-like toxins, including 6-hydroxydopamine, 1-methyl-4-phenylpyridinium (MPP+), 5,6-dihydroxytryptamine, and AF64A, among others, suggests that these proteins have a role in the establishment of selective neuronal vulnerability to exogenous agents. Thus, neurotransmitter transporters represent critical targets for both therapeutic and pathologic alterations of synaptic function.

Active transport of neurotransmitters (and choline) is driven by the transmembrane Na+ gradient (Kanner and Schuldiner 1987), established by Na+/K+ adenosinetriphosphatase (ATPase), in a process analogous to the active transport of glucose across brush border epithelia (Stein 1986). However, unlike intestinal glucose transport, additional ions are required for transport of many neurotransmitters, such as intracellular K+ and extracelluar Cl-. Membrane vesicle (Kanner and Schuldiner 1987) and whole cell patch clamp (Nicholls and Atwell 1990) studies indicate that the effects of these ions on transport are unrelated to their effects on transmembrane potential (Vm), but rather indicate that there is parallel movement of these ions during each translocation cycle. These energetic properties, along with clear pharmacologic differences, distinguish these transporters from the intracellular vesicular carriers used to package neurotransmitters for exocytosis (Cooper et al. 1986; Kanner and Schuldiner 1987). Specific radiolabeled antagonists, including [^{3}H]nisoxetine for the NE transporter (Tejani-Butt et al. 1990), [^{3}H]GBR12909 and [^{3}H]GBR12935 for the DA transporter (Anderson 1989), [^{3}H]citalopram for the 5-HT transporter (D'Amato et al. 1987), and [^{3}H]hemicholinium-3 for the choline transporter (Sandberg and Coyle 1985), have been used to label transporters, both in broken cell preparations and in brain sections, permitting both pharmacologic and anatomic analyses. These tools have confirmed the enrichment of transporter sites on presynaptic terminal membranes and, at least for the choline transporter (Saltarelli et al. 1987), have revealed alterations in carrier density on plasma membranes in response to depolarization.

Despite decades of kinetic and pharmacologic studies on the behavior of neurotransmitter transporters in membrane preparations, structural data relevant to questions of mechanism and regulation have been difficult to obtain. One initial assumption is that all transporters utilizing cotransported Na+ ions, from Escherichia coli (E. coli) to man, are derived from a common ancestral gene, with sequence identity illustrative of common tasks such as Na+ binding or translocation, and sequence divergence responsible for additional ionic and pharmacologic specificities. Homologous, facilitated glucose transporters (which lack the ability to move sugar uphill against a concentration gradient) are found throughout phylogeny as members of an extended gene family (Henderson 1990). The intestinal Na+/glucose (Hediger et al. 1987, 1989) and the E. coli Na+/proline (Nakao et al. 1987) transporters are encoded by single genes and bear sequence similarities absent from the family of facilitated carriers, revealing the presence of a distinct gene family encoding Na+/ symporter proteins. One possibility was that neurotransmitter transporters would bear detectable sequence conservation with this latter gene family. However, attempts to use the limited homology of the Na+/glucose and Na+/ proline transporters to identify novel carriers expressed in the rodent central nervous system met with no success (R.D. Blakely, unpublished data), suggesting a novel gene family might encode neurotransmitter transporters.

EXPRESSION OF NEUROTRANSMITER TRANSPORTERS IN *XENOPUS LAEVIS* OOCYTES

In the absence of sequence information suitable for molecular cloning of transporter cDNAs by conventional hybridization strategies, Blakely and colleagues (1988, 1991a) turned to the *Xenopus laevis* oocyte expression system to characterize and perhaps clone transporter mRNAs. Following the injection of poly(A)+RNA, *Xenopus laevis* oocytes actively accumulated many different transmitter substrates, including Glu, GABA, Gly, 5-HT, DA, and choline (Blakely et al. 1988). Transport of these substrates was time- and temperature-dependent, and uptake was abolished with the removal of extracellular Na+. Regional enrichment of transporter mRNAs followed well the distribution of soma predicted to synthesize each carrier. In addition, pharmacologic sensitivities of oocyte-expressed transporters were similar (Blakely et al. 1991a) if not identical to those observed in brain membrane preparations (table 1). Using mRNA prepared from different brain regions during development, Blakely and colleagues (1991a) revealed a postnatal rise in Glu, GABA, and Gly transporter transcript abundance paralleling the postnatal rise in brain transport activity for these substrates. Most important in the consideration of expression cloning strategies was that mRNAs encoding Glu, GABA, and Gly carriers could be size-fractionated on sucrose density gradients, with minimal loss of activity relative to unfractionated mRNA and with a narrow distribution of peak activities (figure 1, example of Glu transporter size fractionation). These data supported the assumption that neurotransmitter transporters were likely to be encoded by single, separable mRNAs and, therefore, could be cloned utilizing expression as a functional screen for the presence of a single transporter clone in a cDNA library.

MOLECULAR CLONING AND EXPRESSION OF A COCAINE- AND ANTIDEPRESSANT-SENSITIVE NOREPINEPHRINE TRANSPORTER DERIVED FROM A HUMAN NEUROBLASTOMA (SK-N-SH) CELL LINE

Despite the demonstrated ability of the *Xenopus laevis* oocyte expression system to reconstitute neurotransmitter transporters, there were several drawbacks precluding its use for the cDNA cloning of these carriers. Seasonal variability in oocyte viability and expression levels, coupled with an inability to produce a high “signal-to-noise” ratio for monoamine transporters (Blakely et al. 1988), dictated the design and implementation of an alternative strategy (figure 2). This method, based on the ability of COS cells to replicate episomal copies of transfected plasmids bearing SV40 replication origins (Gluzman 1981), has been used in various forms to clone cDNAs for several membrane proteins, including several peptide receptors (Aruffo and Seed 1987; Sims et al. 1988; D’Andrea et al. 1989; Munro and Maniatis 1989). Pacholczyk and

TABLE 1. *Inhibitor sensitivity of oocyte uptake activities induced by adult rat mRNAs*

Substrate	RNA Source	Inhibitor	Concentration	Percent Inhibition
L-Glu	Forebrain	Dihydrokainate	1 mM	61.0 ± 6.9
	Cerebellum	Dihydrokainate	100 mM	16.0 ± 5.3
		D-aspartate	100 µM	91.7 ± 15.5
		Nipecotic acid	100 µM	10.0 ± 1.1
	Spinal cord	Dihydrokainate	1 mM	63.0 ± 8.1
GABA	Cerebellum	D-aspartate	100 µM	8.2 ± 1.4
		Nipecotic acid	100 µM	81.2 ± 9.0
	Spinal cord	Nipecotic acid	100 µM	75.7 ± 10.1
Gly	Spinal cord	Nipecotic acid	100 µM	0.3 ± 0.1
Choline	Spinal cord	Hemicholinium-3	10 µM	82.2 ± 4.0
DA	Midbrain	Nomifensine	10 µM	98.3 ± 12.3
	Adrenal	Nomifensine	10 µM	79.4 ± 6.8

NOTE: *Xenopus laevis* oocytes were injected with 40 ng poly(A^+) RNA and incubated at 18 °C for 2 to 3 days prior to assay. Uptake assays (22 °C) with different [^{3}H]-labeled substrates were performed as described in Blakely and colleagues (1991a) with or without inhibitors at the concentrations indicated. Mean transport inhibition (±SEM) relative to assays conducted with substrates alone is presented.

SOURCE: Blakely, R.D.; Clark, J.A.; Pacholczyk, T.; and Amara, S.G. Distinct, developmentally regulated brain mRNAs direct the synthesis of neurotransmitter transporters. *J Neurochem* 56:860-871, 1991a. Copyright 1991 by Raven Press, Ltd. (New York).

colleagues (1991) reasoned that the uptake of an iodinated analog of NE, meta-iodobenzylguanidine (mIBG), could be visualized in cells successfully transfected with the NE transporter (NET). Prior to embarking on this method, they characterized the transport of mIBG in SK-N-SH cells, an adrenergic neuroblastoma cell line possessing a high-affinity NE uptake system (Richards and Sadee 1986), and found the substrate to be

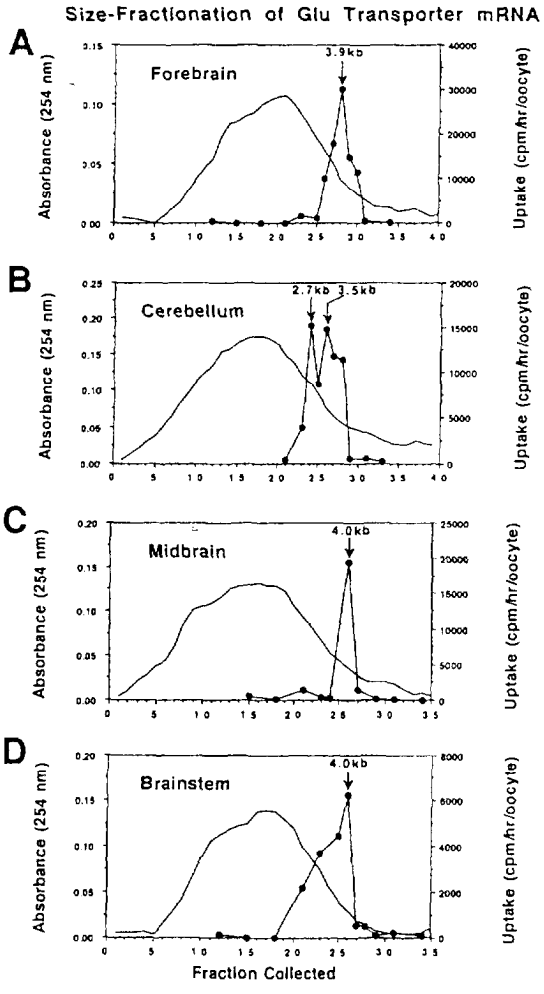

FIGURE 1. *Size-fractionation of Glu transporter mRNA*

SOURCE: Blakely, R.D.; Clark, J.A.; Pacholczyk, T.; and Amara, S.G. Distinct, developmentally regulated brain mRNAs direct the synthesis of neurotransmitter transporters. *J Neurochem* 56:860-871, 1991a. Copyright 1991 by Raven Press, Ltd. (New York).

accumulated in a desipramine-sensitive manner as reported (Smets et al. 1989) and imageable autoradiographically. Because the technique could be performed on large tissue-culture pans, Pacholczyk and colleagues (1991)

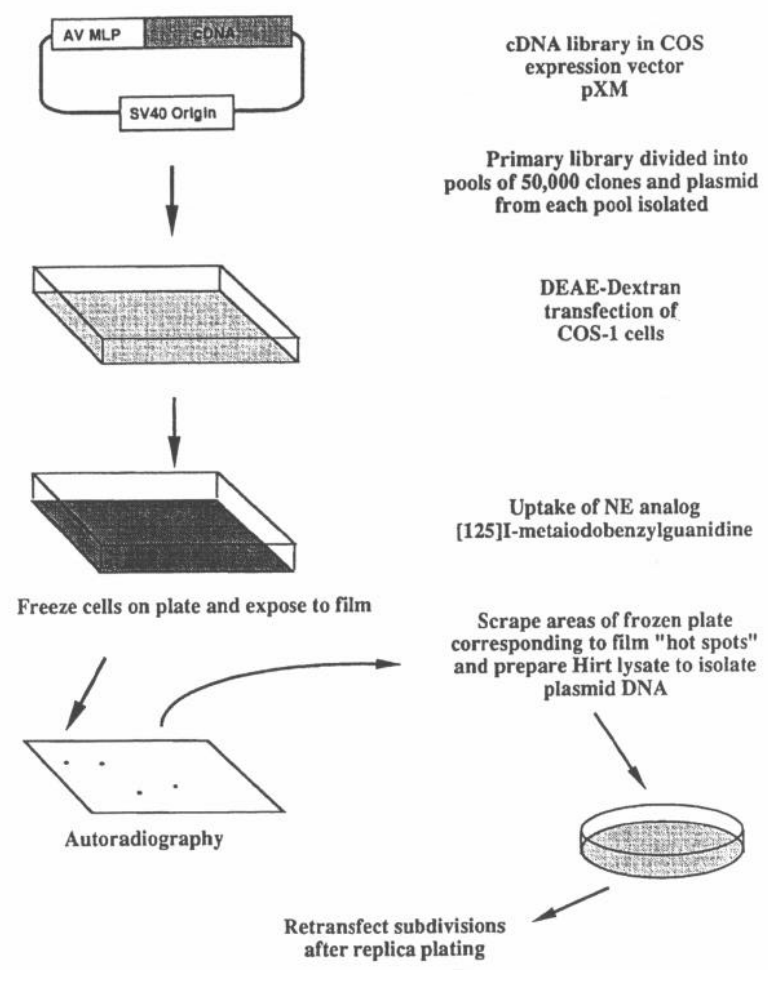

FIGURE 2. *COS cell expression system for cDNA cloning of Na+/L-NET. AV MLP-adenovirus major late promoter.*

were able to screen large pools of cDNA library plasmids derived from SK-N-SH mRNA. Plasmid DNA derived from scraped cells overlying “hot spots” on the film was extracted by the Hirt lysis procedure, and the method was repeated several times on a smaller scale. Finally, a single plasmid was isolated, which was capable of producing a functional mIBG transporter in transfected COS cells, with transport activity abolished when autoradiographic uptake

experiments were conducted in the presence of the NE transport-specific inhibitor desipramine (T. Pacholczyk, unpublished data).

To demonstrate that the single cloned cDNA directed the synthesis of a transporter with the expected ionic and pharmacologic sensitivities of the native human NET (hNET), Pacholczyk and colleagues (1991) cloned the cDNA insert downstream of a T7 RNA polymerase promoter in pBluescript SKII(-) and transfected the construct into transformed human fibroblasts (HeLa) infected with a vaccinia virus encoding T7 RNA polymerase (Fuerst et al. 1986). Blakely and coworkers (1991b) had demonstrated that this expression method gave faithful, rapid, and high-level expression of cloned Na+/glucose and Na+/GABA transporters, thus providing a convenient transient expression method for analysis of the putative hNET. Assays performed on HeLa cells 12 hours after transfection revealed the generation of a high level of Na+-dependent NE transport activity; analysis of substrate-dependence revealed the induction of a single, saturable activity with an NE Kt of 457 nM (figure 3). Assays conducted with a wide range of transporter and receptor antagonists revealed the NE uptake (1) to be markedly sensitive to selective NE transport inhibitors as compared with DA or 5-HT transport inhibitors, (2) to be potently antagonized by both cocaine and D-amphetamine, and (3) to be insensitive to inhibitors of either amino acid and sugar transport or to adrenergic and adrenergic receptor antagonists (figures 4a and 4b, table 2). As expected, reserpine, an inhibitor of the vesicular NE transport inhibitor (Kanner and Schuldiner 1987), also failed to block NE uptake in transfected cells, ruling out any possibility for ectopic expression of vesicular carrier. Thus, all the readily testable pharmacological features of Uptake 1 (Iversen 1967) appear to be encoded by this single cDNA. Given that multiple RNAs are not required to synthesize a functional carrier, these findings also suggest that the membrane-imbedded transporter may be monomeric in composition, as has been argued for the transporter Lac permease (Costello et al. 1987), or may exist as a homomultimer, as envisaged with the Na+/glucose transporter (Stevens et al. 1990).

Sequence analysis of the isolated NET cDNA reveals a large open reading frame encoding a highly hydrophobic 617-amino acid peptide with a predicted molecular weight of ~69 kD. Hydrophobicity analysis (Kyte and Doolittle 1982) reveals ~12 regions capable of forming transmembrane domains. Although this motif of 11 to 13 transmembrane domains superficially resembles the pattern observed with both the Na+/glucose and E. coli Na+/proline symporters, sequence analysis fails to reveal significant conservation in primary sequence. Rather, shared sequences evident with the cloned rat and human Na+/GABA transporters (GAT1) (Guastella et al. 1990; Nelson et al. 1990) reveal the presence of a new transporter gene family, united by the function of their

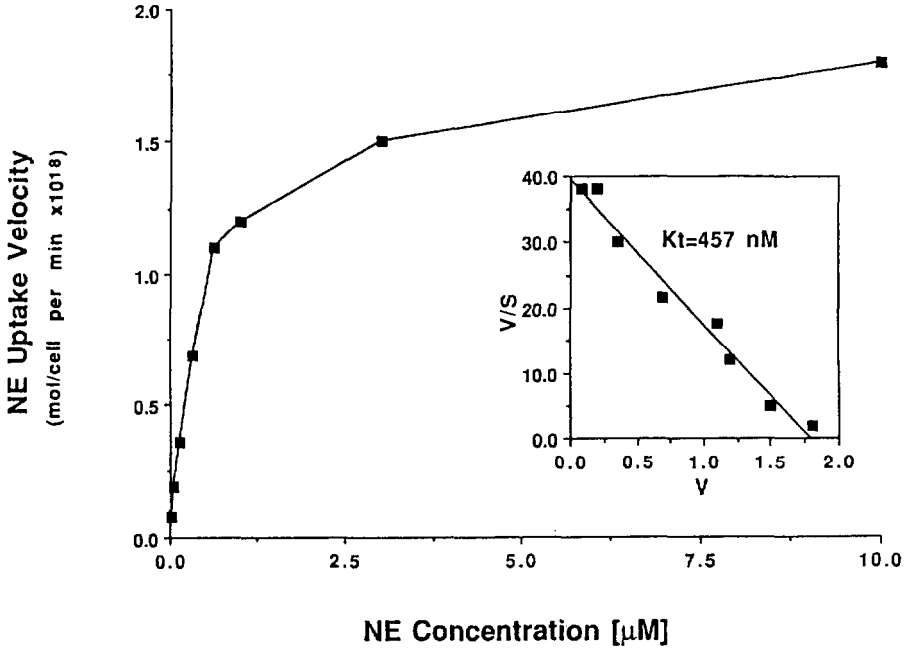

FIGURE 3. *Transport of L-NE in transfected HeLa cells*

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substrates as neurotransmitters. Alignment of the predicted amino acids for GABA and NE carriers reveals 46-percent identity, which rises to 68 percent, allowing for conservative substitutions. Sequences predicted to lie within or adjacent to predicted transmembrane regions contain many of the conserved residues. One striking stretch, lying between putative transmembrane domains 1 and 2, possesses 19 out of 20 residues absolutely conserved. A comparison of their virtually superimposable hydrophobicity profiles gives a clear indication that the GABA and NE transporters are likely to assume similar secondary structures in the plasma membrane (figure 5). As with GAT1, a large hydrophilic loop bearing three consensus sites for N-linked glycosylation is observed between transmembrane domains 3 and 4. When paired with the absence of a detectable signal sequence for membrane insertion, this feature suggests an initial model with both NH2- and COOH-termini in the cytoplasm, as depicted in figure 6.

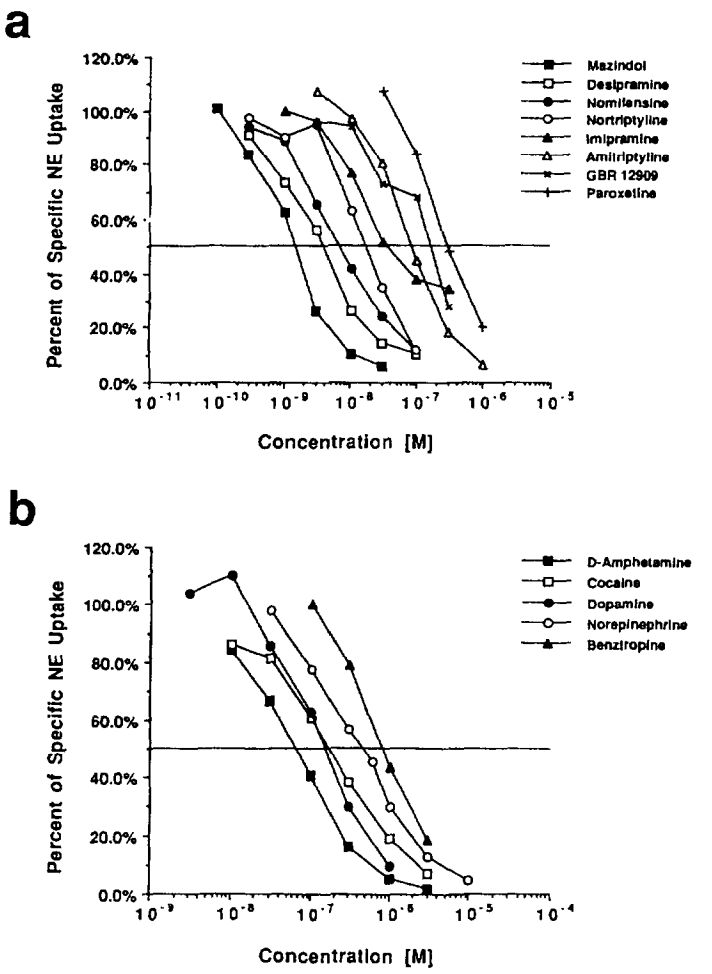

FIGURE 4. *Inhibitor sensitivity of transfected Na+/L-NET*

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TABLE 2. *Inhibitor sensitivity of L-NE uptake in pNET-transfected HeLa cells*

Inhibitor	K_I (nM)	Hill (n_H)
Mazindol	1.36	0.98
Desipramine	3.88	1.22
Nomifensine	7.68	1.17
Nortriptyline	16.5	0.95
D-amphetamine	56.1	1.00
Imipramine	65.4	1.08
Amitriptyline	100	0.75
GBR 12909	133	1.06
DA	139	0.86
Cocaine	140	1.25
Paroxetine	312	0.75
Benztropine	822	0.82
Citalopram	>1,000	—
Propranolol	10,000	—
5-HT	>10,000	—
Yohimbine	>10,000	—
Prazosin	>10,000	—
Hemicholinium-3	>10,000	—
Nipecotic acid	>10,000	—
Phloridzen	>10,000	—
Reserpine	>10,000	—

NOTE: HeLa cells (200,000 to 300,000/well) infected with a T7 RNA polymerase-containing vaccinia virus were transfected with pNE (100 ng) and incubated with 20 nM [2,5,6,^{3}H]-L-NE (New England Nuclear) ±inhibitors for 15 minutes at 37 °C. K_I values and Hill coefficients reflect mean estimates from triplicate determinations of complete uptake inhibition curves, adjusting for substrate concentration after Cheng and Prusoff (1973).

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High-affinity, antidepressant-sensitive NE uptake activity is enriched in projections of brain stem noradrenergic neurons, particularly from the locus coeruleus, as well as in terminals of noradrenergic sympathetic neurons and in the neural crest-derived adrenal medulla. Northern hybridizations with labeled NET reveal a 5.8-kb RNA localized to rat brain stem, rat adrenal gland,

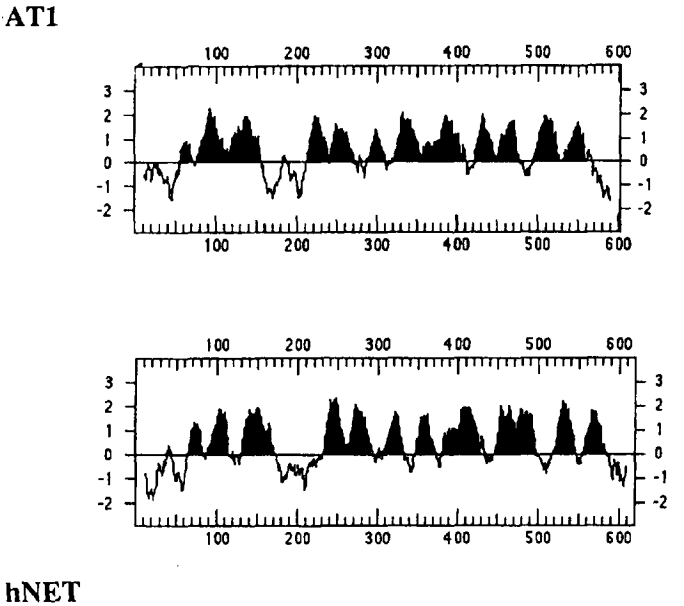

FIGURE 5. *Hydrophobicity comparison of GABA and NET proteins encoded by cloned GAT1 and hNET cDNAs. The transformation is as described by Kyte and Doolittle (1982) with a window of 19 residues. Hydrophobic values are positive in this display with shaded regions indicating the 12 putative transmembrane regions.*

rat PC-12 cells, and the human SK-N-SH cells (Pacholczyk et al. 1991). A more widespread 3.6-kb band is also observed in brain regions and cell lines and may represent a splicing variant of NET or a related and cross-hybridizing transcript. Because desipramine-sensitive NE uptake has been reported in primary cultures of neonatal rat astrocytes, this latter species may also represent a more broadly distributed glial carrier. Also, although both PC-12 and SK-N-SH cells exhibit many noradrenergic traits, their phenotypes can be quite heterogeneous depending on culture conditions, and thus, multiple transcripts may also reflect the mixed character of these cells.

In summary, a single 1,983-bp cDNA clone encoding a 617-amino acid protein is sufficient to transfer upon nonneuronal cells the ability to accumulate NE with high affinity, in a Na+-dependent manner, and with marked sensitivity

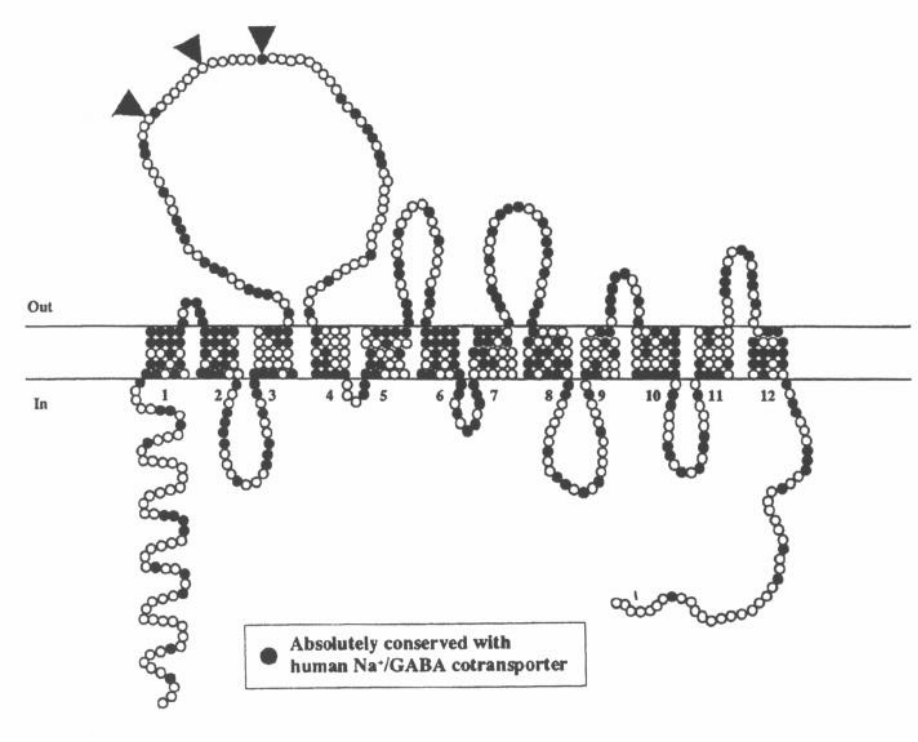

FIGURE 6. *Hydrophobicity-based structural model of the human Na^+/L-NET*

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to specific antidepressants, cocaine, and amphetamine. As these latter agents have profound effects on human behavior, exhibiting both therapeutic and abuse potential, identification of a clone for this transporter may provide a direct route to the rational design of novel therapies. In addition, the availability of a cDNA encoding the NET should allow for an examination of the degree to which structural alterations in the NE carrier gene underlie hereditary patterns of major affective disorders, hypertension, and drug abuse.

EVALUATION OF DIVERSITY WITHIN THE NEUROTRANSMITTER TRANSPORTER GENE FAMILY

The presence of conserved amino acid sequences between GAT1 and NET suggests that other neurotransmitter transporters, such as those for DA, 5-HT, Glu, Gly, and choline, are likely to be members of this family. Indeed, given the

major pharmacologic differences between the transporters encoded by GAT1 and NET, it is likely that the observed 46-percent identity between these two proteins represents the lower limit of sequence similarity to be observed as new members are uncovered. This would seem true for the monoamine transporters, where considerable antagonist overlap exists among NE, DA, and 5-HT uptake systems (Hendley 1984; Cooper et al. 1986; Richelson 1990). One conventional approach to the identification of related gene products is the screening of cDNA libraries by hybridization at reduced stringency. Using this approach, however, Nelson and colleagues (1990) were unable to identify homologs of GAT1 , suggesting that additional family members might exhibit too little overall identity with GAT1 to permit elucidation in this manner.

Recently, with the introduction of the polymerase chain reaction (PCR) (Saiki et al. 1988), it became possible to use short stretches of dispersed sequence identity to identify and clone novel members of an extended gene family (Gould et al. 1989; Libert et al. 1989; Kamb et al. 1989). In the implementation of this strategy, Peek and colleagues (1991) have designed degenerate, inosine-substituted oligonucleotides based on the sequence identity present between GAT1 and NET in the regions connecting transmembrane domains 1 and 2 as well as that observed in transmembrane 6. With these oligonucleotides as primers, Peek and colleagues (1991) amplified rat and human cDNAs and subcloned products of a size similar to that found between these sequences in NET. Sequence analysis of the cloned amplification products revealed multiple, distinct gene products all bearing sequence identity to NET and GAT1. Several of these products bear considerable sequence identity to NET (60 to 80 percent), and others exhibit only the level of identity (40 to 50 percent) observed when comparing NET and GAT1 over these regions. Although these amplification fragments represent partial cDNA clones, they can be used to determine the anatomical localization of the endogenous transcripts by Northern and in situ hybridizations and can be used as high-stringency probes for the identification of their full-length functional sequences by conventional library hybridization. Northern analysis of each of these products revealed several that are broadly distributed across the rodent central nervous system and perhaps difficult to associate with the distribution of a particular neurotransmitter system. However, several fragments hybridize in a regionally selective manner. For example, one clone is enriched in forebrain regions, whereas another is highly concentrated in thalamic, midbrain, and brain stem regions. Particularly striking is the hybridization of two clones selectively to midbrain and brain stem regions. Peek and coworkers (1991) performed in situ hybridization analysis of these clones and found one of these to be localized to the substantia nigra and ventral tegmental area, whereas the other is confined to the midbrain and brain stem raphe complex, identifying these two clones as likely candidates for the DA and 5-HT transporter cDNAs, respectively.

Utilizing oligonucleotides derived from the 5'-end of the PCR fragment encoding the 5-HT transporter candidate, Blakely and colleagues (1991c) isolated a series of overlapping clones from a rat brain stem cDNA library. Sequence analysis of one of these clones reveals the presence of a large open reading frame in register with that of NET. After subcloning the bacteriophage insert into a T7-promoter-bearing plasmid, Blakely and colleagues (1991c) transfected this cDNA into HeLa fibroblasts infected with T7 polymerase-vaccinia virus. After 8 hours, transfected fibroblasts exhibit marked expression of 5-HT transport, which can be abolished by the selective 5-HT transport antagonist fluoxetine (figure 7). Thus, like NET, the 5-HT transporter (SERT) appears to be encoded by a single RNA (Blakely et al. 1991c). The availability of a cDNA clone encoding this carrier should permit a refined analysis of the structural basis of antidepressant interactions with nerve terminals. Sequence comparisons with NET and GAT1 reveal

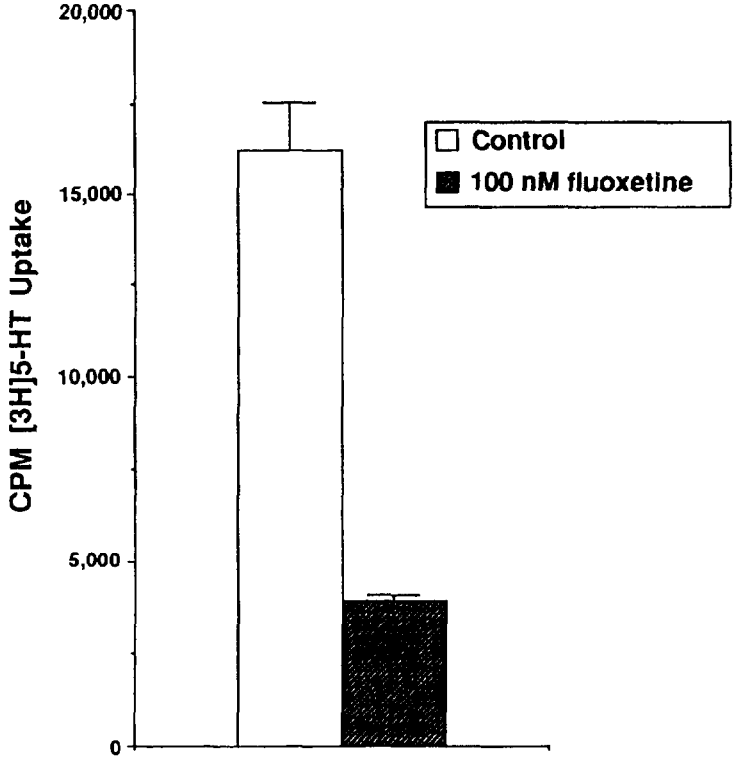

FIGURE 7. *Expression of 5-HT transport in HeLa cells transfected with SERT cDNA*

highly conserved residues likely to be important for common tasks, including substrate translocation and Na+ binding. In addition, with regulation of the 5-HT carrier reported to be altered in depressed humans (Paul et al. 1981; Meltzer et al. 1981), the availability of a cDNA clone encoding this carrier should lead to a direct search for structural changes associated with the human SERT homolog in affective disorders. Recently, the first cloning of a functional rat brain DA transporter cDNA was also achieved (Kilty et al. 1991; Shimada et al. 1991), bearing sequence identity to the substantia nigra localized PCR species described above. Together, the elucidation of the 5-HT and DA transporters broaden understanding of the structural basis for neurotransmitter transporter diversity and provides new tools for the molecular analysis of transporter gene expression and regulation in brain disorders and drug abuse.

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Regulation of Gene Expression by Dopamine: Implications in Drug Addiction

Orla M. Conneely, Ronan F. Power, and Bert W. O'Malley

INTRODUCTION

Cocaine is thought to produce pleasure by increasing dopamine transmission in mesocortical and mesolimbic dopaminergic tracts. Increased dopaminergic transmission results from an inhibition of the dopamine reuptake transporter by cocaine, which also inhibits reuptake of both serotonin and norepinephrine (Ritz et al. 1988). The reward-seeking behavior produced by cocaine self-administration is similar to that produced by electrical self-stimulation of dopaminergic pathways. The observation that chronic cocaine administration decreases electrical self-stimulation reward indices suggests that a down-regulation of dopaminergic reward areas may accompany addiction (Gawin and Ellinwood 1988).

The molecular mechanisms underlying long-term neuroadaptive changes that accompany chronic cocaine abuse have yet to be established. However, it is becoming increasingly evident that neuronal adaptation to repeated perturbation of neurochemical signals involves changes in gene expression. Examples of such genomic regulation of neuronal adaptation include the regulation of gene expression by serotonin during long-term facilitation (Barzilai et al. 1989) and the development of kindling by repeated electrical stimulation of the hippocampus (Jonec and Wasterlain 1979; Shin et al. 1990). Therefore, it seems plausible that the effects of chronic cocaine abuse would be reflected at a level of altered gene expression in dopaminergic and/or dopaminoceptive cells. In this case, elucidation of the mechanisms by which dopamine transduces a neurotransmitter signal to changes in gene expression would be expected to yield valuable information on the molecular nature of neuroadaptation to repeated cocaine administration. To this end, the authors have identified a class of transcription factors that are activated by dopamine. Activation of these factors results in a dopamine-dependent induction of transcription of specific target genes. The activated factors are members of the steroid/thyroid receptor superfamily of transcription factors.

THE STEROID/THYROID RECEPTOR FAMILY OF TRANSCRIPTION FACTORS

The steroid/thyroid receptor family constitutes a subclass of transcription factors that regulate and coordinate complex events in development, differentiation, and homeostasis. These proteins control behavioral and physiological responses by regulating the expression of specific gene networks in response to diverse stimuli (for a review, see Evans 1988). Members of the above family of transcription factors contain three conserved regions of amino acid sequence (McDonnell et al. 1987). The conserved regions consist of a DNA-binding domain responsible for specific binding of the transcription factor to upstream regulatory DNA sequences within the promoter region of responsive target genes (Evans 1988) and two additional regions of conservation located in the ligand-binding domain of the ligand-activated members of this family (McDonnell et al. 1987). The steroid receptors were the first characterized members and serve as prototypes for this class of transcription factors (Hollenberg et al. 1985; Greene et al. 1986; Conneely et al. 1987). These receptors are soluble proteins that upon specific binding to their cognate steroid hormone become active transcription factors capable of regulating the expression of specific target genes (Evans 1988). In addition to the steroid receptors, the ligand-activated members of this family include receptors for retinoic acid (Giguere et al. 1987), vitamin D_3 (Baker et al. 1988), and thyroid hormone (Weinberger et al. 1986); more than 20 additional members have been identified. They have been termed “orphan receptors” since no ligand has been identified that binds to these proteins. However, recent evidence has demonstrated that several members of the steroid/thyroid receptor superfamily can be activated in the absence of ligand by phosphorylation (Glineur et al. 1990; Denner et al. 1990). This observation raises the possibility that the orphan receptors may be activated by direct ligand binding and/or by phosphorylation via indirect signaling pathways.

One such orphan receptor, chicken ovalbumin upstream promoter-transcription factor (COUP-TF) (Wang et al. 1989), participates in the regulation of several genes, including the chicken ovalbumin gene (Wang et al. 1987), the rat insulin II gene (Hwung et al. 1988), and the proopiomelanocortin gene (Drouin et al. 1989). Although COUP-TF is widely distributed in tissues throughout the body, including the brain, at least one of its functions appears to be the regulation of retinal cell development since the Drosophila counterpart of this protein is required for the determination of photoreceptor cell fate (Mlodzik et al. 1990). The present study was initiated in an attempt to identify chemical signals that would lead either directly or indirectly to the activation of COUP-TF. In the context of the studies described below, functional COUP-TF expression is measured as COUP-TF-dependent activation of a specific target gene.

DOPAMINE REGULATES COUP-TF-DEPENDENT GENE TRANSCRIPTION

To search for compounds that activate COUP-TF, the authors replaced the DNA-binding domain of COUP-TF with that of the tissue-specific progesterone receptor (figure 1). Since the enhancer DNA specificity is provided by the DNA-binding domain, the resulting chimera is expected to direct COUP-TF-dependent transcription of progesterone-responsive target genes (Beato 1991). This strategy was employed to prevent competition in the assay from endogenous COUP-TF in transfected cells. Expression plasmids containing the chimeric COUP-TF construct (pADFCOUP) and a progesterone-responsive target gene (PRETKCAT) were cotransfected into a progesterone receptor negative monkey kidney cell line, CV1. The target gene used in these studies contained two copies of a progesterone-responsive enhancer element (PRE) located upstream of the herpes simplex virus thymidine kinase promoter linked to a chloramphenicol acetyl transferase (CAT) gene.

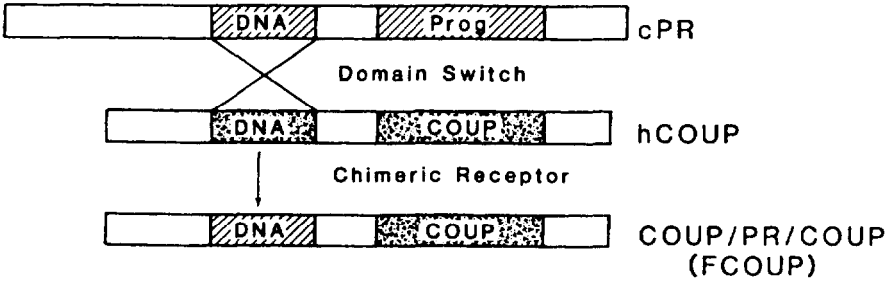

FIGURE 1. *Strategy for generation of the chimeric COUP protein. Schematic representations of cPR and COUP-TF are shown. Prog designates the progesterone-binding domain of cPR. The chimeric COUP-TF (FCOUP) contains the NH_2 and COOH-terminal domains of COUP-TF fused to the DNA-binding domain of cPR. The cPR cDNA (Conneely et al. 1987) was digested with HindIII at nucleotide 1801, repaired, and ligated to Bsml-digested, blunt-ended human COUP-TF cDNA (hCOUP) to fuse the cPR DNA-binding domain to the COOH-terminus of COUP-TF. A polymerase chain reaction was used to generate a Sacl site in the COUP-TF cDNA immediately 5' to the DNA-binding domain. Sacl-digested COUP-TF cDNA then was ligated to Sacl-digested PR cDNA to fuse the N-terminus of COUP-TF to the PR DNA-binding domain.*

SOURCE: Data from Power et al. 1991a

More than 150 compounds and tissue extracts were tested for their ability to activate COUP-dependent CAT gene expression. Cells were maintained in serum-free media, and individual compounds were added immediately after transfection. The cells were harvested after 48 hours, and extracts were assayed for expression of CAT activity. The results of these experiments demonstrated that the neurotransmitter, dopamine, was capable of stimulating COUP-TF-dependent activation of CAT expression. The data shown in figure 2 demonstrate that concentrations of dopamine as low as 3 mM were capable of stimulating CAT gene activity fivefold to tenfold (lanes 2 to 7). Dopamine did not stimulate CAT expression in cells transfected with a parent expression plasmid that lacked the chimeric COUP-TF coding sequences (lane 12). This finding indicated that activation of gene expression did not result from a general increase in transcriptional activity in transfected cells but was dependent on the expression of a chimeric COUP transcription factor.

The activation of COUP-TF by dopamine does not result from direct ligand binding because dopamine binding assays demonstrated no direct binding of COUP-TF to the neurotransmitter. This observation suggested that the activation of COUP-TF resulted from an indirect signaling pathway. Dopamine action is mediated by its interaction with several membrane-bound dopamine receptors. These G-coupled receptors are of two subtypes, D1 and D2, distinguished by their ability to stimulate, D1, and inhibit, D2, adenylyl cyclase, respectively (Kebabian and Calne 1979). Analysis of the ability of selective agonists of these receptor subtypes to activate COUP-dependent gene expression demonstrated that the dopamine receptor agonist α-ergocryptine mimicked dopamine in its ability to activate CAT expression (lanes 8 to 11). These data suggested that a dopamine-receptor-mediated signaling pathway was responsible for the activation of the COUP-TF chimera.

CV1 CELLS POSSESS A DOPAMINE-SENSITIVE ADENYLYL CYCLASE

The presence of dopamine receptors in renal tissue has been documented (Felder et al. 1989). To determine whether a dopamine-dependent signaling pathway of the D1 receptor subtype is operative in CV1 cells, the authors analyzed these cells for expression of a dopamine-sensitive adenylyl cyclase. The results are shown in figure 3. Dopamine elicited a twofold increase in cyclic AMP (cAMP) levels in membranes prepared from these cells. Maximum stimulation was achieved with 100 mM dopamine. Thus, a dopaminergic responsive cyclase system is intact in CV1 cells.

COUP-TF IS ACTIVATED BY PHOSPHORYLATION

The above data taken together suggested that the activation of COUP-TF by dopamine may be a phosphorylation-mediated event. Phosphorylation has

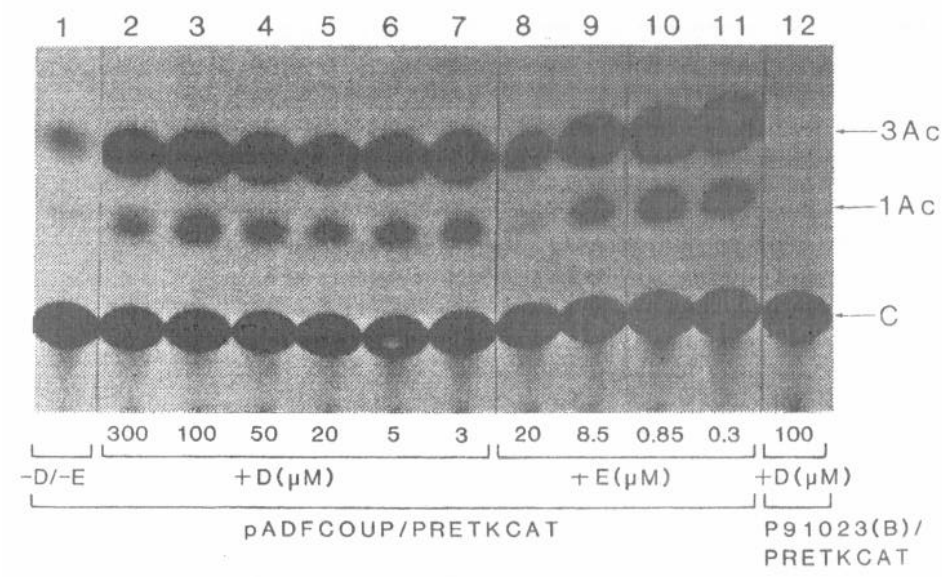

FIGURE 2. *Induction of CAT gene expression by dopamine (D), and α-ergocryptine (E). pADFCOUP (5 μg) and PRETKCAT (5 μg) were cotransfected into CV1 cells as described (Conneely et al. 1987). Cells were cultured for 2 days in serum-free media supplemented with Nutridoma (Boehringer Mannheim: Indianapolis, IN) in the absence (lane 1) or presence of varying concentrations of dopamine (lanes 2 to 7) and α-ergocryptine (lanes 8 to 11). The media was replaced after 24 hours, and fresh compounds were added. As a control, cells were transfected with P91023 (B), which lacked COUP-TF coding sequences, and with PRETKCAT and cultured in the presence of 100 μM dopamine (lane 12). CV1 cell extracts were prepared for CAT assays as described (Conneely et al. 1987), and the assays were performed for 12 hours with 50 μg of protein extract The results shown are representative of at least six separate experiments in which duplicate points were performed. The variation in signals between duplicate points in any one experiment was not more than 5 percent. In all experiments the positions of [^{14}C]chloramphenicol (C) and the 1- and 3-acetylated forms of [^{14}C]chloramphenicol are indicated (1AC and 3AC, respectively).*

SOURCE: Data from Power et al. 1991a

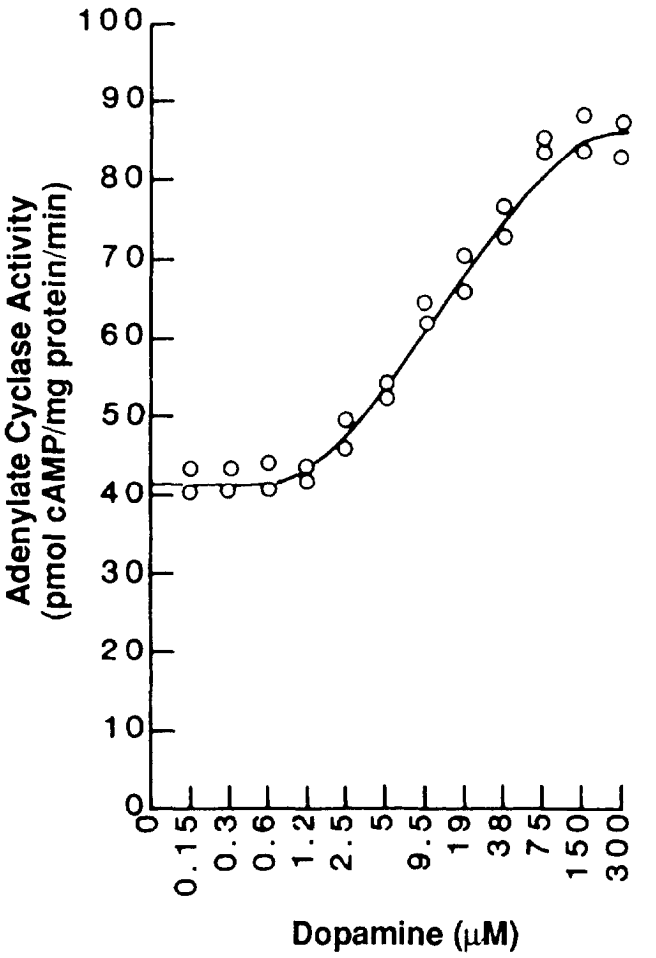

FIGURE 3. *Dopamine-sensitive adenylyl cyclase in CV1 cells. Production of cAMP in homogenates from untransfected CV1 cells cultured in Nutridoma-supplemented media was measured as a function of increasing dopamine concentration. Adenylyl cyclase assays were performed in duplicate for each point. Homogenization of ceils and adenylyl cyclase assays were performed exactly as described (Toro et al. 1987).*

SOURCE: Data from Power et al. 1991b

previously been shown to play a role in activation of several transcription factors, including members of the steroid/thyroid receptor family (Glineur et al. 1990; Denner et al. 1990). Therefore, we examined the ability of 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP) and the protein phosphatase 1 and 2A inhibitor, okadaic acid, to mimic dopamine in the activation of CAT gene expression. The result shown in figure 4 demonstrates that these agents also stimulate COUP-TF-dependent transcription in the authors' assay system and thus support the hypothesis that the dopamine activation of COUP-TF is a phosphorylation-mediated process.

DOPAMINE STIMULATES LIGAND-INDEPENDENT ACTIVATION OF SEVERAL MEMBERS OF THE STEROID/THYROID RECEPTOR FAMILY

The ability to activate COUP-TF by a signaling pathway stimulated by dopamine prompted an examination of the classical steroid receptor members of the same family to determine whether they can be activated by dopamine in the absence of ligand. The results of these assays are shown in figure 5.

The authors selected several members of this family and found that the progesterone (cPRA, cPRB), estrogen (hER), and vitamin D_3 (hVDR) receptors also were activated by dopamine and okadaic acid. Again, CV1 cells were transfected with the appropriate receptor expression vector and a specific reporter gene (HREtKCAT) containing the appropriate receptor responsive enhancer element (HRE) located upstream of the thymidine kinase promoter and a CAT gene. The level of activation attained with dopamine was comparable to that attained with the natural ligand for these receptors (figure 5, panels A to D). Interestingly, not all steroid receptors were activated by this neurotransmitter under our test conditions. CV1 cells cotransfected with a vector for expression of the human glucocorticoid receptor pRShGRa (Giguere et al. 1986) and the PRE/GRETKCAT reporter plasmid were transcriptionally responsive to dexamethasone but were unresponsive to either dopamine or okadaic acid (panel E). Likewise, cells transfected with a vector for expression of the human mineralocorticoid receptor pRShMR (Arriza et al. 1987) showed the expected increase in CAT gene expression in response to aldosterone and dexamethasone but showed only a marginal response to dopamine and okadaic acid (panel F). It has been shown that the progesterone receptor can be activated by 8-Br-cAMP in the absence of hormonal ligand (Denner et al. 1990). The data above are consistent with the notion that the activation of several members of this family by dopamine occurs through phosphorylation of these transcription factors via a dopamine-dependent intracellular signaling pathway. However, confirmation of this mechanism will require direct identification of a dopamine-dependent phosphorylated site on these transcription factors.

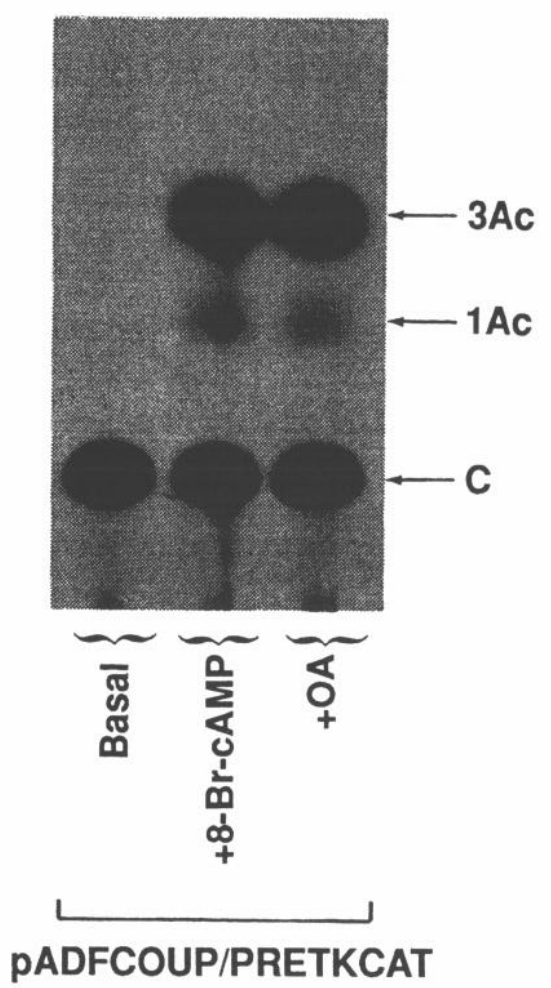

FIGURE 4. *Activation of COUP-TF-mediated transcription by phosphorylation. Cells transfected with pADFCOUP (5 µg) and PRETKCAT (5 µg) were either untreated (basal) or treated with 8-Br-cAMP (10^{-3}M) or okadaic acid (OA) ($5x10^{-8}$M) as described (Denner et al. 1990).*

SOURCE: Data from Power et al. 1991a

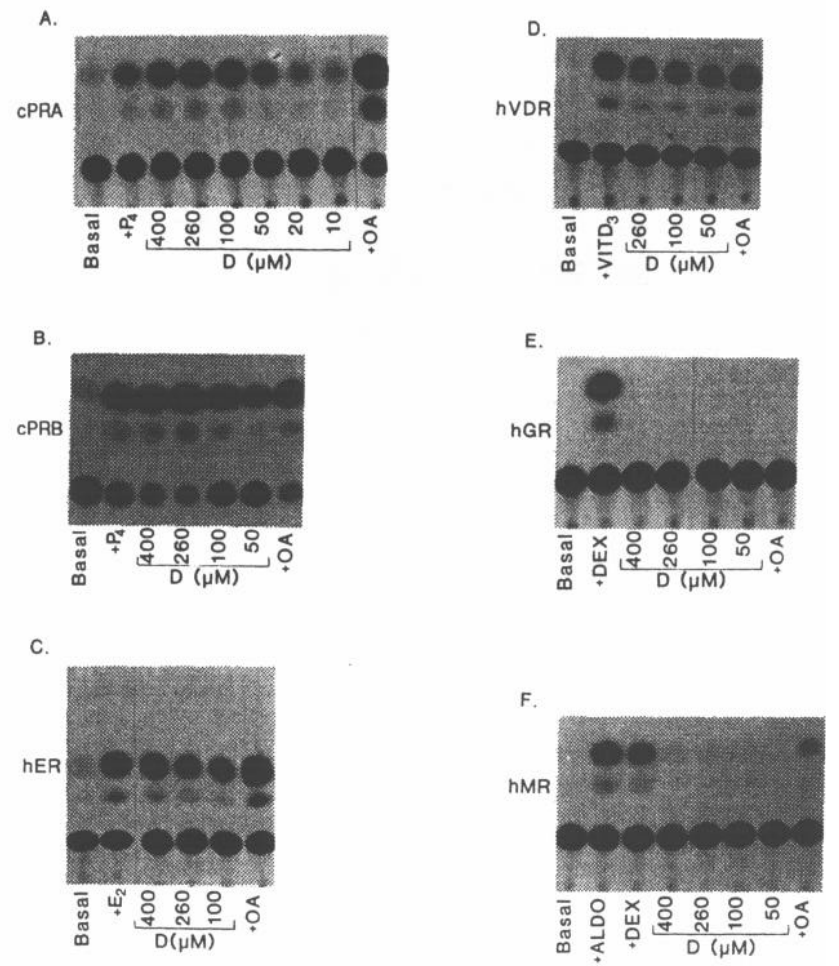

FIGURE 5. *The effect of dopamine on the transcriptional activity of steroid hormone receptors. Expression vectors (5 μm) for the chicken progesterone receptor A and B forms (cPRA, cPRB) (Conneely et al. 1987), human glucocorticoid receptor (hGR) (Giguere et al. 1986), and human mineratocorticoid receptor (hMR) (Felder et al. 1989) were cotransfected with the reporter plasmid PRE/ GRETKCAT (5 μm) (Jantzen et al. 1987). The human estrogen receptor (hER) expression construct (2 μm) was cotransfected with an estrogen receptor-responsive reporter, ERE E1bCAT (5 μm). The human vitamin D_3 receptor construct (1 μm) (McDonnell et al. 1989) was cotransfected with a reporter*

plasmid VDRETKCAT (4 µm) containing three copies of the vitamin D receptor response element fused to the herpes simplex virus thymidine kinase promoter and the bacterial CAT gene. Transfected cells were either untreated (basal) or treated with 10^{-7}M of the relevant ligands for each receptor; progesterone (P_4), estradiol (E_2), 1,25$(OH)_2D_3$ ($VITD_3$), dexamethasone (DEX), and atdosterone (ALDO). Cells also were treated with the indicated concentrations of dopamine (D) and okadaic acid (OA; $5X10^{-8}$M). CAT activity was determined 42 hours posttransfection.

SOURCE: Data from Power et al. 1991b

DOPAMINE REGULATION OF GENE EXPRESSION IS REGULATED BY A RECEPTOR OF THE D1 SUBTYPE

To examine the role of D1 and D2 receptors in the mediation of dopamine-dependent activation of gene expression, we tested the ability of selective D1 and D2 agonists to mimic the dopamine activation of progesterone receptor-dependent gene transcription. CV1 cells, transfected with the cPRA expression vector and the reporter plasmid PRETKCAT, were treated with either progesterone, dopamine, the selective D1 receptor agonist SKF38393, or the selective D2 receptor agonist quinpirole. The relative amounts of CAT activity expressed after treatment of the cells with each compound are shown in figure 6. The D1-selective agonist SKF38393 stimulated CAT-gene expression to a level even greater than progesterone or dopamine. However, no stimulation of CAT activity was obtained afler treatment with the selective D2 receptor agonist quinpirole. Thus, the activation of the steroid receptors by dopamine appears to result from a signaling pathway that is mediated by a D1-adenylyl-cyclase-linked receptor.

DISCUSSION

The authors have demonstrated that the neurotransmitter dopamine can stimulate an intracellular signaling pathway that results in activation of members of the steroid/thyroid receptor family of transcription factors. The ability of dopamine D1 receptor agonists to selectively mimic dopamine in the activation of these factors, together with the demonstration of a dopaminergic-responsive cyclase system in CV1 cells, suggests that the pathway is mediated by a dopamine receptor of the D1 subtype. Furthermore, the activation of COUP-TF and the steroid receptors examined in this study appears to be mediated by phosphorylation. Receptors activated by dopamine

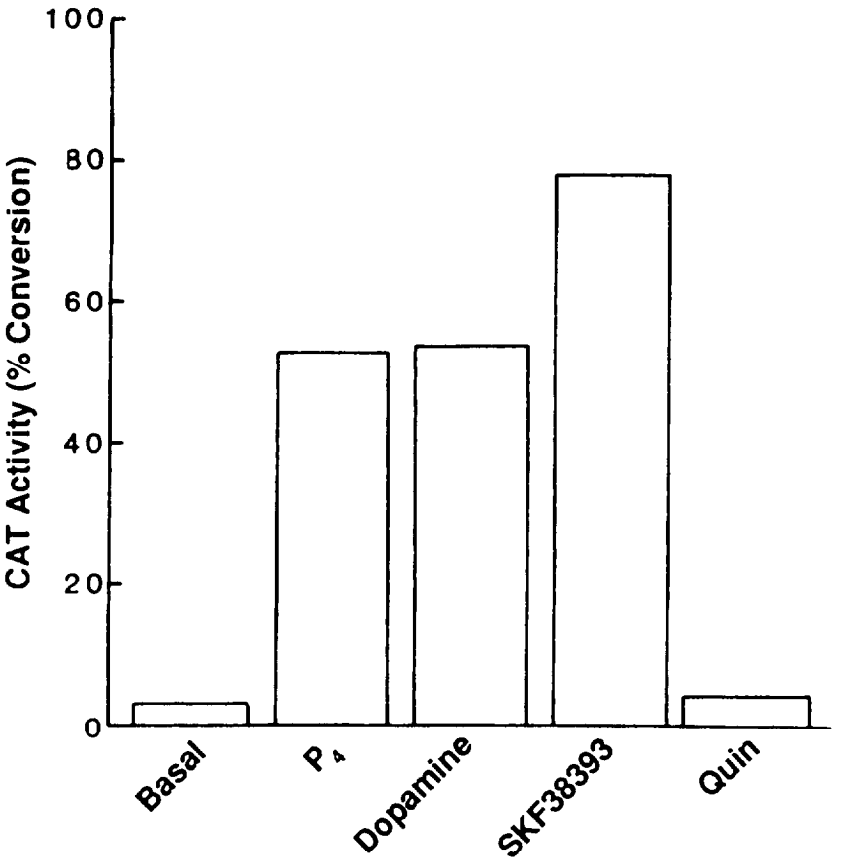

FIGURE 6. *Regulation of progesterone receptor-mediated gene transcription by dopamine receptor agonists. CV1 cells were transfected with the cPRA expression vector and the PRETKCAT reporter as described in the figure 2 legend. Transfected cells were either untreated (basal) or treated with progesterone (P4; 10^{-7}M), dopamine (400 μM), the selective D1 dopamine receptor agonist SKF38393 (100 μM), or the selective D2 dopamine receptor agonist quinpirole (Quin; 20 μM). CAT activity was determined 42 hours posttransfection. Each result represents a mean value obtained from duplicate experiments.*

also are activated by 8-BR-cAMP and the phosphatase inhibitor okadaic acid, whereas other members of the same family of receptors that are not activated by dopamine also are not activated by 8-Br-cAMP and okadaic acid.

The data obtained in this study provide the first demonstration of a link between a dopamine-stimulated signaling pathway and transcription factor activation. The activation of members of the steroid/thyroid receptor family of transcription factors by dopamine provides a means by which dopamine can regulate gene expression in dopaminoceptive cells. Furthermore, the data suggest that the same signaling pathways that mediate short-term cellular responses to dopamine stimulation also may mediate long-term neuroadaptive responses to dopamine by reprograming genomic expression. In this regard, additional studies will be required to substantiate the physiological relevance of this pathway, to identify which members of the steroid/thyroid receptor family are expressed in dopaminoceptive cells, and to identify the specific target genes that are regulated by dopamine.

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Regulation of Opioid Gene Expression: A Model To Understand Neural Plasticity

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PLASTICITY OF OPIOID GENE EXPRESSION: A MODEL TO ANALYZE MECHANISM

Over the past several years, it has become clear that neurotransmitter regulation of gene expression is a critical mechanism underlying neural plasticity (Golet et al. 1986; Montarolo et al. 1986; Goodman 1990; Sheng and Greenberg 1990; Morgan and Curran 1991; Comb et al. 1987). The role of peptide neurotransmitters in this process has received much attention because they act as important regulators of neural signaling and their synthesis and expression is dependent on the transcription of precursor genes in the nucleus. This direct biosynthetic dependence on gene transcription endows peptide transmitters with special "adaptive" properties that distinguish them from classical small-molecule neurotransmitters (Comb et al. 1987). Perhaps the most interesting and best studied example of this process in brain is the activity-dependent switch in opioid peptide expression in hippocampus (Gall 1988; White et al. 1987; White and Gall 1987; Morris et al. 1988). Electrical or chemical stimulation of the rodent perforant pathway, the major input to hippocampus from entorhinal cortex, turns on expression of proenkephalin and turns off expression of prodynorphin in granule cells of the dentate gyrus. These changes in opioid precursor result in dramatic and longlasting changes in proenkephalin and prodynorphin peptides (Gall 1988; White et al. 1987) in mossy fibers and mRNAs (White et al. 1987; White and Gall 1987; Morris et al. 1988; Sonneberg et al. 1989) in granule cell bodies. Because enkephalins have a profound excitatory effect on target hippocampal pyramidal cells, this activity-driven switch may exert major control over hippocampal excitability and may be intimately involved in the molecular mechanisms underlying plastic changes in hippocampal signaling.

As opioid peptides are critical regulators of neural pathways mediating pain, reward, motivation, and hormone release, understanding how their synthesis and expression is regulated takes on great practical as well as theoretical significance. With the realization that neurotransmitters regulate endogenous opioid biosynthesis has come the understanding that drugs that interfere with or alter neurotransmission also may influence the expression of endogenous opioids. Hence, neurotransmitters and drugs of abuse acting through synaptic connections in brain may alter gene expression in the postsynaptic neuron. Such drug-induced changes in gene expression may underlie important components of addiction, withdrawal, and drug-seeking behaviors, which have been particularly difficult to understand at a mechanistic level.

IMMEDIATE EARLY GENE REGULATION IN BRAIN

Seizures and electrical stimulation also trigger a rapid and transient RNA induction of several different immediate early genes (IEGs) within granule cells of the dentate gyrus. For example, RNA encoding different components of the AP-1 transcription factor complex: c-Fos, c-Jun, and JunB are coinduced (White and Gall 1987; Sonneberg et al. 1989; Saffen et al. 1988; Cole et al. 1989; Wisden et al. 1990) together with proenkephalin mRNA in hippocampal granule cells by seizure and electrical stimulation of the perforant pathway. The time course of IEG induction precedes the induction of proenkephalin RNA (White and Gall 1987; Sonneberg et al. 1989). These observations have led to the hypothesis that IEGs may act as “third messengers” to initiate and coordinate programs of gene expression by regulating the expression of appropriate “target” genes, for example, proenkephalin. This argument is strengthened by the observation that binding sites for many of these factors have been found within a compact region of the proenkephalin promoter (figure 1) known to mediate synaptic and second-messenger regulation (Comb et al. 1986, 1988; Hyman et al. 1988; Nguyen et al. 1990; Chu et al. 1991; Kobierski et al. 1991). In addition, these proteins are known transcription factors; the authors show herein that different components of the AP-1 complex have different effects on proenkephalin transcription. Regulation of transcription by the AP-1 complex at the proenkephalin-inducible enhancer represents the first neural model system to understand IEG target gene regulation and promises to outline general mechanisms that may regulate the synthesis of other key neural signaling molecules. The recent discovery that drugs of abuse such as cocaine (Young et al. 1991), amphetamine (Graybiel et al. 1990), and morphine (Chang and Harlen 1990) also activate the expression of IEGs suggests that this process may represent an adaptive response of the brain to drugs of abuse.

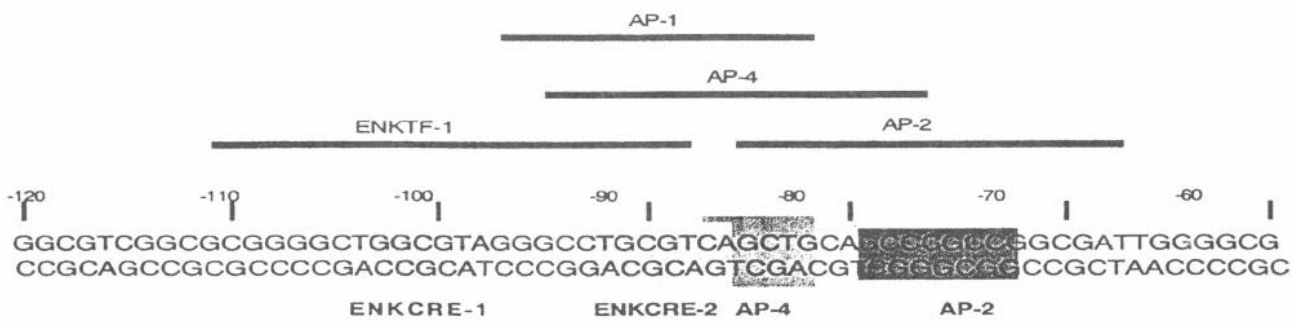

FIGURE 1. *Human proenkephalin gene promoter sequence*

TRANSSYNAPTIC REGULATION OF PROENKEPHALIN TRANSCRIPTION

Studies over the past 5 years have defined a short 50-base-pair region of the human proenkephalin promoter that is necessary and sufficient for transcriptional regulation by adenosine 3':5'-cyclic phosphate (cAMP)-, 12-O-tetradecanoyl-phorbol ester (TPA)-, and CA^{++}-dependent intracellular signaling pathways. Extreme conservation of this sequence between human and rat genes reinforces its critical role in proenkephalin expression and regulation. Two functional elements were localized within this region and shown to interact synergistically to mediate second-messenger regulation (Comb et al. 1986, 1988; Hyman et al. 1988; Nguyen et al. 1990; Chu et al. 1991; Kobierski et al. 1991). Previous studies have identified and characterized transcription factors that interact with these DNA elements. NF-1 (Chu et al. 1991) proteins interact with the CRE-1 element, and AP-1 and AP-4 interact with the CRE-2 element (Comb et al. 1988; Hyman et al. 1988; Nguyen et al. 1990; Chu et al. 1991; Kobierski et al. 1991). Recent identification and characterization of the CREB/ATF transcription factors (Hai et al. 1989; Gonzalez and Montminy 1989) prompted the authors to examine the role of these factors in the regulation of proenkephalin transcription. DNA binding studies (in vitro) suggested that these factors interact poorly with the proenkephalin-inducible enhancer as homodimers and focused our attention on the AP-1 (Fos/Jun) family of transcription factors, which bind the enhancer with high affinity (Golet et al. 1986).

Work from the authors' laboratory (Comb et al. 1988; Kobierski et al. 1991) and Tom Curran's laboratory (Sonneberg et al. 1989) has demonstrated that the AP-1 transcription factor binds the proenkephalin CRE-2 element and that in vitro-translated c-Jun and c-Fos bind the CRE-2 element as Fos/Jun heterodimers. Our laboratory has also shown that two other Jun members, JunD and JunB, bind the CRE-2 element and have opposite effects on proenkephalin transcription (Kobierski et al. 1991). In this chapter, discussion is restricted to the role of the AP-1 factors mediating second-messenger regulation of proenkephalin transcription at the CRE-2 element.

Previous studies have also demonstrated that cAMP rapidly activates proenkephalin transcription, with increases in steady-state mRNA levels apparent within 15 minutes after the addition of forskolin to C6 glioma cells (Kobierski et al. 1991). RNA levels peak at 1.5 to 2 hours following forskolin addition and then rapidly decline to undetectable levels at between 4 and 8 hours. Activation is rapidly followed by a refractory period during which the proenkephalin gene is unresponsive to further stimulation of the cAMP pathway. The forskolin-dependent rapid activation is not affected by protein synthesis inhibitors: however, protein synthesis inhibitors block the decline in mRNA

levels leading to super induction (Kobierski et al. 1991). In addition, forskolin treatment of C6 glioma cells leads to rapid induction of c-Fos and JunB mRNA and a reduction in the level of JunD RNA.

CHARACTERIZATION OF JUND, JUNB, AND C-JUN INTERACTIONS WITH CRE-2

Three observations have focused the authors' efforts on identifying members of the AP-1 complex that bind the CRE-2 element and mediate inducible expression: (1) Multiple copies of this element reconstitute the most essential features of cAMP-, TPA-, and Ca^{++}-inducible regulation; (2) ATF/CREB molecules bind this element poorly if at all; and (3) AP-1 complexes bind the CRE-2 element with high affinity. To test the ability of Fos/Jun molecules to stimulate or repress proenkephalin transcription, we have developed expression vectors and a cotransfection assay to determine the effect of expression of various Fos/Jun molecules on proenkephalin expression. Full-length cDNAs encoding c-Jun, JunB, c-Fos, and JunD have been introduced into a Rous sarcoma virus expression vector. Expression vectors are then cotransfected with pENKAT-12 into F9 cells in the presence or absence of a plasmid expressing high levels of the cAMP-dependent protein kinase (PKA). Results of this type of analysis have focused our attention on two different Jun molecules, JunD and JunB, which have opposite effects on proenkephalin transcription. JunD strongly transactivates proenkephalin transcription in a manner that is totally dependent on the catalytic subunit of the cAMP-dependent PKA (figure 2). JunB has no effect in the presence or absence of PKA and acts to repress JunD-dependent stimulation of proenkephalin transcription (figure 3). Finally, c-Jun strongly activates proenkephalin transcription in a constitutive fashion that is not influenced by PKA (see figure 2). Activation and repression by JunD, JunB, and c-Jun map to the CRE-2 element by cotransfection using plasmids containing multicopy elements and point mutations. We have extended these observations to several other cell lines, including C6 glioma and SK-N-MC cells, that express proenkephalin. These findings clearly demonstrate that transactivation of proenkephalin by JunD is completely dependent on the catalytic subunit of the cAMP-dependent PKA. In addition, as each of these molecules is expressed in the granule cell of the hippocampus, JunD constitutively (Wisden et al. 1990) and JunB and c-Jun in a highly inducible fashion (Saffen et al. 1988; Cole et al. 1989; Wisden et al. 1990), it is likely that these molecules play a critical role in the activation and repression of proenkephalin gene expression in the nervous system.

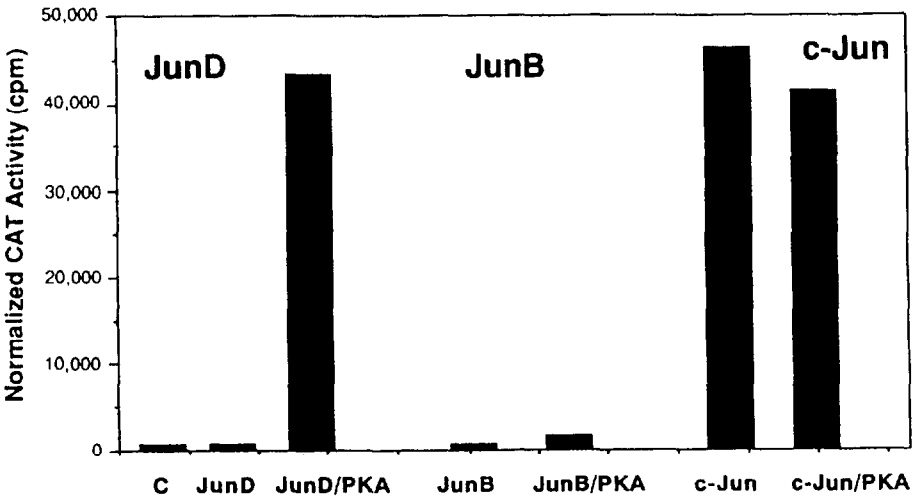

FIGURE 2. *Different effects of Jun proteins on proenkephalin transcription. Plasmids (5 µg each) expressing JunD (RSVJunD), JunB (RSVJunB), and c-Jun (RSVc-Jun) were cotransfected into F9 cells together with a proenkephalin/CAT fusion gene (pENKAT-12 5 µg) in the presence or absence of a plasmid expressing the catalytic subunit of the cAMP-dependent PKA (pMTCalpha 5 µg). In addition, a plasmid, pRSVßGal (10 µg), was also included in each transfection, and ß-Gal expression was used to normalize CAT expression between transfection experiments. Data presented are from one experiment but are representative of at least five different experiments.*

CHARACTERIZATION OF JUND AND JUNB BINDING AT THE PROENKEPHALIN ENHANCER

To examine the binding of JunD and JunB to the CRE-2 element, RNA encoding JunD, JunB, and c-Fos was transcribed and then translated individually and in combinations as described below in rabbit reticulocyte lysates. Control lysates show no specific binding to a 30-base-pair oligonucleotide, spanning the proenkephalin enhancer from -110 to -80. However, lysates programed with JunD RNA produce a shifted band, which is specifically self-competed but not competed by AP-2 or AP-4 oligonucleotides and is very weakly competed by a CRE-1 oligonucleotide. Both TRE and CRE oligonucleotides compete effectively for JunD DNA

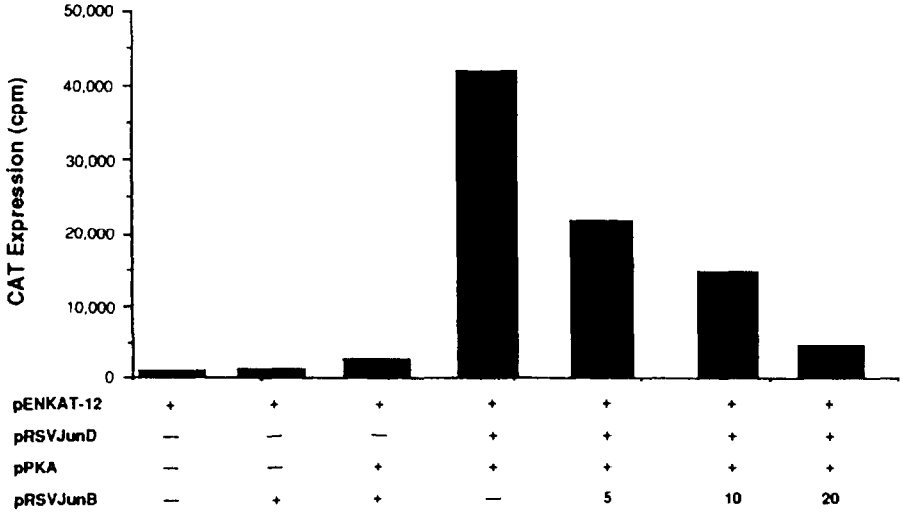

FIGURE 3. *JunB represses PKA-dependent activation of proenkephalin by JunD. Plasmids were cotransfected into F9 cells as described in figure 2. In each case, 5 µg of DNA was used except where indicated for pRSVJunB.*

binding. In addition, mutations within the 3'-end of CRE-2, which have the most profound effect on second-messenger regulation of proenkephalin transcription (Comb et al. 1988), also produce the most severe effect on JunD binding.

In contrast, binding of in vitro-translated JunB to the enhancer is not observed. Because JunB is known to dimerize with JunD and c-Fos (Ryder et al. 1989; Nakabeppu et al. 1988), we also analyzed the interaction of these complexes with the CRE-2 element. Consistent with previous reports using AP-1 oligonucleotides, binding of JunB and JunD to the CRE-2 element is dramatically stimulated in the presence of c-Fos.

CLASSIC CRE AND TRE ELEMENTS ALSO MEDIATE PKA-DEPENDENT TRANSCRIPTION VIA JUND

Because the proenkephalin enhancer contains a hybrid TRE/CRE-like element, CRE-2, we tested the ability of JunD to transactivate "classic" TRE and CRE elements in a PKA-dependent fashion by transiently transfecting F9 cells with plasmids containing the somatostatin CRE, or the collagenase TRE,

together with pRSVJunD and pMTCaneo. JunD strongly transactivates the somatostatin CRE element and transactivates the collagenase TRE to a somewhat lesser extent. These results indicate that JunD can act at both CRE and TRE elements to stimulate transcription in a PKA-dependent fashion.

ANALYSIS IN TRANSGENIC ANIMALS

To extend the analysis of mechanisms underlying activity-dependent regulation of proenkephalin transcription to the intact nervous system, we have made transgenic animals expressing proenkephalin/LacZ fusion constructs. The first construct analyzed contains 3 Kb of human proenkephalin 5'-flanking sequences and 1.5 Kb of 3'-flanking sequences driving expression of the LacZ reporter. Expression of this construct has been examined in three independent transgenic lines. This construct also contains the well-characterized cAMP-, TPA-, and Ca^{++}-inducible enhancer located within the first 110 bases of 5'-flanking DNA (Comb et al. 1986) and is shown to direct correct tissue-specific expression to all (with only two known exceptions, striatum and olfactory tubercle) sites within the adult reproductive (Borsook et al., in press) and nervous systems. Although some variation between founder animals is apparent, there is a remarkable degree of tissue-specific transgene expression observed in the adult mouse brain.

In brain, as in the reproductive system, ectopic expression of the transgene is not seen. As illustrated in table 1, expression on the transgene in various brain regions appears to be remarkably precise as determined by X-Gal staining. LacZ expression has been most carefully examined in founder #3 where X-Gal staining is seen in the spinal cord, thalamus, hypothalamus, hippocampus, entorhinal cortex, cerebral cortex, amygdala, dorsal horn of the periaqueductal gray matter, ventral tegmental area, pons and medulla oblongata, and many other brain regions. As expected, the major sites of hypothalamic transgene expression are the paraventricular nucleus and ventromedial nucleus. High-level expression is also seen in the central nucleus of the amygdala in excellent agreement with previous immunohistochemical studies of cholchicine-treated rats. In addition, correct tissue-specific expression is seen in laminae I and II (substantia gelatinosa) of the dorsal horn of the spinal gray matter and is also observed in scattered cells throughout laminae IV and V-VII. A comparison between LacZ expression and previously reported expression of proenkephalin peptides and mRNA is also shown in table 1. Taken together, these remarkable results suggest that the 30 Kb proenkephalin/LacZ fusion gene directs expression of LacZ to the vast majority of diverse sites where proenkephalin is normally expressed in the adult. The one anomaly in brain expression is striatum. Little or no expression of LacZ is seen in striatum of each of the three independent founder lines examined. The missing or low-level expression

TABLE 1. *Comparison of transgene LacZ expression vs. proenkephalin mRNA (in situ) and peptide immunoreactivity*

Structure	ß-Gal*	In situ	Immunoreactivity
Telencephalon			
Olfactory bulb	—	ND	+++
Cingulate cortex	++	+++	+++
Entorhinal cortex	+++	+++	+++
Olfactory tubercle	—	++++	+++
Caudate-putamen	—	++++	++
Lateral septum	++	++	++
Bed n. stria termin	+	+	
Diagonal band Broca	++	+	++
Preoptic area	+	+	+
Amygdala central	++++	++++	+++
Medial	+++	+++	+
Cortical	+++	+++	+++
Anterior	++	++	+
Lateral	+	+	+
Dentate gyrus	+	+	+
Hippocampal pyramidal	+	+	+
Diencephalon			
Hypothalamus anterior	++	++++	++
Perifornical	+++	+++	+++
Lateral	++	++	+
Suprachiasmatic	—	ND	+/-
Paraventricular	++	++	++
Ventromedial	++++	++++	+
Thalamus lateral geniculate	+++	++	++
Periventricular	+	+	+
Mesencephalon and rhombencephalon			
Periaqueductal gray	+++	+	++
Ventral tegmental	+++	+++	+++
Locus coeruleus	+	+	+
Pontine reticular	—	+	+
Raphe magnus	+++	+++	+++
Gigantocellular reticular formation	++	++	++
Raphe pallidus	+	+	+
Cerebellum (Golgi II)	++	+++	—
Spinal cord (I, II, V, X)	+++	++	+++

KEY (relative intensity of expression): -=none detected; ND=not determined; and increasing intensity=from + to ++++

*ß-Gal staining in mouse; in situ data and immunohistochemical data from rat, adapted from Fallon and Leslie (1986)

in striatum and olfactory bulb suggests that additional element(s) may be necessary for expression at these sites. Alternatively, these elements may be present, yet are more sensitive to the site of chromosomal integration. To further investigate this possibility, the authors are producing and analyzing additional independent founder animals.

SUMMARY AND CONCLUSION

The recent finding that neurotransmitters and drugs that affect neurotransmission have important influences on gene expression suggests that drug-induced alterations in gene expression may underlie many long-term effects of addictive drugs, for example, dependence and drug-seeking behaviors. These long-term adaptive responses to opiate drugs have been particularly difficult to understand at a mechanistic level. Data presented here indicate that the gene encoding the opioid precursor proenkephalin is highly regulated by neural activity, second-messenger pathways, and PKA. These observations raise the possibility that drugs of abuse (e.g., opiates acting through opiate receptors) may act at the genetic level to modulate the expression of endogenous opiates and that these effects may underlie one component of the brain's long-term adaptive response to exogenous opiates. The transgenic animals described above can be used to investigate opiate drug-induced changes in proenkephalin gene expression, allowing rapid analysis of changes in proenkephalin gene expression in highly restricted populations of neurons in a fashion previously impossible. In addition, by analyzing the effects of specific enhancer mutations on tissue-specific and transsynaptic regulation of proenkephalin expression, transgenic models will permit mechanistic investigations within the intact nervous system that cannot otherwise be undertaken.

Investigation of mechanisms underlying this process requires the analysis of intracellular signaling pathways, responsive DNA regulatory elements, and the transcription factors transducing synaptic signals into gene regulation. In the studies described herein, we demonstrate that AP-1 complexes consisting of different Jun proteins differentially regulate proenkephalin transcription at the CRE-2 element. c-Jun constitutively activates proenkephalin transcription, whereas JunD activates in a fashion completely dependent on the activation of second-messenger pathways and the cAMP-dependent PKA. JunB alone has no effect on proenkephalin gene expression, yet this molecule effectively blocks activation mediated by JunD and, hence, may act as a repressor. These data are consistent with a model (figure 4) in which preexisting JunD mediates the rapid cAMP-dependent activation of the proenkephalin enhancer, whereas IEGs such as JunB or c-Fos mediate the protein synthesis-dependent inactivation. Because c-Jun activates proenkephalin transcription constitutively, induction

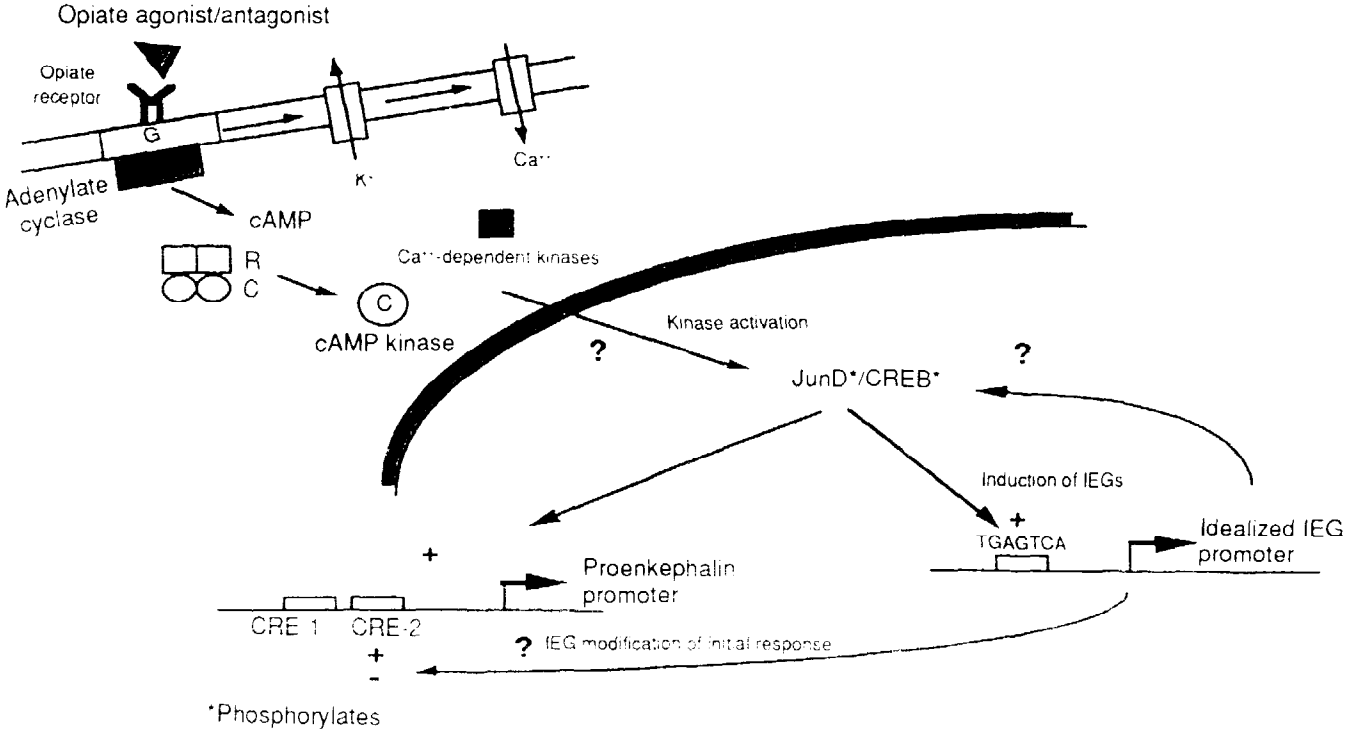

FIGURE 4. *Model for activation and repression of proenkephalin transcription by AP-1 complexes. Neurotransmitters or drugs acting through neurotransmitter receptors activate second-messenger pathways and PKA. Activated PKA rapidly dissociates to the nucleus and phosphorylates substrates, leading to activation of preexisting JunD/CREB-like transcription factors, which in turn rapidly activate proenkephalin transcription. These factors also activate IEGs such as c-Jun and JunB, which may further stimulate or repress proenkephalin transcription.*

of c-Jun may lead to a further and prolonged activation of proenkephalin gene expression. Hence, the ratio of c-Jun to JunB induction may determine whether proenkephalin is repressed or further activated.

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Cellular and Molecular Analysis of Opioid Peptide Gene Expression

Cynthia T. McMurray, Karen M. Pallock, and James Douglass

INTRODUCTION

The molecular mechanisms underlying behavioral and physiological events associated with the intake of addictive drugs such as cocaine, amphetamine, and opiates are poorly understood. The problem of physical dependence to and withdrawal from these drugs is challenging to approach experimentally, as the establishment of these states likely involves a complex cascade of biochemical and electrical events. Such events involve the diverse and widely distributed array of central nervous system (CNS) neurotransmitters, receptors, ion channels, and transporters and extend to complex autonomic and sensorimotor neural networks. In addition, various events associated with drug-seeking behavior and addiction are also influenced by genetic, species, and environmental factors, which only serve to further complicate experimentation in this area.

There is growing evidence that specific changes in neuronal transcription patterns result from the administration of narcotic drugs. For example, it has been reported that the administration of morphine significantly decreases striatal levels of proenkephalin mRNA in the rat (Uhl et al. 1988), and chronic naloxone treatment increases expression of both proenkephalin and protachykinin in the rat striatum (Tempel et al. 1990). In striatum, prodynorphin gene expression is increased following chronic administration of the indirect dopamine antagonists methamphetamine and cocaine (Sivam 1989; Hanson et al. 1987). Thus, it appears that administration of narcotic drugs can induce localized alterations in the synthesis of mRNA encoding striatal peptide neurotransmitters. More recently, evidence has been presented suggesting that the aforementioned transcriptional alterations may be mediated by rapid, transient changes in expression of specific immediate early genes, such as c-*fos.* For example, amphetamine and cocaine can induce drug-specific activation of the c-*fos* gene in striosome-matrix compartments and limbic subdivisions of the striatum (Graybiel et al. 1990). Furthermore, this induction appears to involve the D1 dopamine receptor system (Young et al.

1991). Thus, to continue to gain insight into the nuclear events associated with substance abuse, researchers must understand in greater detail the basic mechanisms that regulate the expression of pertinent transcriptionally active genes in the CNS, such as those mentioned above.

REGULATED EXPRESSION OF THE RAT PRODYNORPHIN GENE

Distribution and Physiological Effects of Dynorphin Peptides

The prodynorphin precursor encodes the dynorphin family of opioid peptides (Kakidani et al. 1982; Civelli et al. 1985). Northern blot and in situ histochemical analysis have determined that the prodynorphin gene is transcriptionally active in a wide variety of cell types within the central and peripheral nervous systems (Akil et al. 1984; Civelli et al. 1985). Such regions include the hypothalamus, striatum, hippocampus, midbrain, brain stem, and cerebral cortex, as well as neurons within the spinal cord and gut. Prodynorphin transcripts are also present at relatively high levels in endocrine tissues, such as the anterior pituitary, adrenal, testis, ovary, and uterus.

The opioid receptor subtype exhibiting specificity for prodynorphin-derived peptides is the κ-opioid receptor (for review, see Mansour et al. 1988). The κ-receptor belongs to the G protein-linked family of receptors and is also coupled to calcium conductances. This latter characteristic serves to biochemically distinguish the κ-receptor from the μ- and δ-opioid receptor subtypes, which are coupled to potassium conductances. Studies utilizing radiolabeled ligands specific for κ-receptor binding have determined that this species of opioid receptor is widespread throughout the CNS and endocrine system.

As with the opioid peptides derived from the proopiomelanocortin and proenkephalin precursors, it has been difficult to assign precise physiologically relevant functional roles to those peptides derived from the prodynorphin precursor. However, numerous studies have suggested that the dynorphin peptides serve to regulate a wide variety of physiological and behavioral responses (Mansour et al. 1988). These roles include the mediation of visceral analgesia, effects on appetite suppression, mediatron of hypotensive cardiovascular responses, inhibition of vasopressin secretron and possibly additional renal functions, involvement in modulation of motor seizure thresholds and intensity and involvement in recovery from spinal cord injury and stroke. A role for dynorphin peptides in narcotic tolerance mechanisms also has been sugested.

Transcriptional Regulation of the Rat Prodynorphin Gene In Vivo

The cloning of rat prodynorphin cDNA and genomic DNA (Civelli et al. 1985; Douglass et al. 1989) has afforded the opportunity to study regulation of prodynorphin gene expression in distinct neuronal cell types following specific surgical or pharmacological manipulations. The spinal cord and hippocampus represent those neural systems in which the most dramatic alterations of prodynorphin gene expression have been observed to date.

In the spinal cord, prodynorphin transcripts and peptides are localized to laminae I-II and V-VI, suggestive of a functional role in endogenous pain recognition and control. Following the onset of unilateral inflammation of the hindlimb, prodynorphin biosynthesis is substantially elevated in the region of the spinal cord receiving sensory input from the affected limb (ladarola et al. 1988; Naranjo et al. 1991). Prodynorphin mRNA levels rise substantially within the first 24-hour period, and maximal stimulation (eightfold to ninefold increase) is observed between 3 and 5 days after the onset of inflammation. By day 14, prodynorphin mRNA levels have approached control values. This time course of induction and subsequent decline closely parallel that of hindlimb edema and hyperalgesia. Spinal cord DynA1-8 levels also rise approximately threefold during the inflammatory period, consistent with an increase in both the rate of synthesis and release of dynorphin peptides from spinal cord neurons. These data suggest the active participation of dynorphin-containing spinal cord neurons in the modulation of sensory afferent input during peripheral inflammatory pain states.

Opioid peptides derived from the prodynorphin precursor are also present at high levels in the rat hippocampus. In this brain structure, dynorphin immunoreactivity exhibits a restricted distribution, being localized to the granule cell/mossy fiber axonal system (McGinty et al. 1983). Over the past several years, numerous groups have documented striking changes in the levels of hippocampal prodynorphin mRNA following the onset of seizure activity. In one study, the acute effects of kainic acid (KA) administration were examined (Douglass et al. 1991). Following a single subcutaneous injection of KA (8 mg/kg), hippocampal prodynorphin mRNA levels rose thirteenfold to fourteenfold within 3 hours, began to decline at 12 hours, and by 48 hours were at or below control values. Although mRNA levels were dramatically stimulated, hippocampal DynA1-8 levels remained at values significantly 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.

Interestingly, other paradigms that induce seizure activity, such as electroconvulsive shock (Xie et al. 1989a), prepyriform cortical kindling (Xie et al. 1989b), and hippocampal kindling (Morris et al. 1987), serve to significantly reduce hippocampal prodynorphin mRNA levels to 25 to 40 percent of control values. Thus, it appears that the prodynorphin gene is capable of being both positively and negatively transcriptionally regulated in the granule cells of the dentate gyrus.

Characterization of the Rat Prodynorphin Gene

The studies described above document changes in prodynorphin mRNA levels in various neuronal cell types and systems. Presumably, these changes are the result of alterations in the rate of transcription of the prodynorphin gene. The authors have isolated and characterized the rat prodynorphin gene (Douglass et al. 1989) to gain an understanding of the molecular events that mediate transcriptional regulation of the gene. Such analysis has led to the identification of the rat prodynorphin mRNA CAP site and promoter element and to the initial characterization of putative transcripitional regulatory elements.

As diagramed in figure 1, the rat prodynorphin gene is composed of four exonic domains. Exons 1 and 2 encode 5' untranslated regions of the mRNA, whereas exon 3 encodes 15 bases of 5' untranslated region and the first 23 amino acids of the prohormone. Exon 4 encodes the remaining 204 amino acids of the prohormone, including the three leucine-enkephalin moieties, as well as the entire 3' untranslated region. In the CNS, exons 1 through 4 are spliced together to generate a mature 2,400-base transcript. However, in the testis (and perhaps adrenal, as well), exon 2 is not contained within the smaller 2,300-base transcript (Garrett et al. 1989). Thus, the 5' untranslated region beginning 15 bases from the translational start site is unique in the two species of prodynorphin mRNA. Sucrose gradient polysome 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 efficiently translated in both tissues (Garrett et al. 1989). The functional significance of this alternate splicing event involving prodynorphin mRNA is currently unknown.

Analysis of the Rat Prodynorphin Gene Promoter

In vivo studies have documented that rat prodynorphin mRNA levels in specific neuronal cell types can be dramatically altered following neural stimulation. These changes presumably reflect alterations in the rate of synthesis of the prodynorphin transcript. Thus, signals received by prodynorphin-expressing neurons must be relayed to the cell nucleus, resulting in altered levels of transcription of the prodynorphin gene. Such transcriptional effects are

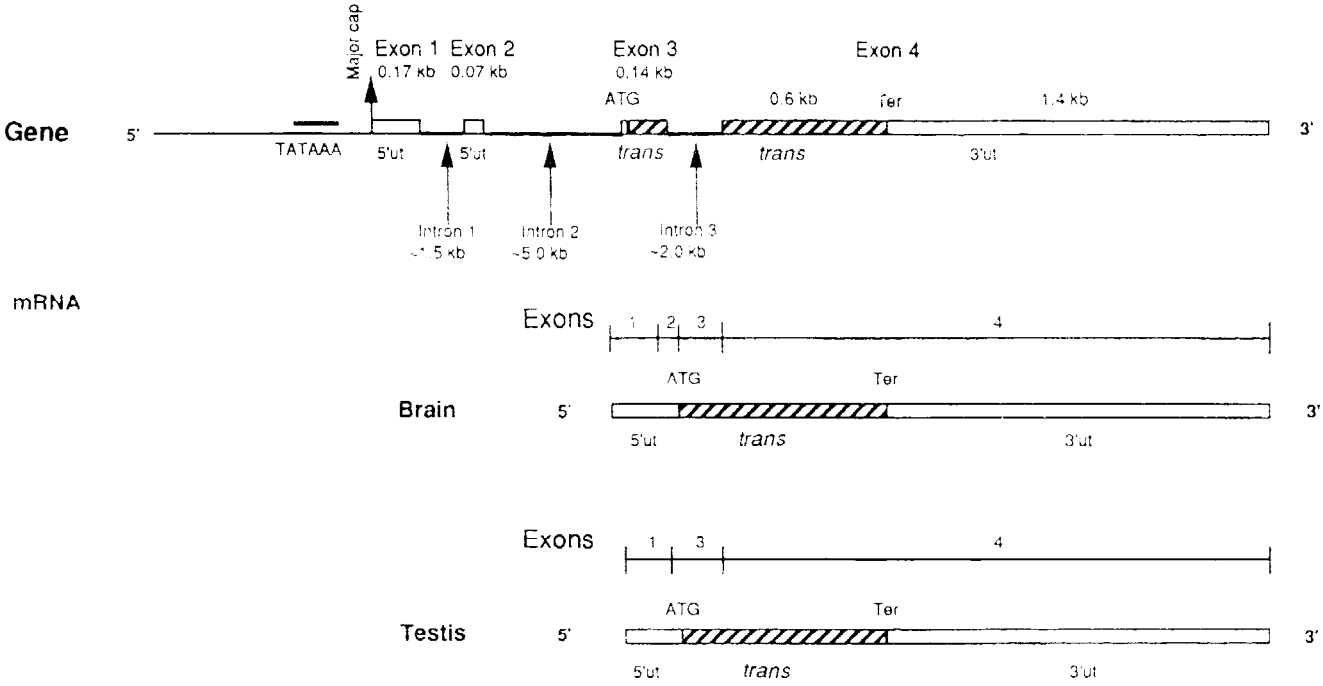

FIGURE 1. *Structure of the rat prodynorphin gene. Alternate splicing of rat prodynorphin precursor mRNA occurs to generate different forms of mature mRNA in the brain and testis.*

presumably mediated through precise interactions between specific DNA binding proteins (i.e., *trans*-acting factors) and their target recognition sequence elements near the prodynorphin promoter region (i.e., *cis*-acting elements). As a first step in characterizing such interactions, the authors have determined the nucleotide sequence of approximately 3.8 kb of rat genomic DNA located 5' to the prodynorphin mRNA CAP site. Figure 2 diagrams the location of potential DNA regulatory elements near the promoter region of both the rat and the human (Horikawa et al. 1983) prodynorphin genes, as identified by computer sequence analysis.

For the rat prodynorphin gene, the nucleotide sequence TAGCGTCAG is present in what constitutes the 5' untranslated region of the transcript at position +62. This element is highly homologous to the cyclic AMP (cAMP) response element present in several neuroendocrine peptide genes (Goodman 1990). At position -102 is the sequence GCCAAT, representing the consensus binding site for the enhancer protein, EBP20 (for review of *trans*-acting protein factors, see Jones et al. 1988). Two potential AP-2 binding sites (AP-2 site=CCCCAGGC) are located at positions -973 and -1826; AP-2 is a 52-kD protein that has been shown to mediate both phorbol ester and cAMP-dependent transcriptional effects. The sequence GGGGCGG at position -1281 represents a potential binding site for the transcriptional activator protein, SP1. Last, the sequence TGCGTCAG at position -1543 represents a sequence identical to that found within the human proenkephalin promoter, referred to as ENKCRE-2. This element is essential for both basal and regulated enhancer function and binds the AP-1 complex with high affinity (Comb et al. 1988). The same sequence element is also present at the human *c-fos* promoter, mediating cell type-specific transcriptional properties (Velcich and Ziff 1990). The human prodynorphin promoter also contains consensus binding sequences for AP-2, SP1, and EBP20, although the sites are not present at the same location relative to the mRNA CAP site when compared with the rat gene. However, the nucleotide sequence from approximately -500 to +100 is highly conserved between rat and human, suggesting that uncharacterized regulatory sequence elements may be localized to this region.

To begin to determine if any of the aforementioned sequence elements are of functional significance, various regions of the rat prodynorphin promoter have been placed 5' to the bacterial chloramphenicol acetyl transferase (CAT) coding region and expressed transiently in CV1 cells. Analysis of several different plasmid constructs is shown in figure 3. A minimal promoter fragment representing nucleotides -122/+135 has relatively weak promoter activity, and low levels of CAT activity are produced. This activity serves as a relative reference point for analysis of other prodynorphin promoter/CAT plasmid constructs. When the promoter region is extended in the 5' direction to -598

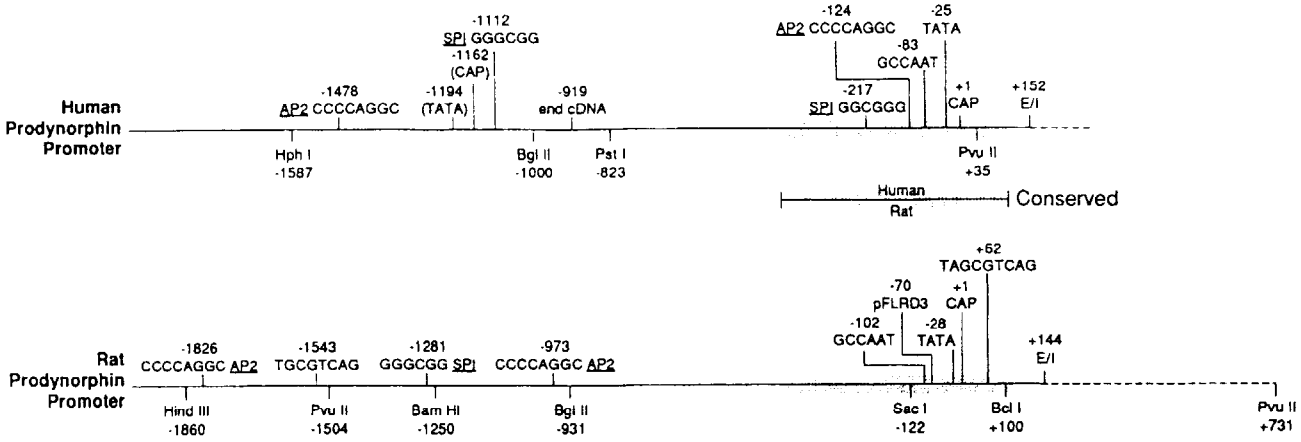

FIGURE 2. *Comparison of rat and human prodynorphin promoter regions. Pertinent restriction enzyme sites are noted. Also noted are the locations of nucleotide sequences that have perfect homology with characterized binding sites of specific DNA binding proteins. CAP sites, TATA elements, 5' ends of characterized cDNA clones, and exon/intron junctions are also shown for both genes. Lastly, the region exhibiting the greatest degree of nucleotide sequence conservation between the two species is shaded.*

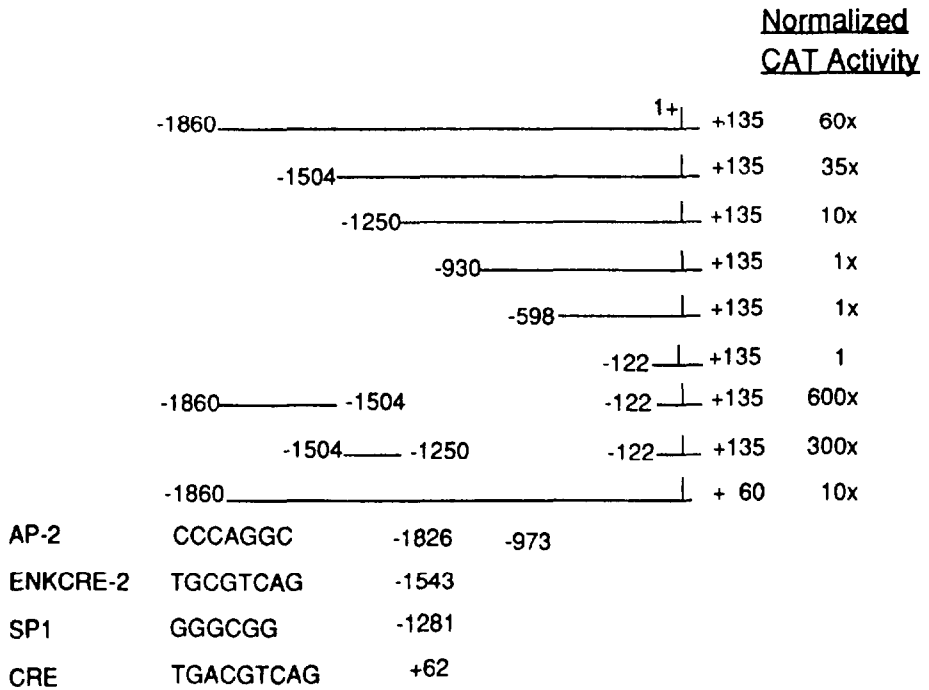

FIGURE 3. *Basal prodynotphin promoter CAT activity levels. Characterization of the ability of rat prodynorphin genomic DNA fragments to serve as transient transcriptional promoters. Nucleotides representing 5' and 3' ends of rat prodynorphin genomic DNA fragments (containing the mapped CAP site at position +1, and the TATAAA promoter element at -28) ligated proximal to the CAT structural gene are shown. The resulting plasmids were transfected into CV1 cells, and cellular CAT activity levels measured. Following standardization, CAT activity levels were normalized against those for the minimal promoter construct. The sequence and location of potential regulatory elements are also noted.*

or -930, again, low levels of CAT activity are observed. However, when the promoter region is extended to -1250, -1504, or -1860, relative levels of CAT activity are induced, 10-, 35-, and 60-fold, respectively. These data suggest that positive transcription sequence elements, such as those described above, are located in this region of genomic DNA. In addition, a positive transcription sequence element appears to be present in the 5' untranslated region of the

prodynorphin transcript between nucleotides +60 and +135, as there is an approximately sixfold reduction in CAT activity when comparing the -1860/+135 promoter region to the -1860/+60 fragment. This positive effect may be mediated through the cAMP-responsive element (CRE)-like element present at +62. Thus, positive sequence elements both 5' and 3' to the CAP site may act in concert to strengthen transcription from the promoter.

To begin to determine if positive-acting elements in the 5' region of the promoter can function outside of their natural context, the -1860/-1504 and -1504/-1250 fragments were placed in front of the minimal -122/+135 promoter. Surprisingly, relative CAT activity levels were approximately 600- and 300-fold elevated, respectively, when compared with -122/+135 CAT levels. Two possible explanations for this observation are that negative transcription sequence elements are present in the region of genomic DNA from -1250 to -122 or that the spacing of the positive transcription elements relative to the promoter plays a role in their efficacy.

HAIRPIN FORMATION WITHIN THE ENHANCER REGION OF THE HUMAN PROENKEPHALIN GENE

The proenkephalin gene encodes the polyprotein precursor to the enkephalin family of opioid peptides. The human proenkephalin gene promoter has been extensively characterized (Comb et al. 1988), and a wide variety of DNA binding proteins are able to interact with high degrees of affinity and specificity with the promoter region from nucleotides -110 to -70. Site-specific mutational analysis has determined that two specific sequence elements designated as ENKCRE-1 (-104 TGGCGTA -98) and ENKCRE-2 (-92 TGCGTCA -86) play a critical role in the transduction of signals transmitted from cell surface receptors to the proenkephalin nuclear transcription complex (Comb et al. 1988). Analysis of the promoter region containing ENKCRE-1 and ENKCRE-2 reveals that these elements are contained within a 23bp imperfect palindrome and that each strand has the potential to form not only a duplex structure but also hairpin structures (figure 4). A unique feature of these hairpin structures is that each would form with mismatched base pairs: the top strand (GT) forming two GT pairs and the complementary bottom strand (AC) forming two AC pairs.

The sequence shown in figure 4 was chemically synthesized to investigate hydrogen bonding properties within each strand as well as the duplex (McMurray et al. 1991). Melting experiments employing the single-stranded oligonucleotides demonstrated that increases in temperature result in a highly cooperative increase in absorbance, indicative of the large degree of base unstacking found in hydrogen-bonded structures upon melting. At neutral pH, 45 °C was the temperature at the midpoint for transition (t_m) of the GT

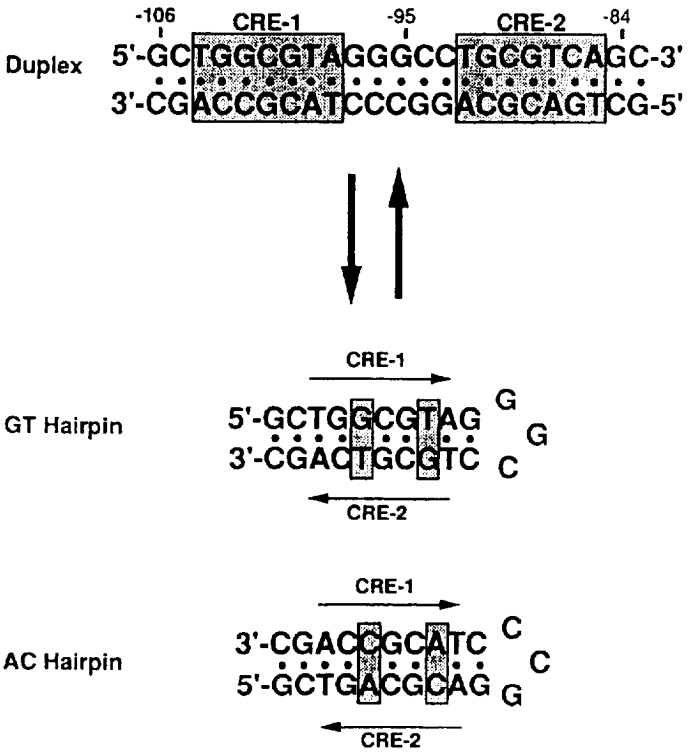

FIGURE 4. *Potential secondary structures formed by the human enkephalin enhancer region. For the duplex structure, the position of nucleotides relative to the endogenous CAP site are noted. The shaded boxed sequences represent elements CRE-1 and CRE-2, which constitute the cAMP-inducible enhancer. For the GT and AC hairpin structures, the shaded boxes indicate the positions of the mismatched base pairs.*

SOURCE: McMurray et al. 1991. Hairpin formation within the enhancer region of the human enkephalin gene. Copyright 1991 by Cynthia T. McMurray (Rochester, MN).

strand, 49 °C for the AC strand, and 63 °C for the duplex. In addition, the hyperchromicity for the melting of each strand was roughly half the value for the melting of the duplex. Thus, in the absence of their partner strand, the individual strands are capable of forming stable hairpin structures, despite the fact that each hairpin forms with two mismatched base pairs.

To determine the possibility of induction of structural transitions within the human enkephalin enhancer, the conformational state of the native enhancer duplex has been examined under a variety of conditions. A G-50 column chromatography/nondenaturing PAGE system has served as the means of analysis of column-purified, radiolabeled oligonucleotides (McMurray et al. 1991), as shown in figure 5. At a solution pH of 7.0, the double-stranded enhancer duplex (first lane noted as D) is easily distinguished from the single-stranded forms (the AC hairpin is sample AC; the GT hairpin is sample GT) by its greatly reduced mobility. In addition, the single-strand forms are observed as doublets, with the faster migrating form corresponding to the hairpin conformation and the slower migrating form corresponding to the linear strand conformation (C.T. McMurray, unpublished results). In solution, the duplex form of the 23bp human enkephalin enhancer is able to dissociate into the AC and GT single-strand forms by altering the pH; lane E represents radiolabeled enhancer duplex in pH 5.5 buffer analyzed on a pH 7.0 polyacrylamide gel. Under conditions in which the solution pH is raised to 7.0, the oligonucleotides once again migrate as a double-stranded duplex form, as shown in sample lane D. This pH-dependent conformation/migration pattern is presumably due to the ability to switch from double-stranded duplex to single-stranded hairpin conformations, as a nonpalindromic 23mer continues to migrate as a duplex under pH 5.5 conditions (sample lane C). Thus, incubation of the enkephalin enhancer oligonucleotide duplex in pH 5.5 buffer results in complete conversion of the duplex form to the single-strand forms. Furthermore, the process is reversible, as the single-stranded forms can convert to the duplex form by adjusting the solution pH to 7.0. It is also noteworthy that the lowest pH condition employed in the study is well above that where significant base protonation generally occurs and is well within the pH range where nucleic acid duplexes are stable.

To further examine the nature of this pH effect, the stability of the enhancer duplex, as well as both of the individual strands that constitute the duplex, was measured as a function of pH (figure 6) (McMurray et al. 1991). Stability was determined by observing the midpoint of the thermal transition (t_m) for each conformation. For the native enhancer duplex (open squares), thermal stability varies only 5 °C over the pH range from 5.5 to 9.0. The duplex exhibits maximal stability in the pH range from 7.0 to 9.0. The GT hairpin (▲) is also quite stable over the same pH range; from pH 5.0 to 9.0 only a 5 °C change in t_m is observed. In marked contrast, however, the stability of the AC hairpin (●) displays the opposite pH-dependent stability profile relative to both the duplex and the GT strand. The AC strand undergoes a 30 °C increase in stability as the pH decreases from 9.0 to 5.5. At pH 9.0, conditions under which both the duplex and the GT strand are most stable, the AC strand is essentially denatured. Between pH 6.0 and 5.5, the stability of the AC hairpin is actually

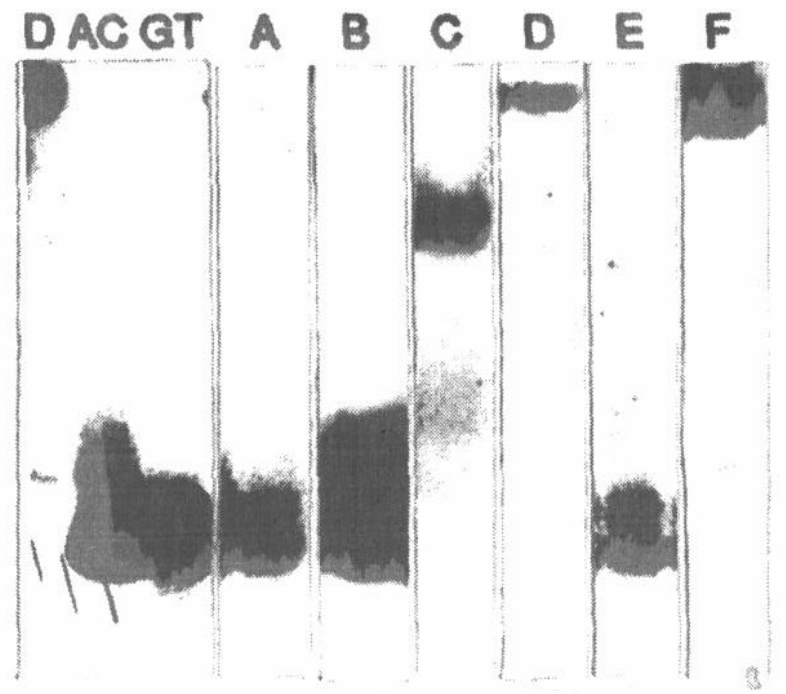

FIGURE 5. *PAGE analysis of G-50 column fractions for the GT and AC strand, a nonpalindromic 23mer duplex, and the human enkephalin enhancer duplex. In the left three lares are standards representing the duplex (D), the AC hairpin (AC). and the GT hairpin (GT). Lane A, GT strand in pH 5.5 buffer analyzed on a pH 7.0 gel ($1x10^{-8}$M base pairs); lane B, AC strand in pH 5.5 buffer analyzed on a pH 7.0 gel ($1.5x10^{-8}$M base pairs); lane C, a nonpalindromic 23mer duplex in pH 5.5 buffer analyzed on a pH 5.5 gel ($1.3x10^{-8}$M base pairs); lane D, the human enkephalin enhancer duplex in pH 7.0 buffer analyzed on a pH 7.0 gel ($1.8x10^{-8}$M base pairs); lane E, the human enkephnlin enhancer duplex in pH 5.5 buffer analyzed on a pH 7.0 gel ($1.8x10^{-8}$M base pairs): lane F, the human enkephalin enhancer duplex in pH 5.5 buffer analyzed on a pH 5.5 gel ($1.8x10^{-4}$M base pairs).*

SOURCE: McMurray et al. 1991. Hairpin formation within the enhancer region of the human enkephalin gene. Copyright 1991 by Cynthia T. McMurray (Rochester, MN).

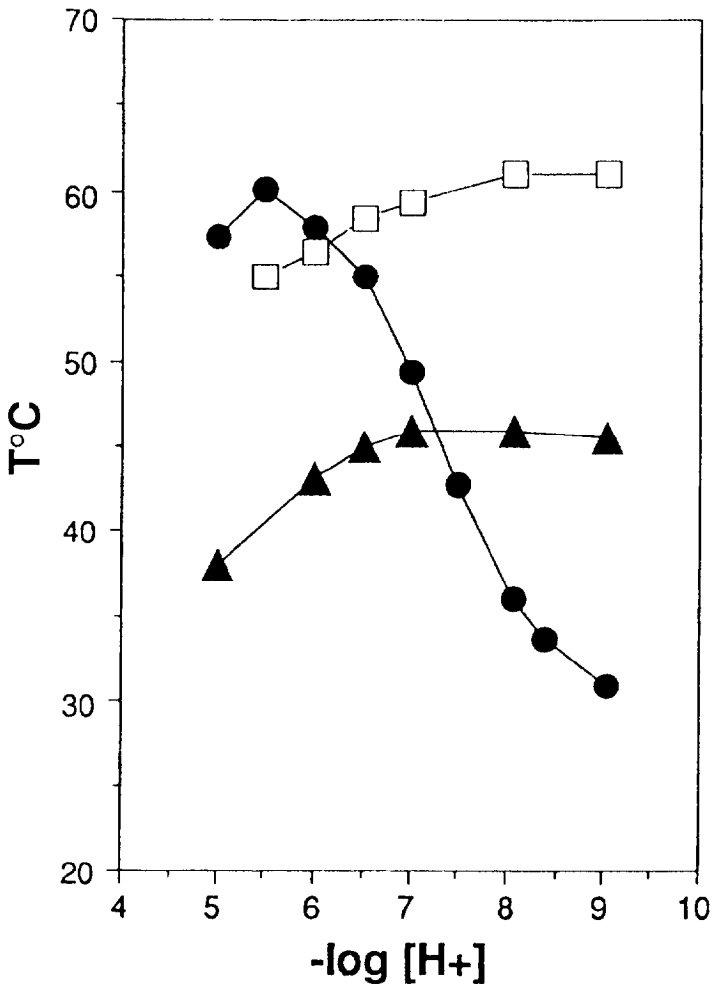

FIGURE 6. *pH dependence of melting transitions for the human enkephalin enhancer duplex (□), the GT strand (▲), and the AC strand (●). T°C represents the temperature at the midpoint for transition.*

SOURCE: McMurray et al. 1991. Hairpin formation within the enhancer region of the human enkephalin gene. Copyright 1991 by Cynthia T. McMurray (Rochester, MN).

greater than that of the double-stranded duplex. Thus, under slightly acidic conditions, the energy difference between the duplex and both of the hairpin forms is small, and under low concentration conditions (data not shown), the hairpin conformation becomes the favored form.

The dramatic pH-dependent increase in stability of the AC strand arises in part from protonation (figure 7), with additional hydrogen bonding presumably occurring from protonation of the N1 of adenine within the AC mismatched pair. From the pH titration curve shown in figure 6, the midpoint for protonation of the AC strand is near physiological pH of 7.2. The importance of measuring the pK_a is that it allows identification of the relevant range of protonation. The AC strand is approximately 80 percent protonated at pH 6.6 and 20 percent protonated at pH 7.8. Thus, changes of only 0.5 of a pH unit create conditions under which the AC hairpin is either largely destabilized or highly stable. A corresponding pK_a shift of this magnitude for protonation of ring nitrogens could be easily mediated by the presence of a nearby charged group, such as local interaction of a charged protein containing regions of acidic or phosphorylated residues. In vivo, the presence of a nearby positive charge may be the driving force to induce the formation of a cruciform structure at this specific region of the enkephalin enhancer. Thus, proton transfer and stabilization of the AC hairpin, at the expense of duplex formation, may be a biological switch for the formation of a cruciform structure.

Whether this transition has subsequent effects on the binding of additional *trans*-acting factors or polymerase subunits that control transcription from the proenkephalin promoter remains to be determined. There is, however, an intriguing piece of information that suggests the functional significance of the ability to form stable cruciform structures. Extensive site-specific mutational analysis of the human enkephalin enhancer region (Comb et al. 1988) has identified two mutations within ENKCRE-1 and ENKCRE-2 that stimulate approximately twofold basal levels of transcription from the promoter, as well as retaining a high degree of inducibility by cAMP. For one of these mutations, specific patterns of protein binding have been characterized and appear to be unaffected. Interestingly, both mutations serve to make perfect base pairs out of the mismatched pairs. Thus, mutations that potentially stabilize the cruciform structure, while having no apparent effect on specific DNA-protein interactions, result in increased expression in vivo.

It is also noteworthy that other cAMP-responsive genes contain CREs within or near sequences capable of forming imperfect palindromes (figure 8) (McMurray et al. 1991). Furthermore, many of these regulatory regions have the potential to form cruciform structures, with AC or GT mismatches representing the major

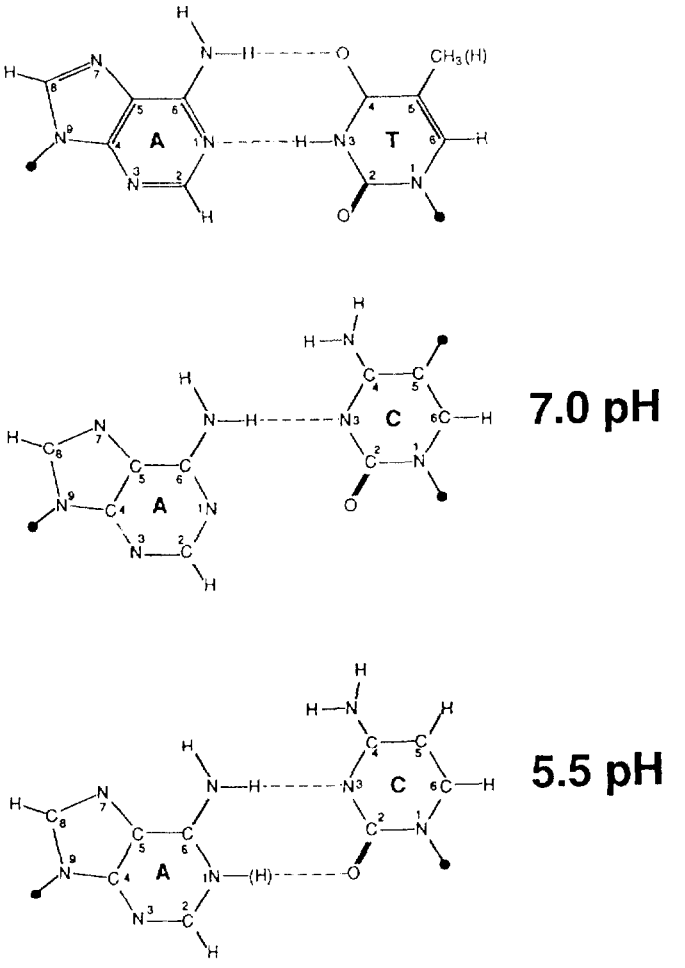

FIGURE 7. *Schematic diagram of the hydrogen binding pattern of an AT base pair and an AC mismatch at pH 7.0 and 5.5. The AC mismatch pair contains one hydrogen bond at pH 7.0 and binds an additional proton under acidic conditions. Protonation can occur at the N1 of adenine, giving rise to an additional hydrogen bond.*

SOURCE: McMurray et al. 1991. Hairpin formation within the enhancer region of the human enkephalin gene. Copyright 1991 by Cynthia T. McMurray (Rochester, MN)

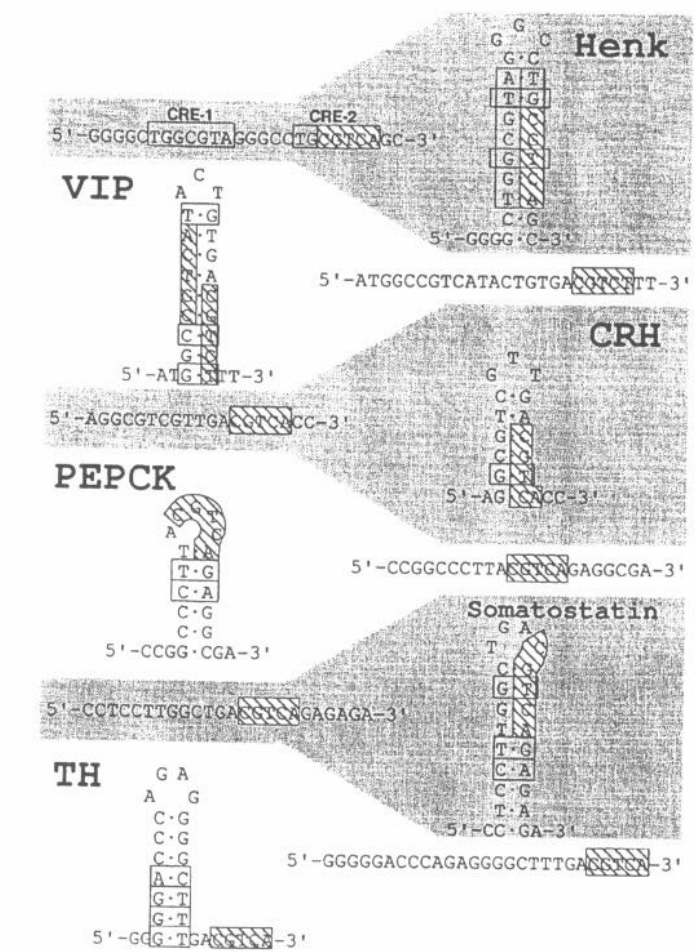

FIGURE 8. *Model for cruciform structures that can potentially form at or near the CREs of several genes. Both linear and potential cruciform structures are shown for the top strand only. Hatched boxes represent characterized CREs for each gene. In the cruciform conformation, the horizontal boxes indicate the position of base pair mismatches. Henk, human enkephalin gene; VIP, human vasoactive intestinal peptide gene; CRH, rat corticotropin-releasing hormone gene; PEPCK, rat phosphoenolpyruvate carboxykinase gene; TH, rat tyrosine hydroxylase gene.*

SOURCE: McMurray et al. 1991. Hairpin formation within the enhancer region of the human enkephalin gene. Copyright 1991 by Cynthia T. McMurray (Rochester, MN).

species of mismatch base pairing. Thus, formation of cruciform structures may be a general feature of certain classes of CREs. This observation, along with the unique physical and thermodynamic properties of the enkephalin enhancer region, suggest a model in which protein-mediated structural changes within the enhancer region DNA play an active role in regulated expression of the human proenkephalin gene via the formation of a cruciform structure. The main challenge for the future will be to determine if such a cruciform structure is capable of forming in vivo when the enhancer region is placed in the context of nuclear DNA.

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The Prohormone and Proprotein Processing Enzymes PC1 and PC2: Structure, Selective Cleavage of Mouse POMC and Human Renin at Pairs of Basic Residues, Cellular Expression, Tissue Distribution, and mRNA Regulation

Nabil G. Seidah, Robert Day, Suzanne Benjannet, Normand Rondeau, Alain Boudreault, Timothy Reudelhuber, Martin K.-H. Schafer, Stanley J. Watson, and Michel Chrétien

INTRODUCTION

Limited proteolysis of precursors at specific pairs of basic residues and/or at single basic amino acids is a widespread mechanism by which the cell expresses a repertoire of biologically active proteins and peptides (Lazure et al. 1983; Mains et al. 1990). Until recently, the tissue-specific and developmentally regulated proteinases responsible for such conversions were not identified at the molecular level. The cloning and cellular expression of the yeast Kex2 gene product demonstrated that this enzyme belongs to the Ca^{2+}-dependent subtilisin family of serine proteinases (Julius et al. 1984; Fuller et al. 1989a) and that it exhibits exquisite selectivity of cleavage at pairs of basic residues in several yeast (Fuller et al. 1988) and mammalian (Bathurst et al. 1987; Thomas et al. 1988; Foster et al. 1991) precursors. Furthermore, the ability of a yeast enzyme to cleave mammalian precursors both in vitro and in vivo reinforced the hypothesis that Kex2 represents a prototype for a mammalian subtilisin-like proteinase(s) physiologically responsible for the cleavage of proproteins at selected pairs of basic residues. The search for the homologous mammalian convertases led to the recent identification and molecular cloning of three distinct proteins. These were called furin (Roebroek et al. 1986; Fuller et al. 1989b; Van den Ouweland et al. 1990), PC1 (Seidah et al. 1990, 1991a; Smeekens et al. 1991, who also called this enzyme PC3), and PC2 (Smeekens and Steiner 1990; Seidah et al. 1990).

CHROMOSOMAL ASSIGNMENT

The chromosomal localization of the genes coding for the convertases PC1, PC2, and furin has been achieved by in situ hybridization of both human and mouse metaphase spreads (Seidah et al. 1991a, 1991b). The data showed that in both species these three genes are not synthenic. Thus PC1, PC2, and furin were located on human chromosomes 5q15-21, 20p11.1-11.2, and 15q25-26 and on mouse chromosomes 13 [C1-C3 band], 2 [F3-H2 region], and 7 [D1-E2 region], respectively. Genetic linkage analysis of each gene in the mouse demonstrated that PC1, PC2, and furin loci map close to the G protein Nras-2, homeobox Pax-1, and fes/fps oncogene Fes (Copeland et al., in press).

STRUCTURAL ANALYSIS

The comparative architectural features of the subtilisin-like proprotein convertases reported so far and those of the subtilisins are presented in figure 1. It is apparent that PC1 , PC2, and Kex2 contain disulphide bridges only within their catalytic domain, whereas furin also exhibits a Cys-rich segment before the putative transmembrane domain (TMD) and two other Cys residues within the TMD and the cytosolic C-terminal segment, respectively. The glycosylation pattern in the mammalian members shows that they each contain three N-glycosylation sites, and unlike Kex2, none appears within the putative prosegment. This figure also shows in the unshaded area a segment (called P-domain) that has been reported to be important for the folding of the proteinase and the elaboration of the full enzymatic activity of Kex2 (Fuller et al. 1991). Finally, although the structures of PC1 and PC2 do not predict the presence of a transmembrane domain, their C-terminal segments code for an amphipathic structure that could interact with membranes in a pH-dependent manner, in a similar fashion to the properties of the soluble carboxypeptidase E (Fricker et al. 1990).

Figure 2 depicts the conservation of sequences around the active sites Asp*, His*, and Ser* as well as the catalytically important Asn* residues of some subtilisins and of all the members of the mammalian subtilisin family reported so far. Aside from the conserved residues in the family (boxed in), certain residues (inverted triangles) are found only in the mammalian subtilisin family, and within these, three of them (Arg, Ser, and Ala, shown in bold) are found only in the Kex2-like convertases and not in the recent structure reported for the human tripeptidyl peptidase II (hTPP II) (Tomkinson and Jonsson 1991). Finally, only in PC2 is an Asp* found instead of the usual Asn* residue. This interesting single point mutation of PC2 (but not PC1 or furin) may have an important bearing on the catalytic efficiency (k_{cat}/K_m) of this proteinase, since

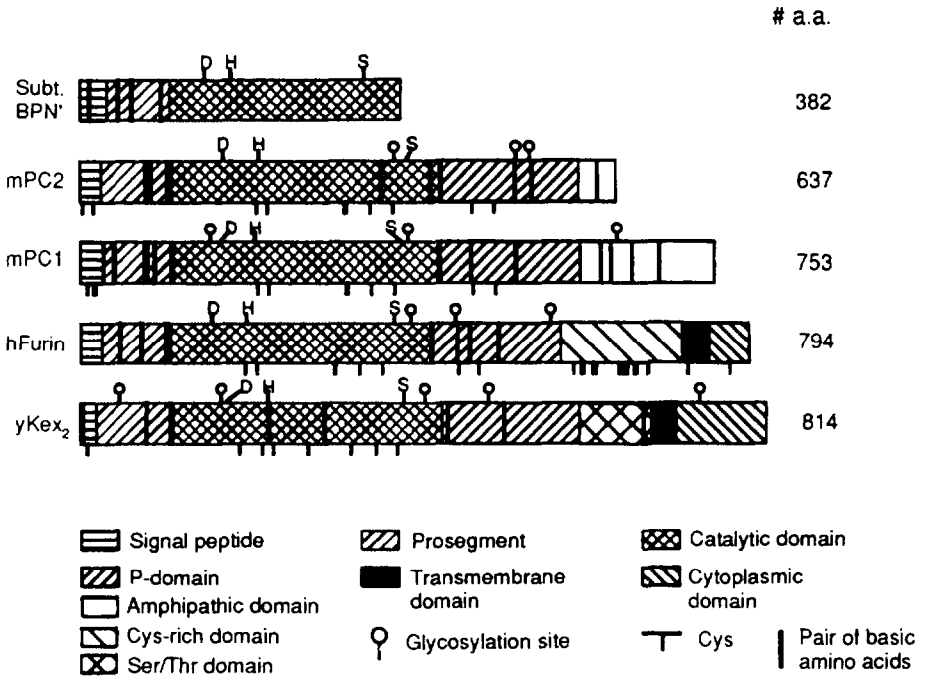
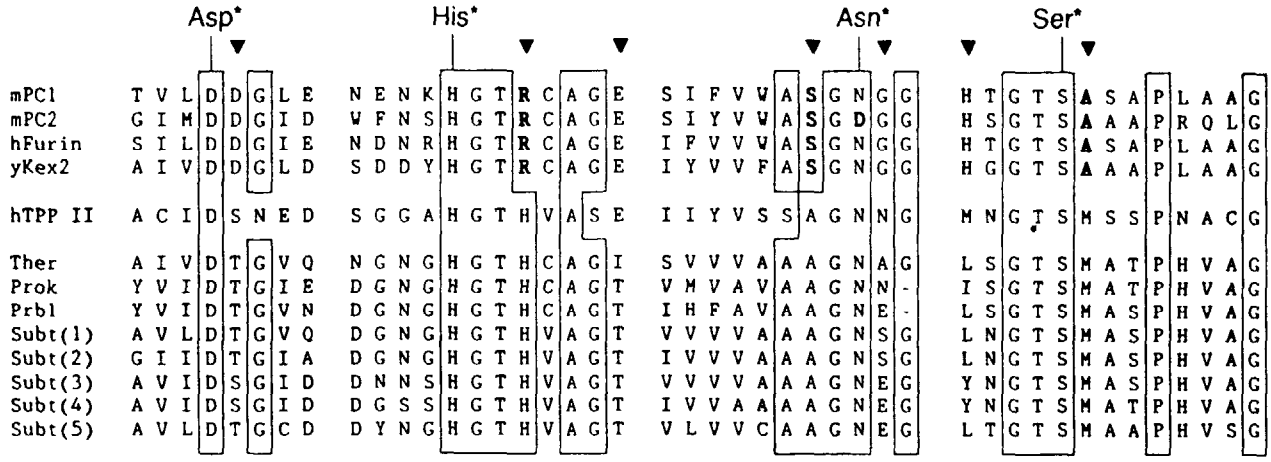


FIGURE 1. *Schematic diagram depicting the various domains of the Kex2-like enzymes and their comparison to the primitive subtilisin BPN enzyme. The number of amino acids (# a.a.) in each primary translation product is shown on the right. The diagram also points out the positions of the active sites Asp*(D), His*(H), and Ser*(S) in each enzyme.*

it has been reported that this Asn* residue forms a hydrogen bond with the developing substrate carbonyl oxygen anion in the transition state complex (Robertus et al. 1972). This interaction was shown to be important, but not essential, for the stabilization of this oxyanion hole in subtilisins (Bryan et al. 1986). From these data and the sequence similarity of the catalytic domains in these various enzymes, it becomes apparent that hTPP II is much closer to subtilisin BPN' than to any other member of the Kex2-like proteinases. This suggests a possible classification of hTPP II as a mammalian degradative enzyme, rather than a Kex2-like specific proprotein convertase, in agreement with its observed broad substrate specificity (Bålöw et al. 1986).



mPC1=mouse prohormone convertase 1; mPC2=mouse prohormone convertase 2; hFurin=human furin; yKex2=yeast Kex2; hTPP II=human tripeptidyl peptidase II; Ther=*Thermoactinomyces vulgaris* thermitase; Prok=*Tritirachium album Limber* proteinase K; Prbl=*S. cerevisiae* vacuolar proteinase B; Subt(1)=*Baccillus subtilis* subtilisin Carlsberg; Subt(2)=*B. subtilis* subtilisin DY; Subt(3)=*B. amyololiquefaciens* subtilisin BPN'; Subt(4)=*B. subtilis var. amylo-sacchariticus* subtilisin S; and Subt(5)=intracellular subtilisin from *B. subtilis* A-50.

FIGURE 2. *Conservation of the sequences around the Asp*, His*, and Ser* of the active sites found in the subtilisins (Moehle et al. 1987), Kex2 (Mizuno et al. 1988), furin (Van den Ouweland et al. 1990), mPC1 (Seidah et al. 1991a; Smeekens et al. 1991), mPC2 (Seidah et al. 1990), and hTPP II (Tomkinson and Jonsson 1991). Boxed amino acids, inverted triangles, and bold residues represent invariant residues, amino acids found only in the eukaryotic members of the family, and conserved amino acids found only in the Kex2-like members of the eukaryotic subfamily, respectively.*

CLEAVAGE SPECIFICITY OF PC1 AND PC2

To investigate the cleavage specificity of PC1 and PC2 the authors used two different precursors as substrate: mouse proopiomelanocortin (POMC) and human renin. Vaccinia virus recombinants of each enzyme were used to coexpress these proteinases with the prosubstrates in several cell lines. Following their purification, the intracellular and secreted peptide products were unambiguously characterized by microsequence.

Vaccinia virus recombinants of the mouse prohormone convertases PC1 and PC2 were coexpressed together with mouse POMC in the constitutively secreting cells, BSC-40, and in the endocrine-derived cell lines PC12 and AtT-20, which exhibit regulated secretion. Figure 3 summarizes the cleavage selectivity of PC1 and PC2 as deduced from the monitoring of the POMC processing. The data demonstrated the distinct cleavage specificities of PC1 and PC2, since in the cell lines analyzed it was found that (1) PC1 cleaves POMC into adrenocorticotropic hormone (ACTH) and ß-lipotropin; (2) PC2 cleaves POMC into biologically active ß-endorphin, an N-terminally extended ACTH containing the joining peptide, and into either α -melanocyte-stimulating hormone (MSH) or des-acetyl α-MSH; and (3) PC2 cleaves POMC at the five pairs of basic residues analyzed, whereas PC1 cleaves preferentially two of them, suggesting that PC2 has a broader spectrum of activity than PC1. These data are consistent with the proposed hypothesis concerning the physiological role of PC1 and PC2 as distinct proprotein convertases acting alone or together to produce a set of tissue-specific maturation products both in the brain and in peripheral tissues (Seidah et al. 1990, 1991a). Interestingly, since POMC processing can also occur in cells devoid of a regulated pathway of secretion, these data also show that POMC processing by either PC1 or PC2 is not dependent on the presence of secretory granules.

Recent data from our laboratory showed that neither PC1 nor PC2 can process human prorenin in a Chinese Hamster Ovary constitutively secreting cell line (data not shown). To test the hypothesis that, unlike POMC, human prorenin processing might require the presence of secretory granules, a somatomammotroph GH4-C1 cell line, which expresses a stable transfectant of human prorenin, was infected with PC1 or PC2 vaccinia virus recombinants. As shown in figure 4, the results of analysis of the secreted renin activity demonstrated that only PC1 can activate human prorenin in this cell line and that neither the endogenous furin nor the exogenous PC2 can activate this zymogen. Pulse chase analysis of the prorenin maturation product confirmed that PC1 cleaved human prorenin at the expected LysArg-zymogen activation site, thereby releasing the N-terminal 45 amino acids prosegment (not shown). These results point out that not all precursors can be processed efficiently in the

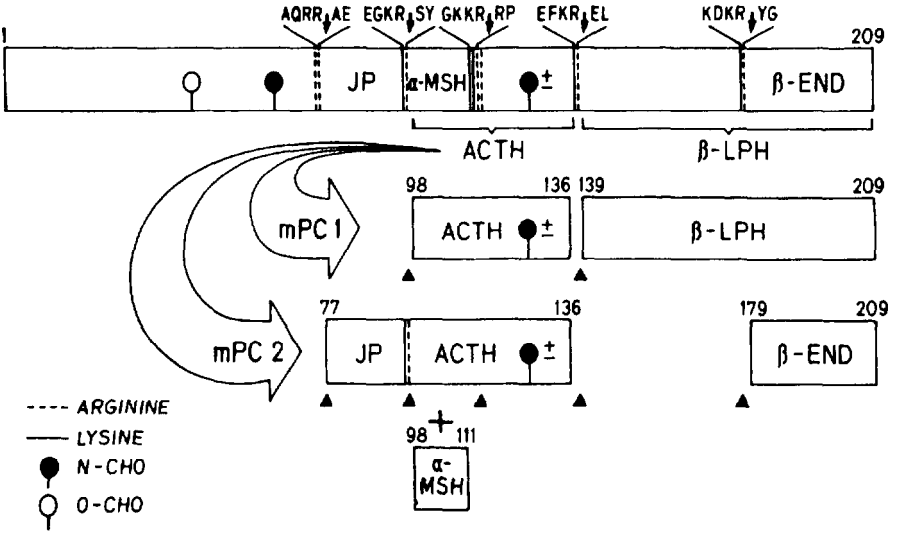


FIGURE 3. *Major end products of POMC processing by PC1 and PC2. The arrows represent the cleavage sites following pairs of basic residues. The dark triangles tally the cleavage sites by each enzyme. The numbers represent the start and end positions of the processed peptides based on the mouse POMC sequence. The N- and O-glycosylation (CHO) sites as well as the pairs of basic residues are emphasized.*

SOURCE: Benjannet et al. 1991

absence of secretory granules and that each case has to be carefully analyzed before general conclusions can be reached. Furthermore, of the three enzymes studied, only PC1 is capable of efficient processing of human prorenin. Neither PC2 nor furin (as shown by work described in this chapter and in Hatsuzawa et al. 1991) can be shown to activate this zymogen.

BACULOVIRUS EXPRESSION OF PC1 AND PC2

To study the physicochemical and kinetic properties of the convertases PC1 and PC2 in vitro, we sought to obtain large quantities of each enzyme using the baculovirus expression system. Recombinant viruses encoding mouse PC1 and PC2 were obtained using the pJV Nhe I transfer vector and by a procedure similar to that previously published (Vialard et al. 1990). Expression of each enzyme was monitored by polyacrylamide electrophoresis

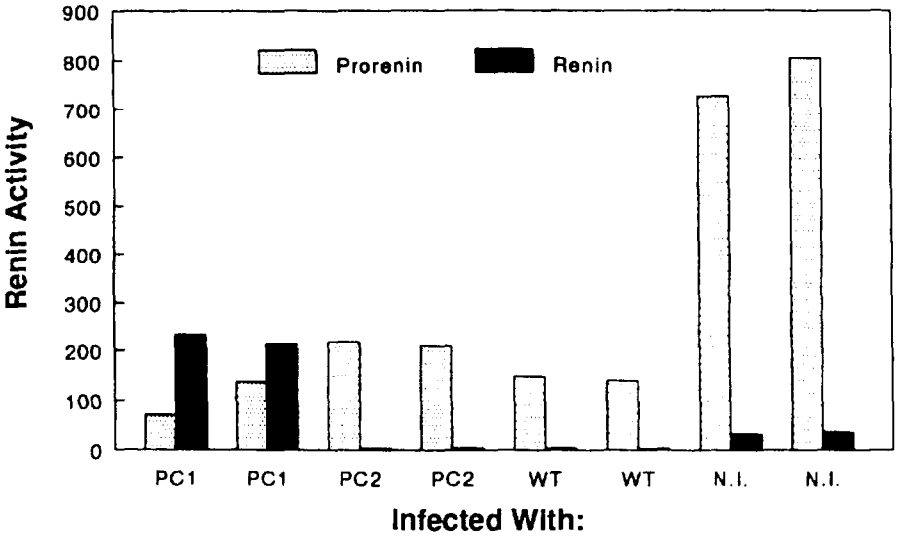


FIGURE 4. *Human prorenin activation by PC1. A stable transfectant of GH4 cells expressing human prorenin (N.I.=noninfected cells) was infected with either 5 pfu of the recombinant vaccinia virus VV:PC1, VV:PC2 or the wild type vaccinia virus VV:WT (Benjannet et al. 1991). The secreted renin activity was measured before (active renin) and after (total renin) trypsin treatment (15 µg for 1 hour at room temperature) by an indirect assay using human angiotensinogen as a substrate for active renin and a radioimmunoassay directed against angiotensin I. The amount of prorenin was calculated from the difference between the renin activity measured after and before trypsin treatment.*

on SDS/PAGE. Surprisingly, the enzymes produced were not secreted from the Sf9 cells but, rather, remained intracellular. The proteins were found to be produced in quantities exceeding 1 mg/L of culture, were associated with membrane components within the cell, and were recovered by centrifugation of the cell lysates in the particulate fraction. On SDS/PAGE, PC1 and PC2 migrate with an apparent molecular weight of 87,000 and 74,000 daltons (not shown). To investigate the enzyme activity of each proteinase, we selected to affinity label the membrane fraction containing either enzyme with a pentapeptide chloromethyl ketone ^{125}I-[D-Tyr]GluPheLysArg-$COCH_2Cl$, of which the GluPheLysArg sequence represents the ACTH/ß-lipotropic hormone junction (Cromlish et al. 1986), which was shown to be cleaved by either PC1 or PC2 (Benjannet et al. 1991). As shown in figure 5, both PC1

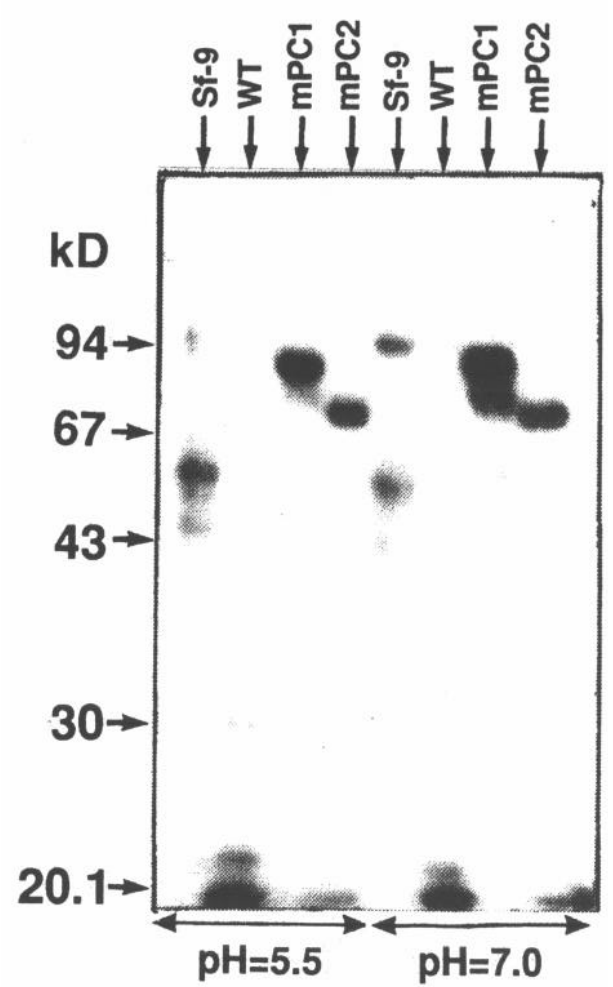


FIGURE 5. *Labeling of the cellular extract of baculovirus-expressed PC1 and PC2 with the affinity label [^{125}I-D-Tyr]GluPheLysArg-COCH$_2$Cl. The labeled proteins migrate exactly at the position of the Coomassie-colored PC1 (87 kD) and PC2 (67 kD), suggesting that these proteins are at least partially active proteinases. This labeling works both at acidic (pH 5.5) and neutral (pH 7.0) pH conditions, and no labeling is seen if the enzyme preparation is first incubated with 1 mM diisopropyl fluorophosphate.*

and PC2 could be specifically labeled with this pentapeptide, and the labeled proteins migrate with a similar molecular weight as the unlabeled proteins stained by Coomassie Blue. However, the specific radioactivity associated with the labeled proteins were found to be much inferior to that obtained with a similar amount of active trypsin or plasma kallikrein (N.G. Seidah, unpublished data). This suggested that either the proteins were synthesized mostly as inactive enzymes in the Sf9 cells or that the presumed prosegment (see figure 1) was not cleaved in this system. The NH_2-terminal sequence analysis of each protein eluted from the SDS/PAGE gel was found to be KRQFVNEWAAEIPGG and ERPVFTNHFLVELHK for mPC1 and mPC2, respectively (figure 6). Aside from confirming the predicted signal peptidase cleavage site in each proteinase (Seidah et al. 1991a), these data revealed that even though both intracellular enzymes retained their N-terminal prosegment (figure 6), they retain partial binding activity toward the pentapeptide chloromethyl ketone, which selectively labels the His* at the active site of each enzyme (Cromlish et al. 1986).

Efforts are now under way to understand the reason for the absence of zymogen activation of PC1 and PC2 in the Sf9 cells, which, under similar conditions, express the active form of the Kex2 proteinase with the concomitant removal of the prosegment (figure 5) (Germain et al. 1992). A possible explanation is that, unlike Kex2 and the subtilisins, PC1 and PC2 might not be capable of autoactivation but, rather, require the presence of another enzyme to start the zymogen activation process.

PC1 AND PC2 IN THE RAT PITUITARY AND BRAIN

In situ hybridization studies in the mouse central nervous tissues and in peripheral organs demonstrated that PC1 and PC2 transcripts are mostly found in endocrine and neuroendocrine tissues and cells (Seidah et al. 1990, 1991a). In contrast, furin was found to be widely distributed both in endocrine and in nonendocrine tissues and cells (Schalken et al. 1987). Figure 7 depicts the comparative in situ hybridization of PC1 and PC2 in the rat pituitary. PC1 transcripts are found in all endocrine cells of the intermediate lobe and in a majority of pituitary anterior lobe endocrine cells. PC2 mRNA is highly abundant in the intermediate lobe, with much smaller amounts found in the anterior lobe. A similar conclusion can also be drawn from Northern blots (figure 7), which show the relative amounts of PC1 and PC2 in both the anterior and the neurointermediate lobes of the pituitary. In the rat, two transcripts are found for both enzymes at about 3 and 5 kb (figure 7), as previously reported in mouse PC1 and PC2 (Seidah et al. 1990, 1991a; Smeekens et al. 1991) and human PC2 (Smeekens and Steiner 1990). The difference between these two mRNAs is not yet fully understood, but preliminary data indicate that the longer forms (5 kb) do not contain an extended coding region for each enzyme (N.G. Seidah, unpublished data).

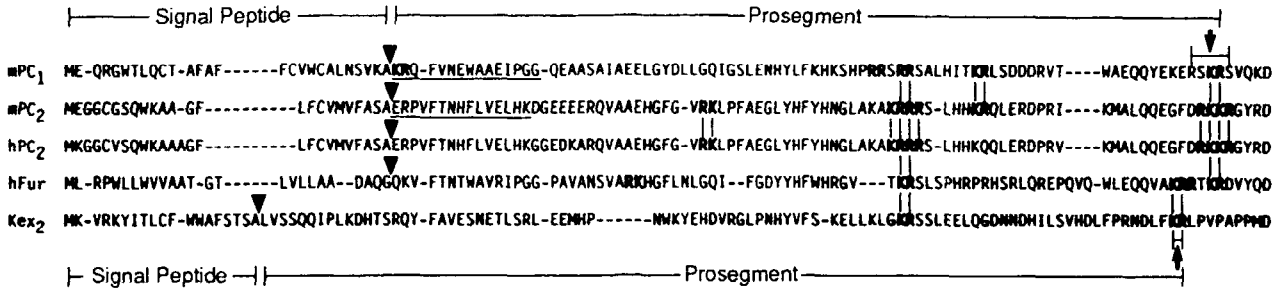


FIGURE 6. *Alignment of the N-terminal segments of the mammalian Kex2-like enzymes. The inverted dark triangles point to the predicted positions of the signal peptidase cleavage sites, based on von Heijne (1986) criteria and confirmed by microsequence analysis of the baculovirus-expressed forms of mPC1 and mPC2 (underlined residues). The computer alignment of the prosegments delineates two regions (shown in bold) where the presumed multibasic cleavage sites exactly align in all cases. By analogy to the position of the prosegment cleavage site in Kex2 (Wilcox and Fuller 1991), the arrows point to the probable equivalent site in the other members of the family.*

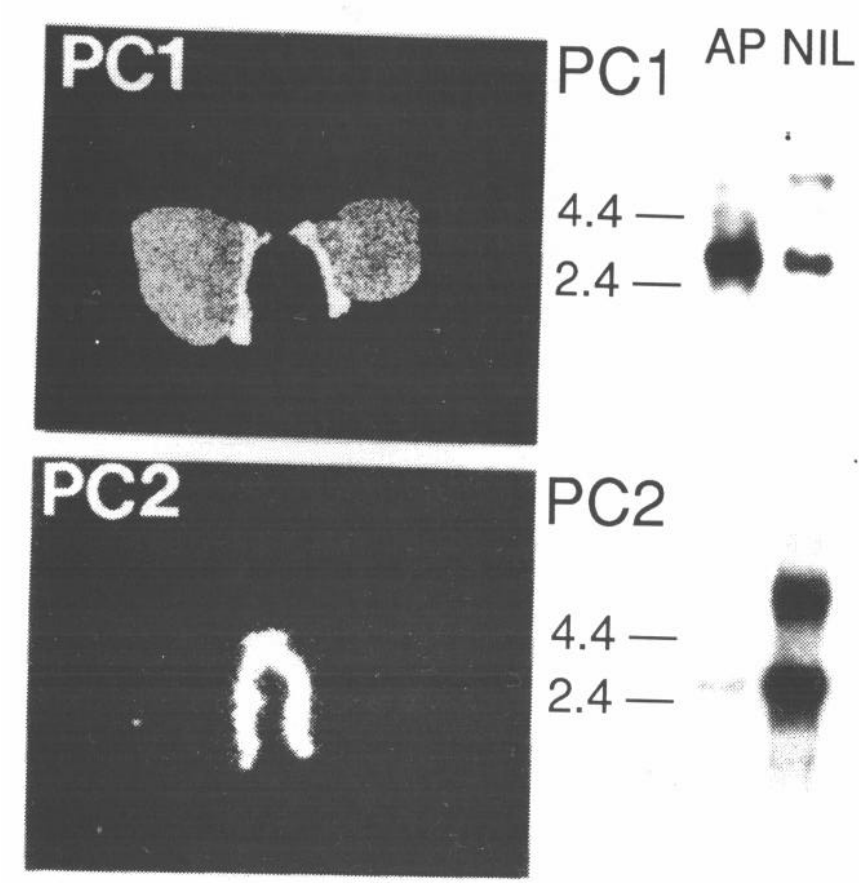


FIGURE 7. *(Left) Autoradiography of the rat pituitary distribution of PC1 and PC2 by in situ hybridization. (Right) Northern gel analysis of PC1 and PC2 mRNA transcripts found in total RNA of anterior (AP; 10 µg) and neurointermediate (NIL; 4 µg) pituitary lobes. X-ray exposures limes are 4 and 16 hours for PC2 and PC1, respectively. Markers are in kilobases.*

Figure 8 depicts a representative photomicrograph of the distribution of PC1 and PC2 mRNA in a coronal section of the rat brain by in situ hybridization. It is clear that these enzymes exhibit distinct distribution patterns in the central nervous system. In general, PC1 has a more restricted distribution, whereas PC2 is widespread. PC1 mRNA is most highly abundant in the hypothalamic nuclei such as the supraoptic, paraventricular, and suprachiasmatic nuclei.

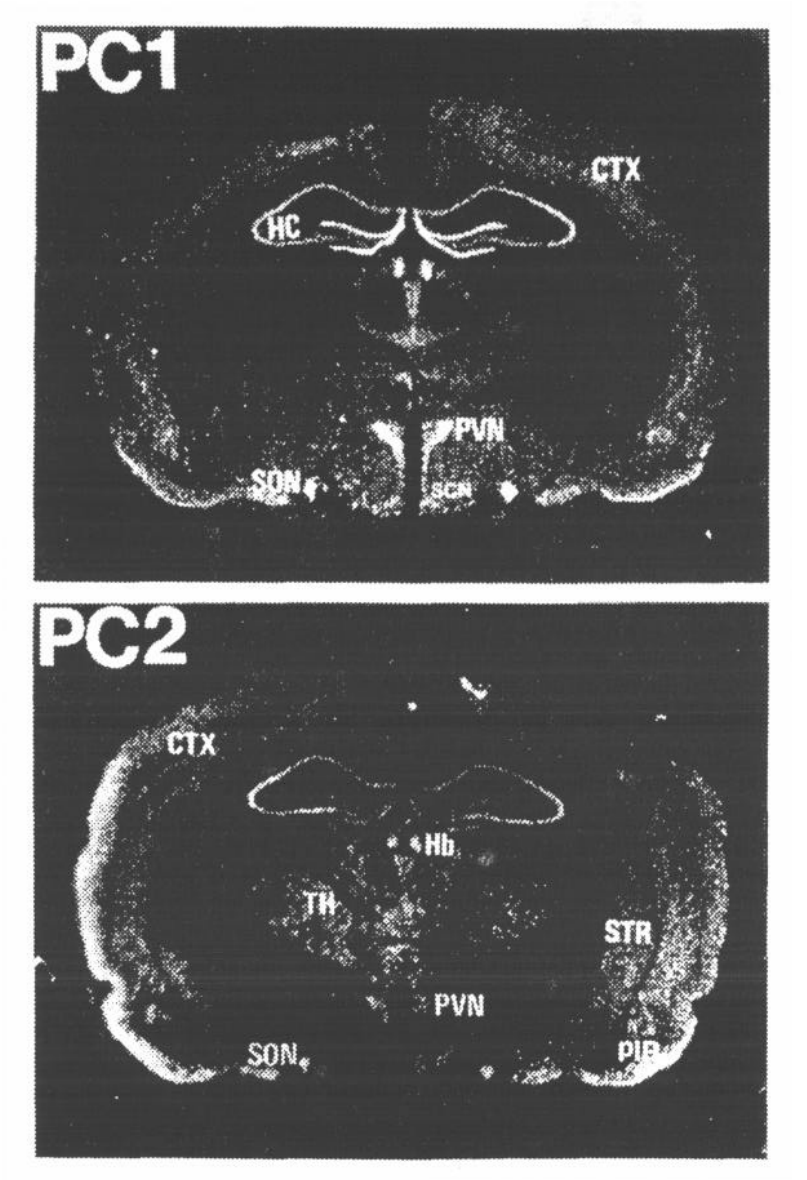


FIGURE 8. *Autoradiography of the rat brain distribution of PC1 and PC2 by in situ hybridization. CTX=cortex; HC=hippocampus; PVN=paraventricular nucleus; SON=supraoptic nucleus; SCN=suprachiasmatic nucleus; Hb=habenula; TH=thalamus; STR=striatum; Pir=piriform cortex.*

The habenula and the hippocampus represent extrahypothalamic areas rich in PC1. Although present throughout the hippocampus, PC1 is expressed with high abundance in the dentate gyrus. PC1 is also more abundant in the deeper cortical layers compared with the superficial ones. On the other hand, PC2 is widely expressed in many brain areas, the most striking example of which is the thalamus, whose numerous subnuclei all appear to express intermediate to high levels of PC2. The thalamic distribution of PC1 is much more restricted. PC2 is also moderately abundant in both the superficial and deeper cortical layers as well as in the striatum. In the hippocampus, PC2 is found mainly in the CA1, CA2, and CA3 regions and, to a much lesser extent, in the dentate gyrus. These unique localization patterns of PC1 and PC2 indicate the differential roles of these convertases in the brain and may have some significance in terms of understanding tissue-specific posttranslational processing.

DOPAMINERGIC REGULATION OF PC1 AND PC2 mRNAs IN PITUITARY PARS INTERMEDIA

PC1 and PC2 are expressed in all pituitary intermediate lobe cells (figure 7). The rat intermediate lobe melanotrophs, a highly homogeneous cell population, are highly abundant in POMC and are under inhibitory dopaminergic control. Figure 9 shows a representative experiment demonstrating the up-regulation of PC2 in the neurointermediate lobes of rats chronically treated with the dopaminergic antagonist haloperidol. Also shown is the down-regulation effect of the dopaminergic agonist bromocryptine on PC2 mRNA in the neurointermediate lobes of chronically treated rats. Parallel effects were observed for PC1 mRNA. In the present study, POMC mRNA levels also increased with haloperidol and decreased with bromocryptine treatments (data not shown), as previously reported (Chen et al. 1983). Accordingly, in intermediate lobe melanotrophs, the authors' data demonstrate the coregulation of the gene expression of POMC with that of PC1 and PC2.

CONCLUSION

The data presented in this chapter show that PC1 and PC2 are distinct mammalian subtilisin-like proteinases that originate from two different genes and that are expressed in neuronal and endocrine tissues in a discrete fashion. Even though both enzymes cleave POMC at specific pairs of basic residues, only PC1 can activate human prorenin in cells containing secretory granules. In the brain, PC2 is generally more abundant and more widely expressed than PC1. In the pituitary, PC2 mRNA is more abundant than PC1 in the intermediate lobe, whereas the reverse is true in the anterior lobe. Similar to POMC, in the intermediate lobe of the pituitary both enzymes are under

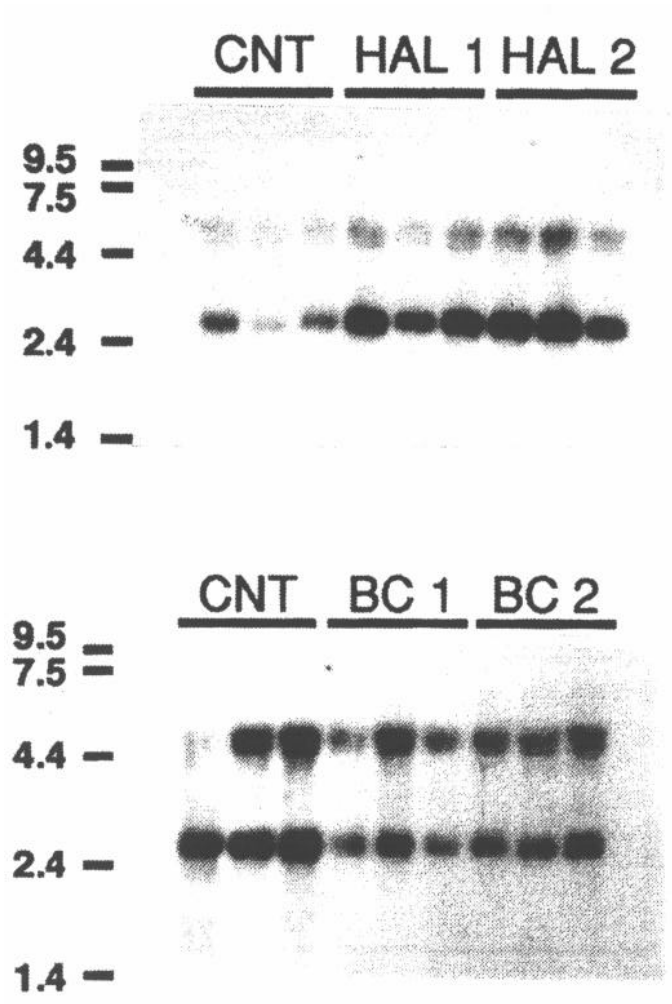


FIGURE 9. *Northern gel analysis of total RNA (2 µg per lane) of neurointermediate lobes of rats chronically treated with the dopaminergic antagonist haloperidol (CNT=vehicle; HAL 1=5.0 mg/kg; HAL 2=10 mg/kg) and the dopaminergic agonist bromoctyptine (CNT=vehicle; BC 1=2.0 mg/kg; BC 2=10 mg/kg). X-ray exposure times are 4 and 16 hours for PC2 and PC1, respectively. Markers are in kilobases.*

negative dopaminergic control. The data from the baculovirus-expressed enzymes suggest that the zymogen form of PC1 and PC2 is only partially active and that this form is not secretable. Recent pulse chase experiments demonstrated that the intracellular activation of PC1 is more efficient than that of PC2 and that neither enzyme can undergo autocatalytic activation (Benjannet et al. 1992). Therefore, the zymogen activation of PC1 and PC2 may require the presence of yet another enzyme, which together with PC1 and PC2 will fashion in a tissue-specific manner the final molecular products generated from a given proprotein.

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