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Table of Contents

Introduction: Molecular Approaches to Drug Abuse Research

Theresa N.H. Lee

The Extramural Molecular Biology and Genetics Program was launched in 1988 in the Division of Basic Research of the National Institute on Drug Abuse (NIDA) to encourage and support investigator-initiated basic research to employ molecular approaches to drug abuse research on all abused substances. In the past 6 years, the program has reached many milestones and achieved numerous successes. The progress made prior to 1991 was best documented in NIDA Research Monograph Series volumes 111 and 126 based on the proceedings of the first and second conferences on "Molecular Approaches to Drug Abuse Research" held in 1989 and 1991, respectively. The third conference on "Molecular Approaches to Drug Abuse Research" was held on March 22 and 23, 1994, at the Bethesda, MD, campus of the National Institutes of Health. This conference intended to capture the recent major advances in this field and to look at the future directions of the program.

The Molecular Biology and Genetics Program has indeed come a long way since 1988. NIDA not only has persuaded an unprecedented number of outstanding scientists to join the extramural scientific community engaging in drug abuse research, but recently celebrated the molecular cloning of the opioid receptors that had eluded numerous dedicated drug abuse researchers for more than 15 years. With the completion of the tedious task of gene cloning of all the pertinent receptors, transporters, channels, and regulatory proteins, a new era has begun. Using these powerful tools, researchers can now employ approaches previously unimaginable and concentrate their efforts and resources on elucidating the basic mechanism of each abused substance as well as the molecular and cellular mechanisms underlying tolerance, dependence, and withdrawal to generate better strategies for effective treatment, education, and prevention.

This conference was organized into three technical sessions: transgenic/ knockout animal models and other genetic approaches, studies on three families of transporters, and a whole session devoted to the rapidly progressing field of opioid receptors. Thirteen accomplished scientists presented their cutting-edge research, which

included many of the major breakthroughs in the field. Due to the ceiling on the number of speakers to invite for the conference, only one example from each category or superfamily could be included rather than encompassing all the recent accomplishments. The proceedings of this conference are presented in the following chapters of this monograph. By the time this monograph is published, virtually all the speakers at this conference will be members of the NIDA extramural scientific community.

Under the able leadership of Dr. James Patrick, significant consensus among the speakers has also been reached during the discussion session of this conference. Some of the highlights are: (1) refining tools for producing transgenic/knockout animals, especially conditional or tissue-specific knockouts; (2) developing effective expression systems for drug receptors; (3) expanding research on the polymorphism and diversity of drug receptors, transporters, and other relevant proteins; (4) developing clinically useful, safe opioid drugs employing the knowledge obtained from recent opioid receptor advances; and (5) using proper animal models to systematically screen nongenetic factors for clues leading to treatment of addiction.

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Immunological Approaches to Nicotine Receptors

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Nicotine is a drug whose abuse results in approximately 400,000 deaths per year in the United States alone (Surgeon General 1988). It is by many accounts the most addictive drug available without prescription; nearly 40 percent of those currently addicted have tried to stop using the drug (Schellingt 1992). Although many are successful in overcoming their dependence, 80 percent of those who stop using return to the drug within 24 months (Schellingt 1992). New drug replacement protocols are increasing the success rate, but pharmacokinetic problems of dosage, route, and side effects remain. Clearly, new drugs and new protocols that would help overcome addiction to nicotine would be valuable and might reduce the loss of life that results from its use.

Drugs of abuse such as nicotine exert both short-term and long-term effects through their interactions with specific molecular targets such as receptors, ion channels, and transporters. A powerful approach to the study of these drugs has been the identification and study of their specific binding sites and mechanisms of action. This approach has the straight-forward and valid rationale that if one understands the mechanism of action of a drug one can eventually understand and reduce its potential for abuse. In addition, if one understands the binding site one can perhaps design drugs with great receptor specificity and lower abuse potential, which could be used to break dependence.

Early biochemical data supported this idea that the sites of action of drugs were homogeneous. However, the idea of a single class of sites for each drug essentially precluded the idea of designing ligands that might distin-guish between binding sites associated with abuse and binding sites for the same drug that might not be associated with abuse. Although the apparent homogeneity of binding sites seemingly facilitated biochemical and binding studies, it moved the authors' explanations of drug action from the drug and its interactions with its receptor to some more complex, interactive, but ill-defined aspect of the nervous system. The potential for critical analysis of drug function was essentially limited to those few cases in which the drug-binding sites were restricted to one or a few specific

brain nuclei or cell types. Only under these conditions could one associate the actions of a drug with the function of specific central nervous system (CNS) nuclei.

It is now known that the diversity of drug/receptor interactions far exceeds scientists' earlier estimates. The diversity is found at two levels. First, binding sites previously thought to be homogeneous are now known to be heterogenous (for reviews see Hollenberg 1991 and Scholfield et al. 1990). For example, nicotine was once thought to bind to a homogeneous class of binding sites in brain; it is now known that there are many different oligomeric receptors that bind and are activated by nicotine (for a review see Luetje et al. 1990b). Drugs that bind to gamma aminobutyric acid (GABA) receptors in brain extracts are now known to distinguish between dozens of functionally different GABA receptors (Stephenson and Duggan 1991). Second, drugs bind to specific identified receptors that were not previously thought to bear the appropriate binding sites. Curare, a drug that was once thought to bind exclusively to, and be diagnostic for, nicotinic acetylcholine receptors, is now known to have its highest affinities for serotonin (5-HT3) receptors (Andres et al. 1991). Phencyclidines are no longer thought to be specific for any receptor but appear to block a wide variety of ligand-gated ion channels (Amador and Dani 1991). Even glycine is no longer considered specific for glycine receptors, but is known to be required for activation of one class of the glutamate receptors (Johnson and Ascher 1987). Both the diversity of receptor types and the cross-modality of drug/receptor interactions is likely to become more significant as more is learned.

The consequences of drug use are also more diverse than anticipated. It was once thought that drug use produced changes in the number of specific binding sites, changes in the efficacy of the drug inactivation mechanisms, or perhaps a change in the abundance of an endogenous ligand. The diversity of drug receptors now forces a consideration of changes in the actual structure of the receptor molecules or changes in the distribution of these molecules on the surface of a neuron. One must now consider the possibility that drugs of abuse have long-term effects that result from the expression of specific genes that are activated as a consequence of the action of the drug on one of its many different receptors. Dopamine modulation of progesterone-regulated gene expression is an important example because it may tie either abuse potential or drug need to variations in normal physiological parameters such as endocrine function (Power et al. 1991a, 1991b). Finally, the potential for long-term activity-dependent modification of synaptic function and the roles these

mechanisms play both in the adult and during development is appreciated more fully. This offers potential for under-standing and perhaps ameliorating some adverse effects of drugs on the fetus during development.

There have been many elegant studies of nicotine binding sites in the CNS, both in extracts and in brain slices (Baneerjee et al. 1990; Clarke et al. 1985; Lippiello and Fernandes 1986; Marks et al. 1986; Martino-Barrows and Kellar 1987; Pabreza et al. 1991; Raja et al. 1988; Wonnacott 1987). These studies show that both high and low affinity binding sites are abundant, and that they are widely distributed in the brain. A key contribution from these studies is the observation that the number of binding sites changes with chronic exposure to nicotine (Marks and Collins 1983; Norbert et al. 1983; Schwartz and Kellar 1983). In addition, the toxin alpha-bungarotoxin, which played a major role in the impressive progress made on understanding the nicotinic acetyl-choline receptor present at the neuromuscular junction (Fambrough 1976), also binds to brain membrane preparations and to cells known to contain neuronal nicotinic acetylcholine receptors (Brown and Fumagalli 1977; Carbonetto et al. 1978; Duggan et al. 1976; Jacob and Berg 1983; Patrick and Stallcup 1977a, 1977b). Although more is known about this toxin-binding component (Couturier et al. 1990; Schoepfer et al. 1990; Seguela et al. 1992), its role on neurons is still unknown.

Antibodies against the Torpedo nicotinic acetylcholine receptor recognize proteins in the CNS and have been used to determine the distribution of the antigenic determinants in the chicken brain (Lindstrom et al. 1987). They have also been used to purify the molecules that carry the antigenic deter-minants (Whiting and Lindstrom 1986) and one subunit of these oligo-meric molecules binds the affinity labeling reagent MBTA (Whiting and Lindstrom 1987). There is also good evidence in chick ciliary ganglion neurons that at least one of the antibodies recognizes a functional receptor (Smith et al. 1986). It is also the case that the amino terminal sequence of one of the proteins purified on the basis of its immunological cross-reactivity corresponds to the sequence of a functional neuronal nicotinic receptor deduced from an expressible cDNA clone (Whiting et al. 1987).

An alternative approach to understanding nicotine function in the CNS was based on the idea that nucleic acids encoding muscle nicotinic acetyl-choline receptor subunits would hybridize, at low stringency, to nucleic acids encoding neuronal nicotinic acetylcholine

receptor subunits. This work resulted first in a clone encoding an alpha subunit expressed in the PC12 cell line (Boulter et al. 1986) and subsequently in the isolation of clones encoding nine members of the rat neuronal nicotinic acetylcholine receptor subunit gene family (Boutler et al. 1990; Deneris et al. 1987, 1988; Duvoisin et al. 1989; Goldman et al. 1987; Lamar et al. 1990; Wada et al. 1988) and a rat alpha-bungarotoxin binding component (Seguela et al. 1992). Other labs have isolated clones encoding chicken nicotinic receptor subunits (Nef et al. 1987, 1988) and two chicken alpha-bungarotoxin binding components (Couturier et al. 1990; Shoepfer et al. 1990).

Nine clones define nine genes that are included in the neuronal nicotinic acetylcholine receptor gene family on the basis of their similarities in sequence. These genes are expressed in unique but overlapping sets of anatomical loci in the brain. Using in situ hybridization the combination of neuronal nicotinic receptor genes expressed in over 500 brain loci has been determined (Miller and Patrick 1992; Wada et al. 1989, 1990).

Some but not all of these genes encode subunits that participate in the formation of oligomeric nicotine-gated ion channels. Oocyte expression studies have established four functional alpha subunits (alpha2, alpha3, alpha4, and alpha7) and two functional beta subunits (beta2 and beta4) (Boulter et al. 1987; Deneris et al. 1987; Duvoisin et al. 1989). Expression of functional nicotine-gated ion channels in Xenopus oocytes results when DNA encoding beta2 or beta4 is injected in pairwise combination with DNA encoding either alpha2, alpha3, alpha4, or when alpha7 is expressed alone. Other members of the gene family, alpha5, alpha6, alpha8, and beta3, have not yet to been shown to participate in the formation of functional ligand-gated ion channels.

Different combinations of alpha and beta subunits produce receptors with strikingly different responses to agonists and antagonists. Receptor sensitivities to agonists and antagonists are dependent upon the subunit composition of the receptor, and both the alpha and the beta subunits confer pharmacological and electrophysiological characteristics to these receptors (Boulter et al. 1987; Duvoisin et al. 1989; Luetje and Patrick 1991; Luetje et al. 1990a, 1993; Papke et al. 1989). These receptor-specific characteristics are not simply quantitative differences in agonist sensitivity but are qualitative in nature. A ligand that is an agonist on a receptor composed of one combination of subunits can be an antagonist on a receptor composed of another combination of subunits (Leutje and Patrick 1991). There

are also striking quantitative differences. Nicotine differs by about one hundredfold in its ability to activate certain receptor combinations. These ligands, therefore, provide structural backbones for chemical modifications that would enhance their ability to discriminate between receptor subtypes. In principle, the potential to make subtype-specific agonists and antagonists exists. Such drugs, coupled with knowledge of the known anatomical location of expression of individual subunits in the CNS, will provide a strong rational basis for whole-animal experiments designed to dissect out the roles that different nicotinic receptors may play in behavioral responses to nicotine.

The neuronal nicotinic receptors differ from one another and from the muscle-type nicotinic receptor in two additional ways. They have a substantial permeability to calcium ions (Mulle et al. 1992; Seguela et al. 1992; Vernino et al. 1991) and their function is regulated by calcium ions acting on the outside of the cell (Vernino et al. 1991). The hetero-oligomeric receptors comprised of both alpha and beta subunits have permeabilities to calcium that are about five times that of the muscle receptor and about one-fifth that of the N-methyl-d-aspartate (NMDA) receptor. The homooligomeric alpha7 receptor has a permeability to calcium that is greater than that of the NMDA receptor (Seguela et al. 1992). This suggests that these nicotine receptors play an important role in regulating calcium-dependent cytoplasmic processes and may contribute to activity-dependent neurotoxic cell death. Furthermore, the activation of these receptors by nicotine is regulated by external calcium over physiologically relevant concentrations (Vernino et al. 1991). This regulation by external calcium provides yet another mechanism for control of receptor function. Clearly, these studies on the pharmacology, permeability, and regulation of the various nicotine receptors provide an important foundation on which to build scientists' understanding of the cellular basis of the behavioral and addictive effects of nicotine.

Exploitation of the diversity of these receptors requires reagents to identify specific combinations of receptor subunits in the brain, tools to quantitate these receptors in specific brain loci, techniques to localize receptors on the neuronal cell surface, and mechanisms for testing specific hypotheses that might account for the nicotine-induced regulation of nicotine binding sites. The following paragraphs describe progress in expressing the subunits of neuronal nicotinic receptors in bacterial expression systems, the production of antisera, and the selection and testing of subunit-specific antibodies.

Nine cDNA clones that encode subunits of the neuronal nicotinic acetyl-choline receptors expressed in rat brain have been cloned and sequenced. These cDNA clones provided the primary structure for these proteins and produced functional receptors in single-cell expression systems such as the Xenopus oocyte. Although these clones are extremely valuable they do not, in and of themselves, readily provide access to the in vivo function of the proteins they encode. Neither do they provide biochemical quantities of protein or even biochemical access to the proteins expressed in the CNS. Therefore, they must be used in an expression system to generate reason-able quantities of protein corresponding to each of the nine cDNA clones that encode the members of this gene family. The pET expression system has several advantages (Rosenberg et al. 1987; Studier et al. 1990). It is available in three reading frames and the amount of sequence added to the expressed protein can be both known and controlled. Most important, the system is well designed to protect the inserted coding sequences from selective processes such as deletion until synthesis is initiated. The system is also known to produce large quantities of protein.

Choice of the region of the subunit to be used for antibody production is extremely important and involves several considerations. It would obviously be easier to obtain subunit-specific antibodies if nonconserved regions were used. This would also increase the likelihood of obtaining a good immune response as those sequences not conserved among subunits are generally not conserved across species. The disadvantage to this approach is that the obvious nonconserved sequences are in the cyto-plasmic domains of the receptor and are thus not accessible to the anti-bodies applied to the outside of the cell. Antibodies to this domain will not stain living cells. More importantly, antibodies to this domain cannot be used to distinguish between the receptors on the cell surface and the precursor receptors, targeted for the cell surface, which exist in vesicles inside the cell. This is because the cytoplasmic domains of the cell surface receptors and their precursors are in the same topological space (i.e., the cytoplasm). However, antibodies to extracellular domains will stain living cells and can be used to distinguish between surface and precursor receptors. The disadvantage of the extracellular domains is that they are very conserved and it is difficult to obtain subunit-specific antibodies.

Antibodies were made against the extracellular domain of each subunit and peptides were used to generate specificity as described below. This approach allows production of polyclonal antibodies

against the extra-cellular domain of the protein and affinity purification of antibodies that are specific for each subunit. In addition, it also provides tools for a quantitative mechanism for assessing both the specificity of the anti-bodies and the abundance of the antigen in intact tissue.

Figure 1 shows the regions of the subunits used as immunogens and the sequences chosen for peptide synthesis. Figure 2 shows the sequences of the peptides used to select the subunit-specific antibodies from the poly-clonal sera. It is important that the peptides used to acquire specificity be derived from homologous regions of each subunit so the peptides can be used to demonstrate nonreactivity with peptides derived from the other subunits. Peptides corresponding to this specific region of the protein were chosen for several reasons. First, it is extracellular and the specific sequence offers substantial variability between subunits. Second, it is a region known to promote antibody synthesis in the case of the muscle nicotinic receptor (Bellone et al. 1989; Tzartos et al. 1988) and antibodies directed against this sequence are known to react with the native protein (Tzartos and Lindstrom 1980). Each of the indicated constructs except alpha7 were made and checked for proper insertion and reading frame by sequencing through the ligation sites. Bacteria were transformed with the pET vector containing the indicated insert and induced to make protein. Induced bacteria were harvested and the protein purified by extraction of the inclusion bodies out of detergent solutions. The coomassie-stained gel in figure 3 shows the bands corresponding to each subunit except alpha7.

The authors' approach to generation of specific antisera by making anti-bodies first against the protein expressed in bacteria and then by acquiring specificity through affinity purification is not the usual one. Many laboratories have tried to make antibodies against peptides or to make monoclonal antibodies against bacterially expressed protein. The anti-bodies against the peptides frequently recognize the amino or carboxy-terminal sequences and thus fail to recognize the native protein. Although there are many successful applications of each of these approaches, the production of antibodies against the very similar extracellular domains of neurotransmitter receptors has been difficult. The results in figure 4 show the reaction of anti-beta2 antibody with bacterially expressed proteins and the specificity attained following purification with peptide. The affinity-purified antibody has lost its cross-reactivity to beta4 and retained activity on beta2. The authors have obtained the same results with antibodies directed against each of the other subunits.

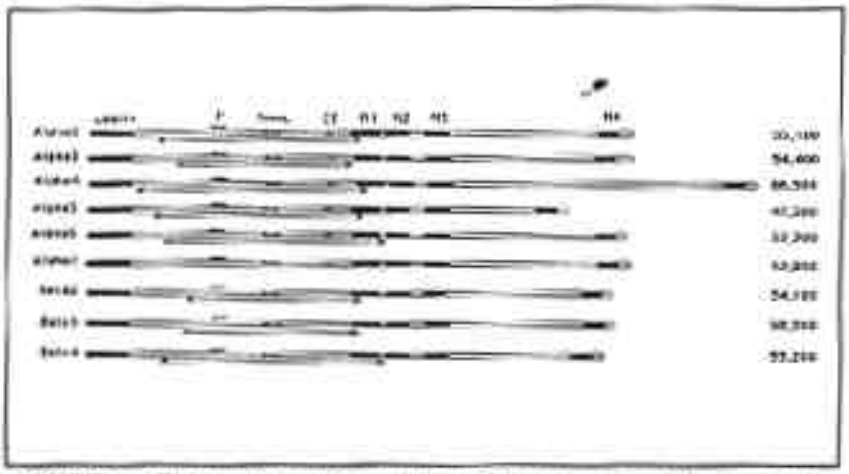

FIGURE 1. *This figure represents the coding sequence of the neuronal nicotinic receptor subunits. Characteristic regions of the proteins are indicated. The double arrow shows the region of the extracellular domain chosen for synthesis in the pET bacterial expression system and the dark bar labeled "P" indicates the region corresponding to the peptide used for purification of subunit-specific antibody.*

ALPHA2	DPAEFGNVTSLRVC
ALPHA3	KPSDYQGVEFMRVC
ALPHA4	DPGDYENVTSIRIC
ALPHA5	NPDDYGGIKIIRVC
ALPHA6	DPTEYDGIETLRVC
ALPHA7	NMSEYPGVKNVRFC
BETA2	KPEDFDNMKKVRLC
BETA3	NPEEYGGINSIKVC
BETA4	NSSCYEGVNILRIC

FIGURE 2. *Synthetic peptides corresponding to amino acids 68-81 plus C-terminal cysteine.*

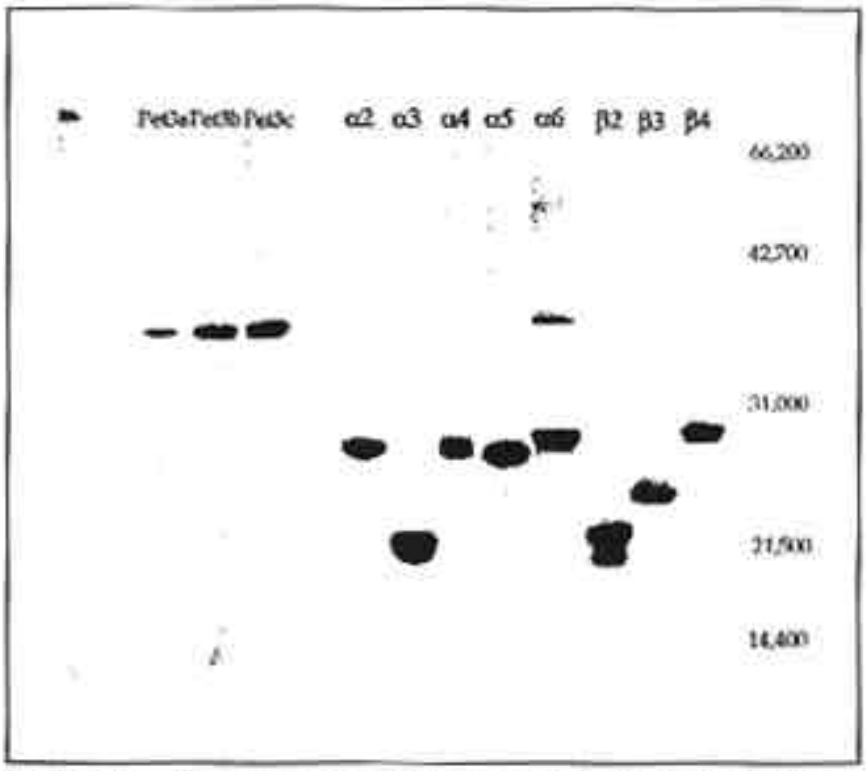

FIGURE 3. *Bacterial expression of the amino-terminal extracellular domain of neuronal nAChR's. Bacteria containing the pET vectors and constructs were grown to an $O.D._{600}$ of 0.4 and induced with 1mM IPTG for 3 hr. Bacteria were pelleted, treated with lysozyme (1 mg/mL, 20 min. on ice), sonicated, treated with DNAse I (1 mg/mL, 30 min, 37° C), and sedimented. The insoluble pellet was washed in 1x PBS containing 1 percent deoxycholate, 1 percent b-mercaptoethanol, and 1 percent Triton X-100 and resedimented. The resulting insoluble pellet was washed in the above solution and resuspended in 1x PBS. Proteins were resolved on 15 percent SDS-PAGE using 15 mg of the detergent-insoluble pellet fraction.*

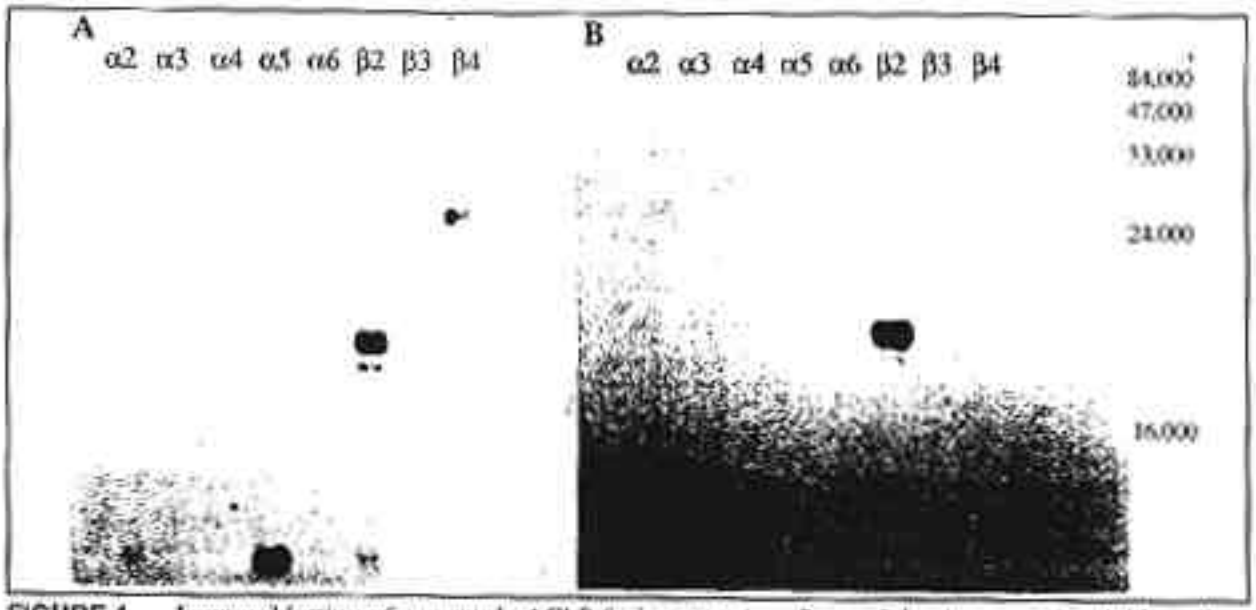

FIGURE 4. *Immunoblotting of neuronal nAChR fusion proteins. 2 mg of the detergent-insoluble pellet fraction from induced cultures of E. coli transfected with one of the eight pET constructs was dissolved in SDS PAGE sample buffer, separated by 15 percent SDS-PAGE, and transferred to nitrocellulose. Blots were reacted with (A.) antisera raised against the b2 pET construct or (B.) b2 antisera affinity purified against the b2 peptide. In order to reduce background staining of bacterial proteins and vector-derived proteins, antisera were absorbed against an acetone precipitate of E. coli cultures transfected with the appropriate parental vector and induced for vector-derived protein production.*

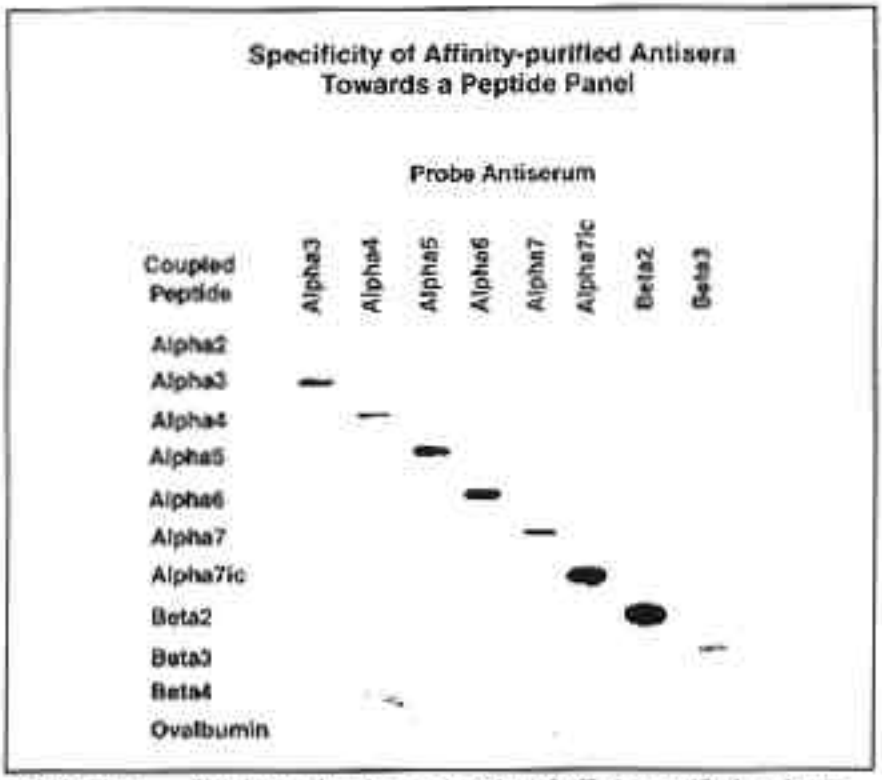

FIGURE 5. *Slot-blots showing specificity of affinity-purified antisera toward a peptide panel. Peptides corresponding to amino acid residues 68-81 of the alpha subunit of muscle nAChR were synthesized for each neuronal nAChR subunit and coupled to ovalbumin according to Yoshitake and colleagues (1979). Five ng of each peptide conjugate was slotted onto a nitrocellulose membrane and reacted with antisera purified using the appropriate synthetic peptide. Immunoreactivity was observed only for the corresponding affinity-purified antiserum.*

The authors used affinity chromatography on peptide columns to prepare a subunit-specific antibody for each of the subunits and tested the product on all of the peptides. The data in figure 5 show a slot blot of peptide coupled to ovalbumin and reacted with each of the antibodies (and with an antibody directed against the intracellular domain of alpha7). The results show reactivity with the appropriate peptide and nonreactivity with the inappropriate peptides. These results show that the affinity-purified antibodies distinguish between peptides but do not address the issue of whether they react with native

protein. These antibodies have been used to detect neuronal nicotinic receptors on the surface of transiently expressed COS cells indicating that they do recognize the native protein (Neff et al. 1995).

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The Dopamine D4 Receptor

Hubert H.M. Van Tol

INTRODUCTION

The observation that receptors which mediate their signals through heterotrimeric guanosine triphosphate (GTP)-binding proteins (G-proteins) share a considerable amount of sequence similarity has resulted in the rapid molecular characterization of this receptor superfamily. Moreover, it became clear that several receptor families consisted of more receptor subtypes than originally anticipated from pharmacological studies. The dopamine (DA) receptor family, which was originally thought to comprise two receptor types (D1 and D2), includes to date five different DA receptor genes (Bunzow et al. 1988; Dearry et al. 1990; Sokoloff et al. 1990; Sunahara et al. 1990, 1991; Tiberi et al. 1991; Van Tol et al. 1991; Zhou et al. 1990). The major difference between the D1-like receptors D1 and D5/D1B lies in their distribution, but pharmacologically and functionally these two receptors are almost indistinguishable (Sunahara et al. 1991; Tiberi et al. 1991). The D2-like receptor family includes two alternatively spliced forms of the D2 receptor (Dal Toso et al. 1989; Giros et al. 1989; Grandy et al. 1989; Monsma et al. 1989), and the D3 (Sokoloff et al. 1990) and D4 (Van Tol et al. 1991) receptors. Apart from clear differences in distribution profiles, the D2-like receptors also display pharmacological differences. The cloned D2 receptor is able to inhibit adenylyl cyclase (Albert et al. 1990), and is also able to activate several other signal transduction pathways (Elsholtz et al. 1991; Kanterman et al. 1991; Vallar et al. 1990). Moreover, the two alternatively spliced forms of the D2 receptor are not identical in their functional activity (Hayes et al. 1992; Montmayeur et al. 1993).

Because of the increased complexity of the DA receptor system, the role of this system in self-rewarding behaviors has become potentially more complex and might need a reevaluation of the roles played by the individual receptor systems. The observation that D3, D4, and D5/D1b receptors are relatively more concentrated in the limbic system than the D1 and D2 receptors suggests a role for these newly recognized receptors in the etiology of addiction. This is illustrated by the observation that dopamine agonists with higher potencies for D3 receptors seem to be relatively more effective in decreasing cocaine self-administration in rats (Caine and Koob 1993).

MOLECULAR CHARACTERIZATION OF THE D4 RECEPTOR

After the initial cloning of the D1 and D2 receptors, it was speculated that there were several other DA receptor types. These speculations were based on several observations, particularly that dopamine receptors from different tissues or brain areas displayed pharmacological and functional profiles that were not in agreement with the predominant D1 and D2 receptor types described for brain tissue (Anderson et al. 1990; Nisoli et al. 1992; Sokoloff et al. 1984).

The search for novel DA receptor subtypes was based on the presumed homology between these receptor subtypes with the D1 and D2 receptors. The search was begun by scanning several tissues and cell lines for the presence of ribonucleic acid (RNA) species that would hybridize to a D2 receptor probe encoding the putative transmembrane regions VI and VII under lower stringency conditions, but not under high stringency conditions. Using this approach, D2 positive hybridizing RNA species (but not D2 itself) were identified in mouse neuroblastoma NB41A3 and N4TG1, hamster kidney cells BHK-21, and the human neuroepithelioma SK-N-MC. In the subsequent screening of a cDNA library from the cell line SK-N-MC the author succeeded in the isolation of a partial cDNA clone (750 bp) that displayed good homology with the D2 receptor and could detect a 5.2 kb RNA species in the SK-N-MC cell line and rat brain (Van Tol et al. 1991). Screening of several human and rat brain cDNA libraries did not result in the isolation of full-length clones, although several similar partial cDNAs were isolated. However, genomic clones from human and rat were isolated that encoded the entire coding region for this putative receptor (Asghari et al. 1994; O'Malley et al. 1992; Van Tol et al. 1991) (figure 1). Pharmacological analysis established that the isolated clones were indeed novel DA receptor subtypes (see below), which were called the DA D4 receptor (Van Tol et al. 1991). Based on the homology with the D2 and D3 receptors, the entire coding sequence for the D4 receptor was identified in a 4.5 kb genomic DNA fragment.

The coding sequence is interspersed by three introns for which the donor/acceptor splice junction sites were conserved at identical positions as seen for introns in the D2 and D3 receptors. Introns 2 and 3 were positively identified by comparative analyses of the genomic clones with

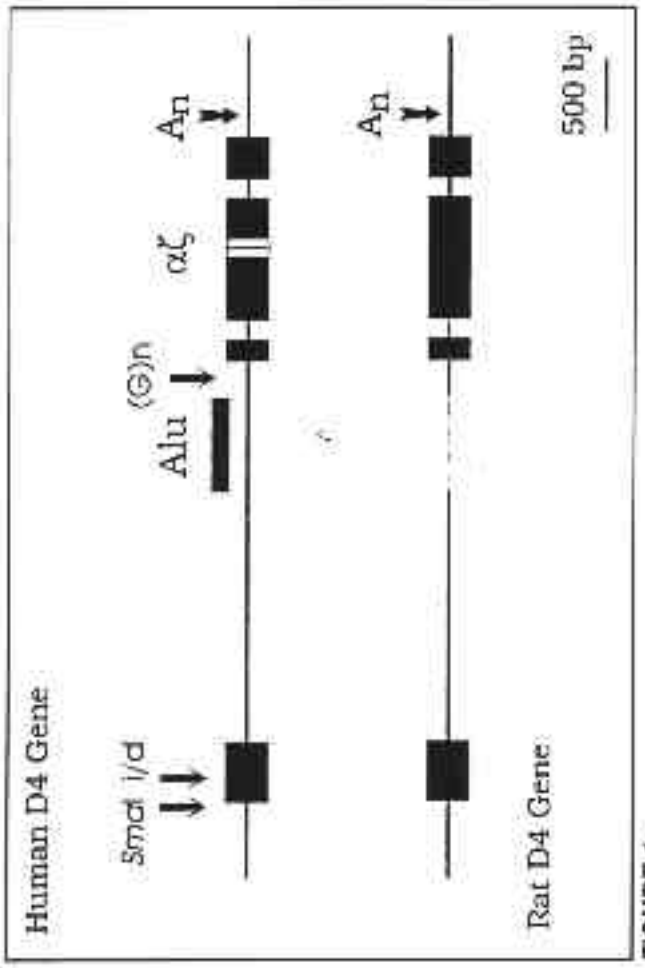

FIGURE 1.

FIGURE 1. *Genomic organization of the human D4.2 and rat D4 receptor genes. The coding regions are represented by the blocks and the noncoding regions by the connecting line. The repeat units alpha and zeta are indicated as two white boxes in the coding block. The position of the polyadenylation site is indicated with An and an arrow. An Alu sequence has been recognized within the first intron of the human gene that is not present within the rat gene. The dotted line within the first intron of the rat gene indicates where the Alu sequence is inserted in the human gene. Apart from the 48 bp repeat units in the third exon, three other polymorphic markers are indicated: (1) a polymorphic SmaI site located immediately upstream from the initiation codon (Petronis et al. 1994a, 1994b), (2) an insertion/deletion polymorphism of 4 amino acids in the first exon (Catalano et al. 1993), and (3) a polymorphic G Mononucleotide repeat within the first intron (Petronis et al. 1994a, 1994b).*

the isolated partial D4 cDNA clones. The position of all three introns was confirmed by exon trapping, a technique through which a full-length D4 cDNA clone was also isolated (Van Tol et al. 1992).

Translation of the coding sequence into amino acid sequence revealed that the isolated D4 receptor clones encode a polypeptide with seven stretches of hydrophobic amino acids that could span the membrane. These putative transmembrane spanning domains display the highest amino acid sequence identity with the putative transmembrane regions of the D2 and D3 receptors, especially transmembrane domains 3, 6, and 7,which have 60percent to 80 percent sequence similarity to the homologous domains in the D2 and D3 receptors. The overall sequence similarity between the D4 and the D2 and D3 receptors is about 40percent. The sequence similarity is least preserved in the intra- and extracellular loops and tails, which supports the notion that the transmembrane domains form the ligand-binding pocket, as has been shown for adrenergic receptors. This is also supported by the fact that an Asp residue in transmembrane 3 and two Ser residues in transmembrane5, which have been identified as crucial amino acids for catecholamine binding in several catecholaminergic receptors, are conserved in the D4 receptor (Strader et al. 1988, 1989). In analogy to the models created for other G-protein-coupled receptors, the amino terminus is located extracellularly and contains one putative N-linked glycosylation site. The carboxy terminus ends in a Cys, as is seen for D2 and D3 receptors, and can serve as a potential substrate for palmitoylation (O'Dowd et al. 1989). Within the third cytoplasmic loop of the human D4 receptor there are only a limited number of residues that potentially can be used for phosphorylation, in contrast to the rat D4 receptor where there are several more such sites (Asghari et al. 1994; O'Malley et al. 1992; Van Tol et al. 1991). An unusual structural feature of the human D4 receptor is the presence of a polymorphic 16 amino acid repeat sequence in the third cytoplasmic loop, which is not found in the isolated rat gene (see below; figure 1). The location of this repeat sequence corresponds to the location of the alternatively spliced 29 amino acid sequence of the D2 receptor.

Through the cloning of several D4 genes and cDNAs it became clear that a region within the third cytoplasmic loop of the human D4 receptor was polymorphic (Van Tol et al. 1992). Subsequent detailed analyses of this region by Southern blot, polymerase chain reaction, and sequence analysis demonstrated that the polymorphic sequence occurs as a 48 bp

tandem repeat of 2 to 10 repeat units (figures 1 and 2). The different polymorphic repeat variants of the D4 are classified according to the number of repeats D4.2 to D4.10. Thus far 18 different repeat units have been identified which display over 90 percent sequence similarity among each other, and which have been identified by different Greek letters (Lichter et al. 1993). Although the first and last repeat units are always the so-called alpha and zeta units, respectively (there is one exception: an allele has been identified in which the last unit codes for a xi unit that is identical in amino acid sequence to the zeta unit), the other units can be found in any position between the alpha and zeta units. This has resulted in the identification of 27 different alleles in over 200 analyzed chromosomes (Asghari et al. 1994; Lichter et al. 1993; Van Tol et al. 1992) (figure 2).

In Northern blot analysis of cells transfected with the human D4 gene, several D4 cDNA variants indicate that the repeat sequence is not spliced out of the D4 gene, but is part of the coding sequence. This is supported by the fact that there are no sequences in or surrounding the repeat, which could be used as splice donor and acceptor sites. Moreover, exon trapping failed to excise this sequence. Thus the 27 variant alleles would actually code for 20 different D4 receptor variants. The putative amino acid sequence for the different repeat regions demonstrate a high Pro content in this region (30 percent to 40 percent). Recently, such Pro-rich segments have been identified as potential SH3-binding domains (Ren et al. 1993; Yu et al. 1994). On the other hand, it might be postulated that the variation in the third cytoplasmic loop sequence might have consequences for the specificity and/or efficacy in signal transduction as seen for the two alternatively spliced forms of the D2 receptor. Thus far, there is only limited evidence to support this speculation (Asghari et al., in press).

PHARMACOLOGY

The most extensive pharmacological characterization of the D4 receptor has been done by transient expression of this receptor in COS-7 cells. The pharmacological characterization of receptors derived from the expression of the entire gene or cDNA in COS-7 does not show any differences, except for the fact that higher levels of expression can be obtained with the cDNA than with the gene cloned into identical expression vectors. Transient expression of the D4 receptor in COS-7

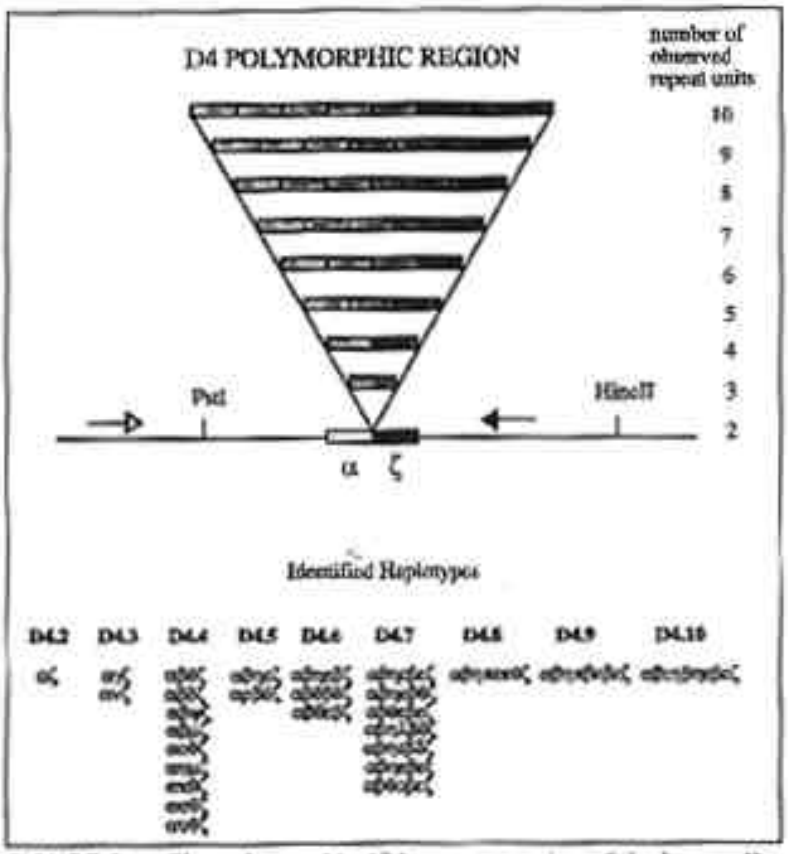

FIGURE 2. *The polymorphic 48 bp repeat region of the human D_4 receptor gene. The polymorphism can be detected by Southern blot analysis using genomic DNA digested with HincII/PstI (Van Tol et al. 1992) or by PCR analysis using specific primers (indicated by arrows) (Lichter et al. 1993). By sequence analysis of this D_4 repeat region of different individuals 18 different 48 bp repeat units have been identified (marked by different Greek letters), which can give rise to 27 different observed haplotypes (Asghari et al. 1994; Lichter et al. 1993).*

cells resulted in the detection of concentration-dependent and saturable [3H]spiperone binding with an affinity dissociation constant of approxi-mately 100 picomolars (pM). The [3H]spiperone binding could be competed with dopamine (inhibition constant (Ki) approximately 27nano-molars (nM)), but less efficiently by norepinephrine and serotonin (Ki>2micromolars (µM). Dopamine competition of [3H]spiperone binding revealed the presence of a high affinity site with a dissociation constant of 500 to 1,000 pM and a low affinity site of 10 to 50 nM. Inclusion of 200µM Gpp[NH]p in the binding buffer resulted in the conversion of the biphasic dopamine competition curves to a single affinity site of approximately 10 to 50 nM (Asghari et al. 1994; Van Tol et al. 1991, 1992). These binding data suggest functional coupling of the D4 receptor in COS-7 cells.

Dopamine D4 receptor binding characteristics have been determined for several D2 agonists and antagonists. This revealed a D4 pharmacological profile that has several similarities with the D2 receptor; however, some remarkable differences have also been detected (figure 3). Probably the most striking is the relatively high affinity of the atypical neuroleptic clozapine for the D4 receptor (Ki 10 to 20 nM) as compared to the D2 receptor (Ki 100 to 200 nM), and the poor affinity of the D2 antagonist raclopride for the D4 receptor (1 to 2 µM) (Asghari et al. 1994; Van Tol et al. 1991, 1992). Furthermore, the D4 receptor demonstrates stereo-selectivity for (+)- and (-)-butaclamol, several (+)- and (-) aporphines, (+)and (-) PHNO, (+) and (-) quinpirole, but not for (+) and (-)-sulpiride (Seeman and Van Tol 1993).

Thus far, no compound has been reported that is selective for the D4 receptor. Although clozapine seems to be tenfold more selective for the D4 receptor as compared to other dopamine receptors, the muscarinic, 5-HT2, and 5-HT7 receptors have similar affinities for clozapine as the D4 receptor. However, by making use of the differential affinities of the benzamides [3H]emonapride and [3H]raclopride for the different D2-like receptors, the density of D4-like sites in brain tissue can be indirectly determined (Seeman et al. 1993). Scatchard analysis with [3H]emonapride will detect and allow determination of the total density of all D2-like receptors (D2, D3, D4), while such analysis with [3H]raclopride will only detect and reveal the density of the D2, D3 receptor pool. Therefore, the difference in maximal density detected by both radioligands in scatchard analysis will reflect the density of a receptor pool that is D4-like.

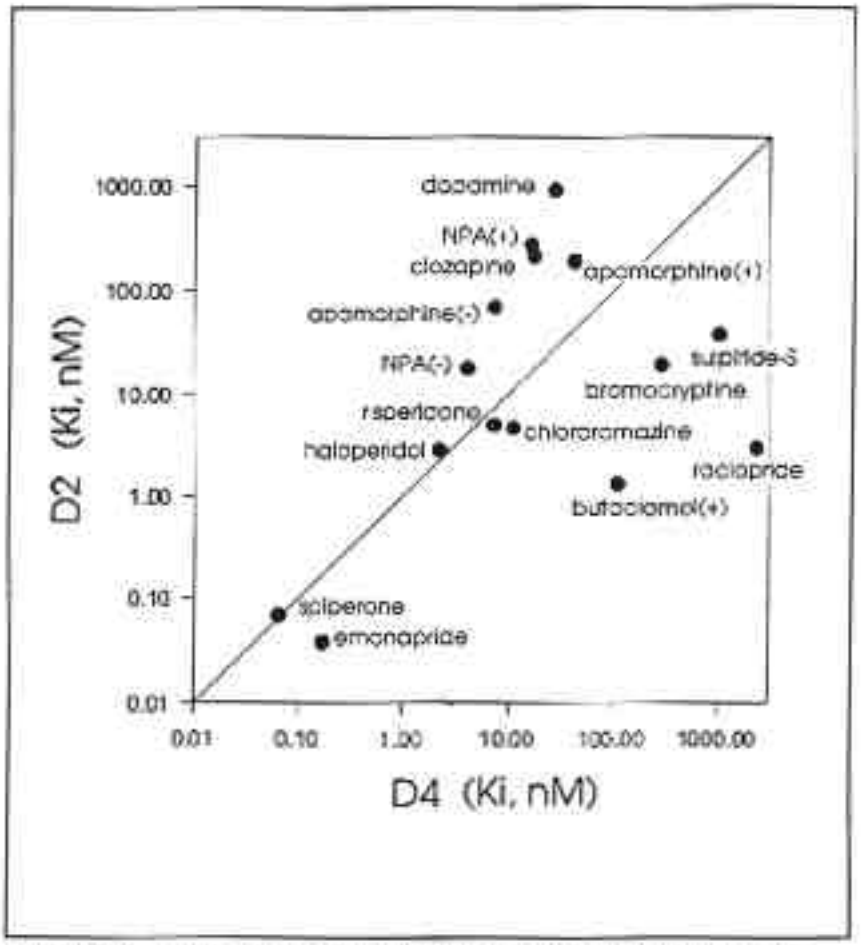

FIGURE 3. *Correlation plot for the affinities of several dopamine agonists and antagonists at the cloned D_2 and D_4 receptor expressed in COS-7 cells. The affinity dissociation constants are determined by competition analysis of [3H]spiperone binding as described previously (Van Tol et al. 1991). For reference a line indicating the position for equal affinities is drawn within the plot.*

Seven different polymorphic repeat variants of the human D_4 receptor have been characterized for their affinities to clozapine, emonapride, haloperidol, raclopride, spiperone, and dopamine (Asghari et al. 1994). Thus far, none of the tested ligands has shown any major differences in

their affinity for several D4 receptor variants. Moreover, for all variants tested, two similar affinity states for dopamine binding were observed that were sensitive to the inclusion of Gpp[NH]p. Under standard DA receptor binding conditions, no major differences have been observed in pharmacological characteristics between variants of the D4 receptor. However, small differences in the sensitivity for sodium chloride on clozapine binding have been observed between D4 variants (Asghari et al. 1994; Van Tol et al. 1992). As yet, it is unclear whether these observed differences are indicative of differences in receptor function. Genetic association studies have thus far not indicated a good correlation between differences in clozapine responsiveness in the patient population and the different D4 isoforms (Shaikh et al. 1993). However, inclusion of four different polymorphic markers for the D4 receptor gene (figure 1) in these analysis gives some ability to predict clozapine responsiveness (Kennedy, personal communication, July 1994).

COUPLING TO ADENYLYL CYCLASE

In order to determine whether the D4 receptor possesses the ability to block adenylyl cyclase activity, the D4.2 variant was cloned by homologous recombination into vaccinia virus. This recombinant vaccinia virus could infect several cell types which would, upon infection, express D4 receptors that display a pharmacological profile identical to that described previously. Approximately 2 to 3 days after infection, cells express D4 receptor levels, as determined by [3H]spiperone binding, of approximately 1 pmol/mg protein. Functional analyses of GH4C1 cells, mouse fibroblast L cells, and Rat-1 cells demonstrated that all these cells, upon infection with recombinant D4 vaccinia virus, could block adenylyl cyclase activity by dopa-mine (Bouvier et al. 1993). Detailed analyses of infected Rat-1 cells demonstrated that dopamine could block forskolin-stimulated adenylyl cyclase activity and increased intracellular cyclic adenosine monophos-phate (cAMP) levels with a 50 percent effective concentration (EC50) of about 10 nM. This activity could be blocked by spiperone and clozapine, but not by raclopride, which was in agreement with the affinities of these drugs for the D4 receptor (Bouvier et al., in press).

As well, several forms of the human D4 receptor were stably transfected into Chinese hamster ovary cells (CHO-K1). These cell lines express the dopamine D4 receptor at concentrations of approximately 200 to 400 fmol/mg protein. Stimulation of these cells

by various concentrations of dopamine did not significantly change the intracellular cAMP levels, compared to nontransfected cells and nonstimulated cells. However, in the D4-expressing cell lines, DA could reduce forskolin-induced intracellular cAMP increases by up to 90 percent (Asghari et al., in press). This reduction was concentration dependent with an EC50 of approxi-mately 15 nM (figure 4). Comparative analysis of various cell lines expressing the D4.2, D4.4, or D4.7 variants failed to show any major differences in either efficacy or EC50 of DA to block adenylyl cyclase activity, although the potency of DA for D4.7 was slightly reduced (Asghari et al., in press). The D2 antagonists emonapride, haloperidol, and clozapine could inhibit the dopamine-induced cAMP changes in forskolin-stimulated cells in a concentration-dependent manner and with a rank order that was in close agreement with the rank order seen for the affinities of these compounds for the D4 receptor. Raclopride was not able to block dopamine's activity at the D4 receptor at concentrations less than 5 µM, which is expected considering the low affinity of this ligand for the D4 receptor. The D4.2, D4.4, and D4.7 all displayed similar functional profiles for these four D2 antagonists.

EXPRESSION OF THE D4 GENE

Dopamine D4 receptor messenger RNA (mRNA) has been detected in various brain regions from humans, monkey, and rat. Northern blot analysis of several dissected brain regions has shown a distribution for this receptor that is dissimilar to the D2 and D3 receptors. A regional distribution study in monkey brain showed relatively high levels for D4 mRNA in frontal cortex, amygdala, midbrain, and medulla (Van Tol et al. 1991). Lower levels were detected in striatum and hippocampus. Although D4 mRNA is detectable by Northern blot analysis in poly A+ enriched RNA preparations, D2 mRNA can be monitored easily by the same methodology in total RNA preparations, suggesting two orders of magnitude difference in the density of both RNAs (Bunzow et al. 1988). This is confirmed by the relatively low signal that is detected by in situ hybridization. In situ hybridization data basically confirm the locali-zation seen by Northern analysis, although cellular localization is more detailed and thus revealed the presence of D4 mRNA at relatively high levels in dentate gyrus (Mansour et al. 1991; Meador-Woodruff et al. 1991, 1994; O'Malley et al. 1992). Interestingly, D4 receptor mRNA has also been detected in relatively high levels in rat heart (O'Malley et al. 1992) and retina (Cohen et al. 1992).

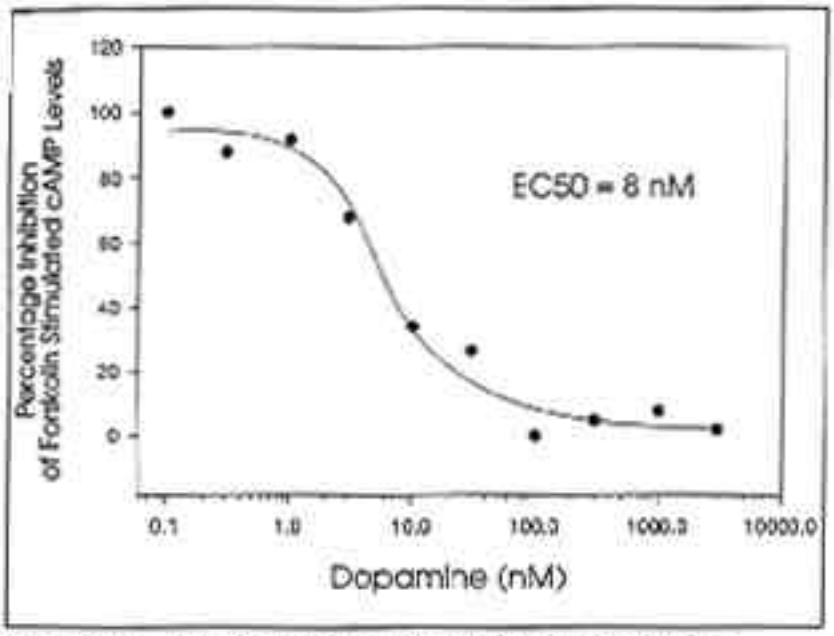

FIGURE 4. *Inhibition of forskolin-stimulated cAMP levels in CHO-K1 cells stably expressing the human D_4 receptor. The cells express approximately 300 fmol/mg protein D_4 receptors as determined by [3H]spiperone binding. Cyclic AMP levels were stimulated with 10 μM forskolin or 10 μM forskolin with varying concentrations of dopamine for 20 min. Cyclic AMP levels were determined by radioimmunoassay essentially as described by Albert and colleagues 1990.*

Alternatively, the author attempted to monitor the location of D4 binding sites. By using the differential binding characteristics of [3H]raclopride and [3H]emonapride for D2, D3, and D4 receptors (see above), D4-like binding sites were detected in caudate putamen of human and rat but not in frontal cortex. This can be interpreted to indicate that a large number of the D4 mRNA containing cortical neurons have their projections with D4 sites in noncortical areas, including caudate putamen. Less likely is that this RNA is not translated into a functional protein. Preliminary data using in vitro autoradiography of [3H]emonapride in the presence of an excess of unlabeled raclopride (> 100 nM) confirms the location of these raclopride "insensitive" [3H]emonapride binding sites seen by scatchard analysis, and also revealed several D4-like binding sites in other rat brain areas, including entorhinal cortex, colliculi, and central

gray area (Nobrega, personal communication, June 1994). Whether these sites are genuine D4 receptors awaits further characterization.

Little is known as yet about whether, and how, D4 receptor density is regulated. Experiments in which rats have been treated for a prolonged period with haloperidol indicate a twofold increase in both D4 mRNA and D4 binding sites, as defined by emonapride and raclopride, in caudate putamen (Schoots et al. 1995). Interestingly, the number of dopamine D4-like binding sites is also considerably increased in postmortem caudate putamen tissue of schizophrenics (Seeman et al. 1993). In the schizophrenic tissues the D4 receptor levels were elevated approximately sixfold over control tissues. Although the majority of the tested tissues originated from schizophrenic individuals who were treated with neuroleptics, similar increases were also seen in the drug-naive individuals, while no significant changes were measured in tissues obtained from patients with Huntington's disease who were treated with neuroleptics. Furthermore, as described above, rats treated with neuro-leptics only showed a twofold change in receptor levels, suggesting that at least a certain proportion of the increased receptor density must be attributed to the disease. On the other hand, the relation of the D4 receptor to schizophrenia is unclear. Genetic linkage and association studies of schizophrenia and bipolar affective disorder with several polymorphic markers for the D4 receptor have not supported this receptor as the primary cause for these disorders (Barr et al. 1993; Macciardi et al. 1994). Similar results have been obtained for the D2 and D3 receptors (Kennedy 1994). This, however, does not rule out any of these receptors as therapeutic targets to control these disorders, since drugs with different D2-like binding profiles and clinical profiles, like raclopride, haloperidol, and clozapine are all effectively used in the treatment of these diseases.

CONCLUDING REMARKS

The role of the dopamine D4 receptor in the DA system with respect to addiction is unclear. Although the DA system has been clearly established as a modifier of self-reward behavior, the recognition of several new DA receptor genes warrants a reevaluation of several components of the system. The absence of a wide variety of agonists and antagonists that are highly selective for the different receptors has prevented the evaluation of the functional role of these receptors in drug addiction. From studies done by Caine and Koob (1993) it is clear that different DA receptors might contribute differently to self-

reward behavior. With respect to the D4 receptor, one published study indicates a higher frequency of the occurence of the alleles D4.3 and D4.6 in alcoholism (George et al. 1993); however, another study failed to demonstrate such an association (Adamson et al. 1995).

To establish unequivocally that the D4 receptor is not a genetic factor contributing to addiction, other paradigms might also have to be tested. As for the roles of the D4 receptor and other newly recognized DA receptors in addiction behavior, researchers are largely ignorant about the different behavioral functions of these receptors. Although the development of highly specific D4 agonists and antagonists might be of great value, alternative approaches should be investigated. As shown for the 5-HT1B receptor (Hen et al., this volume), the use of DA receptor gene "knockout" mice might be of great value for unraveling the functional roles of the dopamine receptors for different behavioral paradigms.

One of the most interesting molecular features of the human D4 receptor is probably the extensive polymorphic repeat sequence in the coding sequence of this receptor. The identification of at least 27 different haplo-types for the repeat encoding 20 putative different D4 receptor proteins has revealed another level of receptor diversity which is thus far unprecedented within the G-protein-coupled receptor superfamily. Until now it was recognized that receptor diversity was generated by the existence of several genes coding for different subtypes or by alternative splicing. Large structural polymorphisms as described for the D4 receptor might account for differences in drug responsiveness and susceptibility to neuropsychiatric disorders, including addiction. It would be of interest to see whether such extensive interindividual differences in coding sequence also exist for other proteins, or whether this is a unique feature of the D4 receptor.

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CANADA

Behavioral Characterization of Mice Packing the 5-HT_{1B} Receptor

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INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is a neuromodulator that is involved in a number of mood disorders such as depression, anxiety, and impulsive violence. In an attempt to dissect the contribution of individual 5-HT receptor subtypes to behavior, the authors have generated, by homologous recombination, mutant mice lacking the 5-HT1B receptor. These mice did not exhibit any obvious developmental or behavioral defect; however, the hyperlocomotor effect of the 5-HT1A/5-HT1B agonist RU24969 was completely absent in mutant mice, indicating that this effect is mediated by 5-HT1B receptors. Moreover, when confronted with an intruder, isolated mutant mice attacked the intruder faster and more intensely than wild-type mice, suggesting an involvement of 5-HT1B receptors in the modulation of aggressive behavior. These data might be related to the fact that a class of 5-HT1 agonists, termed serenics, have antiaggressive properties, and with the findings that certain impulsive aggressive behaviors are associated with deficits in central serotonin.

5-HT is a biogenic amine that is involved in a wide range of physiological functions including sleep, appetite, pain perception, sexual activity, memory, and mood control (for a review see Wilkinson and Dourish 1991). A central serotonin deficit has been associated with behaviors such as suicidality, impulsive violence, depression, and alcoholism (Eichelmann 1992; Roy et al. 1991), and serotonergic drugs are used in the treatment of a number of pathological states including migraine, depression, and anxiety (Sleight et al. 1991). The multiple actions of serotonin are mediated by the interaction of this amine with at least 14 receptors (for a review see Saudou and Hen, in press), most of which belong to the guanosine triphosphate binding protein (G-protein)-coupled receptor family.

The purpose of this study was to determine the contribution of one of these receptors, the 5-HT1B subtype, to the various behavioral responses elicited by serotonin and serotonergic drugs. The 5-HT1B receptor, which is the rodent homolog of the human 5-HT1Db receptor, is expressed in a variety of brain regions, including motor control centers such as the basal ganglia, as well as structures involved in mood control such as the central gray, hippocampus, and raphe nuclei (Boschert et al. 1993; Bruinvels et al. 1993; Maroteaux et al. 1992). Pharmacological studies using poorly specific agonists have suggested that activation of 5-HT1B receptors might lead to an increase in anxiety and locomotion (Griebel et al. 1990; Pellow et al. 1987) and to a decrease in food intake, sexual activity, and aggressive behavior (Fernandez-Guasti et al. 1992; Kennett et al. 1987; Koe et al. 1992; Olivier et al. 1987). In particular, a class of 5-HT1 agonists have been termed serenics because of their antiaggressive properties in several rodent aggression models (Flannelly et al. 1985; Mos et al. 1992; Olivier et al. 1986, 1989). However, it is not clear to what extent the effects of the serenics are mediated by 5-HT1B receptors because these drugs also activate 5-HT1A receptors and possibly some of the recently discovered 5-HT receptors. In addition, the consequences of a blockade of 5-HT1B receptors, or of their human counterpart the 5-HT1Db receptors, are unknown, since there are no specific antagonists for these receptors.

In order to study the function of the 5-HT1B receptor, the authors have generated by homologous recombination homozygous mutant mice lacking both copies of the gene encoding this receptor. These mice are viable and fertile, and they were analyzed for a variety of behaviors that are thought to be modulated by 5-HT1B receptors such as locomotion, anxiety, and aggression.

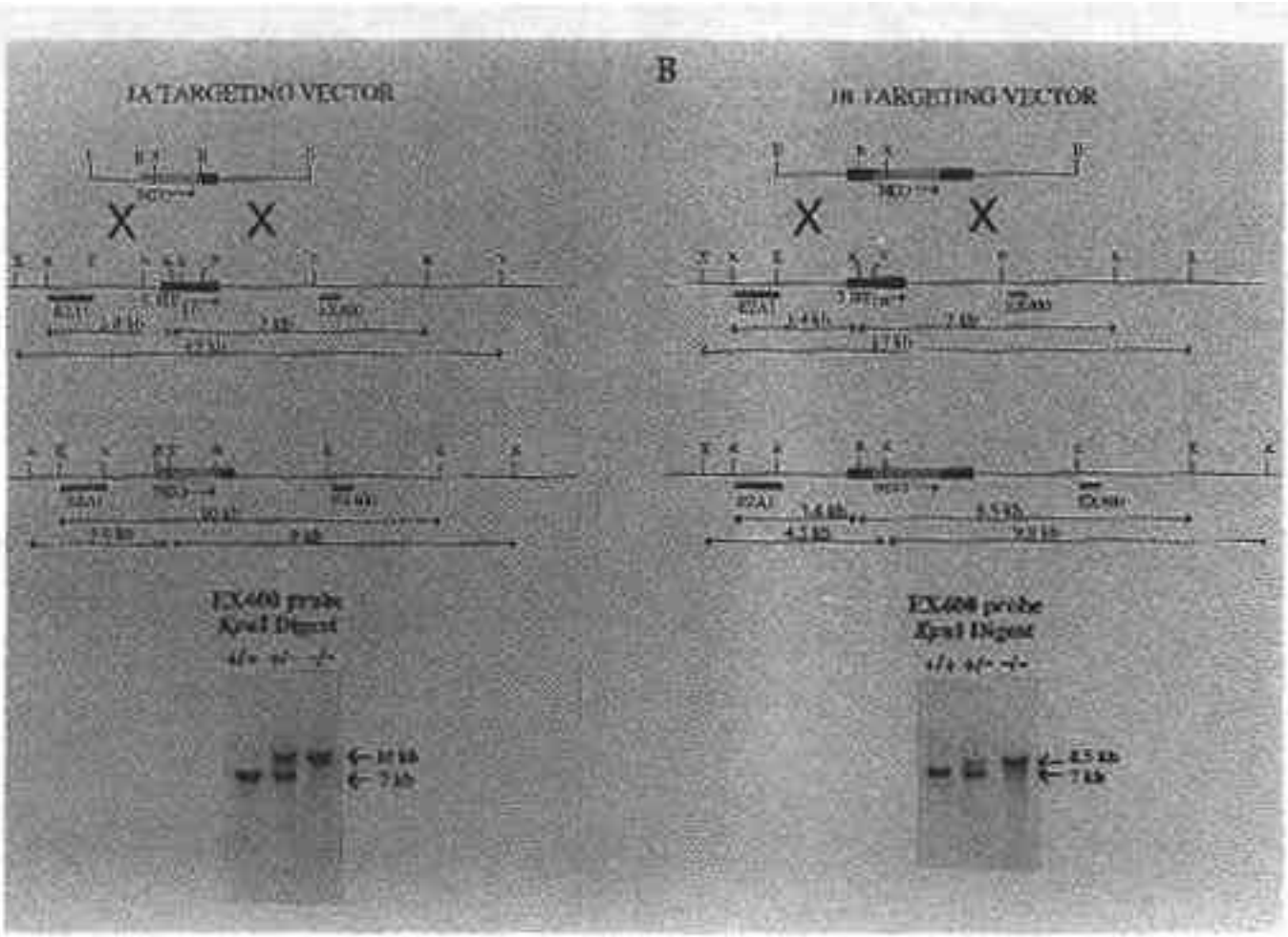
5-HT1B RECEPTOR GENE TARGETING

The 5-HT1B receptor gene was disrupted by homologous recombination (Capecchi 1989; figure 1). The JA construct consisted of 6.0 kilobase-pairs (kb) of genomic sequence in which part of the 5-HT1B coding sequence was replaced by a neomycin phosphotransferase gene (neo) under the control of the GTI-II enhancer (Lufkin et al. 1991; figure 1A). In the JB construct, the neocassette was inserted in the coding sequence of the 5-HT1B gene (figure 1B). The two linearized targeting vectors were electroporated into D3 embryonic stem (ES) cells and G418 resistant colonies were screened by Southern blotting (figure1). The EX400 probe identified

positive clones with the expected 10 and 8.5 kb Kpn I fragments for the JA and JB constructs respectively (figure 1). Four positive clones were obtained with both constructs yielding a targeting frequency of 1/15 (JA) and 1/12 (JB) (table 1). Southern analyses using Xba I digests and the E2A1 probe or the neo probe confirmed that accurate targeting occurred and that no additional integration took place (data not shown). Cells from the positives clones JA7 and JB13 were microinjected into 3.5-day C57BL/6 mouse blastocysts. The two clones gave rise to highly chimeric mice that were bred with C57BL/6 females in order to test for germline transmission of the mutated 5-HT1B receptor gene (table 1). The positive chimeras were bred with females from the 129/Sv-ter inbred strain to obtain heterozygotes on the 129/Sv-ter genetic background. Heterozygous mice were phenotypically normal and fertile. Homozygous animals deriving from both cell lines were generated by heterozygote crossings. In 243 JA offspring and 213 JB offspring, the expected 1:2:1 ratio of wild-type, heterozygous, and homozygous mutant progeny was observed. The homozygous mutants did not display any obvious developmental or behavioral abnormality and were fertile. Although the average lifespan has not yet been determined, no spon-taneous deaths occurred during the first 12 months of life. All the analyses presented here were performed on animals having a pure 129/Sv genetic background. There were no differences between the mice derived from the JA7 and JB13 targeted cell lines.

EFFECTIVENESS OF 5-HT1B RECEPTOR ABLATION

In order to ensure that disruption of the 5-HT1B receptor gene was effective, the authors performed autoradiographic studies (figure 2) on brains of wild-type, heterozygous, and homozygous mutants using the radiolabeled ligand 3[125I]iodocyanopindolol ([125I]CYP). When used in the presence of appropriate masking agents, this radioligand binds specifically to the 5-HT1B receptor (Hoyer et al. 1985; Offord et al. 1988; Pazos and Palacios 1985). In wild-type mice, [125I]CYP binding sites were found in the globus pallidus, substantia nigra, cerebellar nuclei, subiculum, lateral geniculate nucleus, central gray, and colliculi, while no specific binding was observed in homozygous mutants (figure 2). These results demonstrate that effective disruption of the 5-HT1B gene occurred and that in these experimental conditions, [125I]CYP binding sites correspond exclusively to 5-HT1B receptors. Heterozygous mice displayed the same level of binding sites as wild-type mice (figure 2), although they have only one functional allele of the 5-HT1B gene.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1A TARGETING VECTOR
1B TARGETING VECTOR
EX400 probe
XpnI Digest
EX400 probe
XpnI Digest

FIGURE 1. *Homologous recombination at the 5-HT1B locus. Schematic representation of the targeting event using JA* ***(A)*** *and JB* ***(B)*** *constructs and Southern analysis of JA7* ***(A)*** *and JB13* ***(B)*** *mutant mice. The upper panels correspond to the targeting vectors, genomic structure of the 5-HT1B gene, and predicted structures of the mutated alleles after homologous recombination. The black box corresponds to the coding sequence of the 5-HT1B receptor and the hatched box to the neocassette. Arrows on the neocassette (purified from p 581 (Lufkin et al. 1991)) and on the 5-HT1B gene indicate the direction of transcription (from left to right). The locations of the probes E2A1 and EX400 used in Southern analysis are shown. E2A1 and EX400 probes were used to screen neomycin resistant clones after XbaI and KpnI digests, respectively. Bottom, Southern blot analysis. Tail DNA from wild-type, heterozygous, and homozygous JA7* ***(A)*** *and JB13* ***(B)*** *mutant mice were cut by KpnI and hybridized with the 3' end EX400 probe.*

KEY: E=EcoRI; B=BalI; X=XbaI; K=KpnI; V=EcoRV; +/+=wild-type; +/-=heterozygous; and -/ =homozygous mutant.

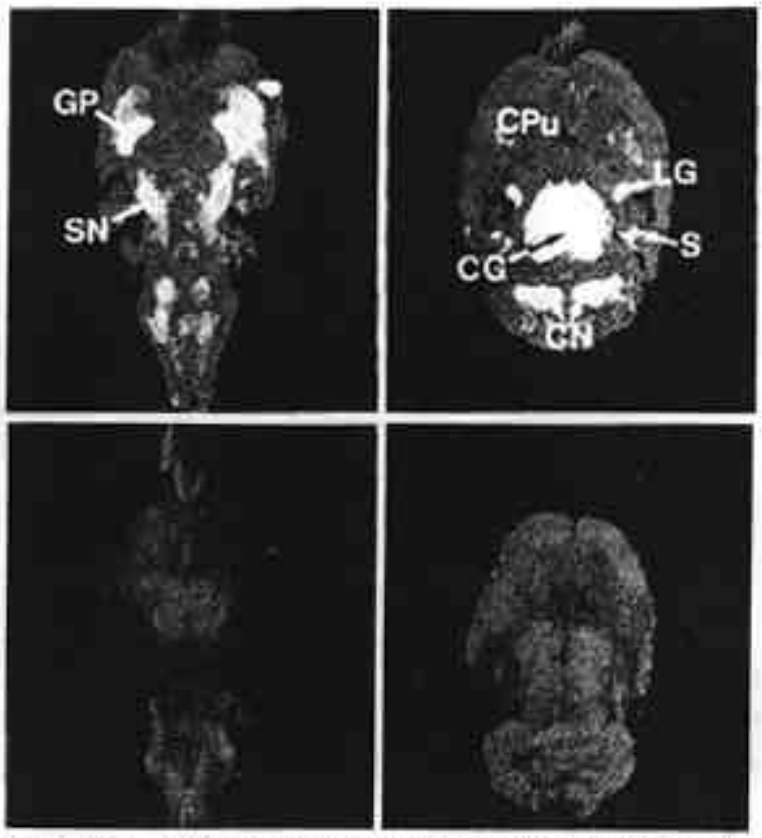

FIGURE 2a. *5-HT$_{1B}$ receptor autoradiography in wild-type and mutant mice. 3[^{125}I]iodocyanopindolol was used to label 5-HT$_{1B}$ receptors in mouse horizontal brain sections. Upper panels correspond to successively more dorsal brain sections of wild type mice and lower pannels correspond to similar levels of sections of homozygous mutant mice (Boschert et al. 1993). Arrows indicate the main sites of 5-HT$_{1B}$ receptor expression. The differences were significant between mutant and wild-type and between mutant and heterozygous mice in all brain regions tested.*

KEY: CG=central gray; CN=cerebellar nuclei; CPu= caudate putamen; GP=globus pallidus; LG=lateral geniculate nucleus; S=subiculum; SN=substantia nigra; **=P<0.001.

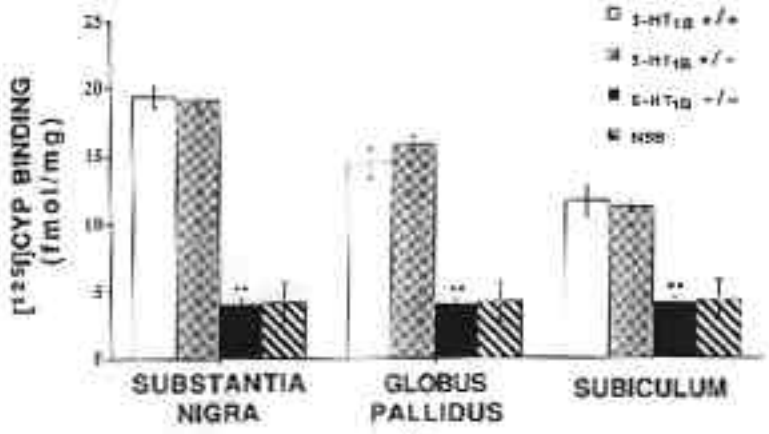

FIGURE 2b. *Graph, density of [^{125}I]CYP binding sites (mean±SEM; n=3) in different brain regions for wild-type (5-HT$_{1B}$ +/+), heterozygous (5-HT$_{1B}$ +/-), and homozygous mutant mice (5-HT$_{1B}$ -/-). Student's t-test revealed no difference between wild-type and heterozygous mice, and between mutant mice and non specific binding (NSB). The differences were significant between mutant and wild-type and between mutant and heterozygous mice in all brain regions tested.*

Locomotion

As shown in figure 2, the 5-HT1B receptor is localized in motor control centers such as the globus pallidus, substantia nigra, and deep cerebellar nuclei. Furthermore, pharmacological studies have suggested an involvement of 5-HT1B receptors in the control of locomotor activity (Green et al. 1984; Oberlander et al. 1986, 1987). The activity of the mice in an open field was analyzed with a video tracking device. No significant differences were detected between the mutant mice and their wild-type littermates (figure 3). Administration of the 5-HT1 agonist RU24969 stimulated locomotor activity in the wild-type mice, while it had no effect in the mutants (figure 3). These results indicate that the hyperlocomotor effect of RU 24969 is mediated by 5-HT1B receptors.

TABLE 1. Homologous recombination of 5-HT1B gene. Analysis of the recombination events and generation of homozygous mutant mice.

Targeting Vector		JA	JB
Length of homology		4.5 kb	6 kb
Neomycin-resistant colonies		62	49
Positives colonies		4	4
Positive colony used		JA7	JB13
Chimeric males		23	2
Chimeric males analyzed		8	2
GLT chimeric males		5	2
Offspring	Total	243	213
from	+ / +	61	54
heterozygote	+ / -	130	99
crosses	- / -	52	60

KEY: GLT = Germline transmission.

Anxiety

5-HT1 agonists such as RU 24969, eltoprazine, fluprazine, or 1-(3-trifluromethylphenyl)piperazine (TFMPP) have been reported to induce anxiogenic responses in rats and mice (Griebel et al. 1990; Pellow et al. 1987). To evaluate the level of anxiety of the 5-HT1B-minus mice, the light/dark choice test (figure 4) was used. The time spent in the lit compartment, as well as the number of transitions between the dark and the lit compartments, have been considered as indices of anxiety since they are increased by anxiolytic drugs. There were no significant differences between mutants and their wild-type littermates for either parameter, suggesting that the mutants have the same level of anxiety as

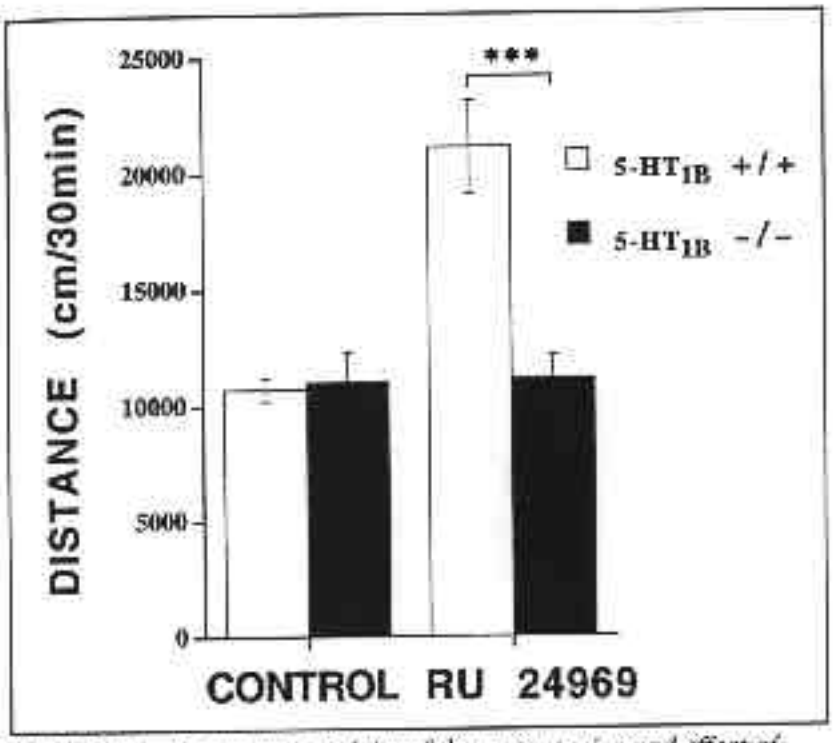

FIGURE 3. *Locomotor activity of the mutant mice and effect of RU 24969 (mean±SEM). Male mice were 12 weeks old at time of testing. They were housed alone in a standard cage with food and water, and kept on a 12/12-hr light dark cycle with light onset at 0700 hr. The mice were tested between 1000 and 1600 hours during the light phase. A video tracking device was used to measure the distance traveled by the animals during a period of 30 min in an open field. The two left columns correspond to the locomotor activity in control conditions of wild-type (N=12) and mutant mice (N=10) and the two right columns to the effect of RU 24969 (5mg/kg bodyweight, 40 min before testing) injected in the same mice 10 days after the first test. There was no significant difference between the wild-type mice and the mutant mice in control conditions as revealed by t-test analysis ($t_{(20)}$=0.22; not significant, NS). However, after RU 24969 treatment there was a significant difference between the two groups ($t_{(20)}$=4.18; ***=P<0.001).*

the wild-type mice in this test (figure 4). In the wild-type mice, RU4969 increased significantly the number of transitions between the dark box and the lit box, but it had no significant effect on the time spent in the lit box (figure4). In the mutants, RU24969 did not modify these parameters. The larger number of transitions displayed by the wild-type mice are probably a result of the increase in locomotion induced by RU-24969. The lack of response of the mutants demonstrates that the effect of RU24969 is mediated by 5-HT1B receptors. In contrast with earlier reports, RU 24969 had no anxiogenic effect. Such an effect might have been masked in the present experiment by the hyperlocomotor effect of this drug. A dose-response curve, as well as other anxiety tests, might reveal an effect of RU24969.

Aggression

A class of 5-HT1 agonists including eltoprazine and fluprazine have been termed serenics because of their antiaggressive properties (Flannelly et al. 1985; Mos et al. 1992; Olivier et al. 1986, 1989) and their effects have been suggested to be mediated by 5-HT1B receptors. The authors therefore investigated the aggressiveness of 5-HT1B-minus mice in a classical aggression test. After an isolation period of 4 weeks, test mice (resident) were analyzed for intermale aggression after introduction in their cage of a wild-type mouse that had been reared in a group (intruder) (figure 5). In this test, the latency of attack and the number of attacks performed by the resident during a 3-minute period were used as aggression indices. The mutant residents attacked the intruder faster than the wild-type or heterozygous residents (figure 5A). Furthermore, the number of attacks in the mutant group was significantly higher than in the wild-type or heterozygote groups (figure 5B). In addition, the intensity of attacks of the mutant residents was higher, as well as the number of tail rattlings preceding the attacks (not shown). Similar results were obtained in two tests performed 1 week apart. The level of aggressiveness was higher in the second test with both the wild-type and the mutant animals, in good agreement with previous reports showing that aggression increases with fighting experience (Lagerspetz and Lagerspetz 1971). A qualitative analysis of the attacks during the 3-minute test period revealed additional marked differences between wild-type and mutant mice (figure6). In the first test, 29 percent of the mutant residents attacked the intruder within less than 10 seconds after introduction of the intruder in the cage (impulsive attacks, figure 6A), while no wild-type or hetero-zygous mice attacked the intruder during that time interval. Conversely, 75 percent of the wild-type mice and only 21 percent of the mutants did

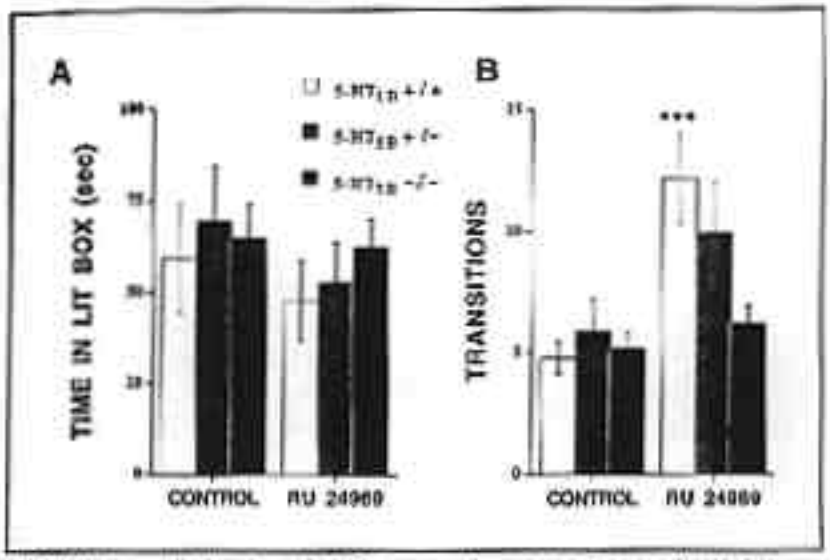

FIGURE 4. *Behavior of wild-type and mutant mice in the light-dark choice test. The mice were tested during the dark phase. (A) time spent by mice in the lit box (time in lit box: mean±SEM) (B) number of tunnel crossings from the dark box to the lit box (transitions: mean±SEM). The left columns (control) correspond to wild-type (N=22), heterozygous (N=12), and homozygous mutant mice (N=26) injected with saline vehicle and the right columns correspond to a different series of wild-type (N=23), heterozygous (N=12), and mutant mice (N=26) injected with RU 24969. ANOVA (factors=genotype and treatment) revealed no significant differences among groups for time spent by mice in the lit box (A): genotype [$F_{(2,111)}$=0.9, NS]; treatment [$F_{(1,111)}$=1.31, NS]; genotype x treatment interaction [$F(2,115)$=0.25, NS]. In contrast, there were significant differences among groups for the number of transitions (B): genotype [$F_{(2,111)}$=3.38, $P<0.05$]; treatment [$F_{(1,111)}$=15.62, $P<0.0001$]; genotype x treatment interaction [$F_{(2,111)}$=4.12, $P< 0.05$]. There were no significant differences among control groups for the transitions [$F_{(2,70)}$=0.49, NS] but the treatment with RU 24969 increased significantly the number of transitions in wild type mice [$t_{(42)}$=3.54, $P< 0.001$], not quite significantly in heterozygous mice [$t_{(22)}$=1.63, $P= 0.11$], and not in homozygous mutant mice [$t_{(50)}$=1.01, NS].*

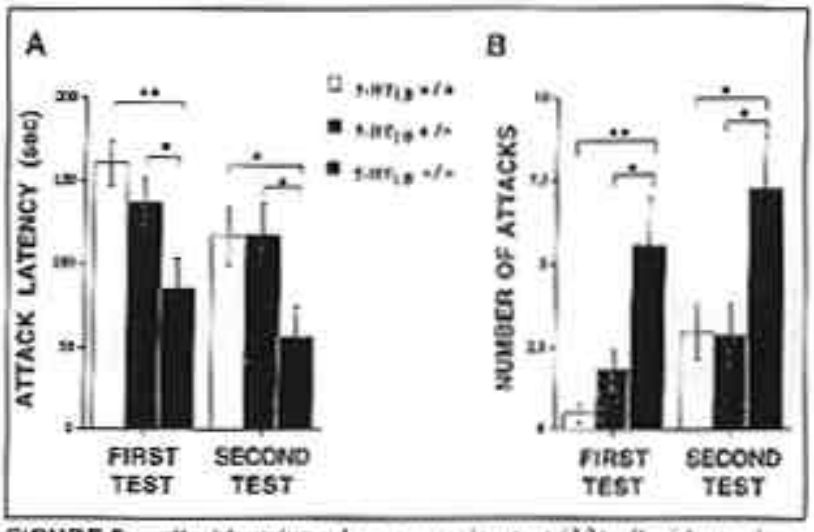

FIGURE 5. *Resident-intruder aggression test (13). Resident mice were: wild-type (N=12), heterozygotes (N=16), and mutant mice (N=14) and isolated during 4 weeks in transparent cages. (A) Attack latency (mean ± SEM): time between the introduction of the intruder and the first attack by the resident. ANOVA revealed significant differences for the attack latency both in the first test [$F_{(2,39)}$=5.28, P<0.01] and in the second test [$F_{(2,37)}$=3.49, P<0.05]. Further statistical analyses revealed significant differences between wild-type and mutant mice (first test, [$t_{(26)}$=3.19, P<0.01]; second test, [$t_{(23)}$=2.38, P<0.05]), heterozygotes and mutant mice (first test, [$t_{(28)}$=2.17, P<0.05]; second test, [$t_{(26)}$=2.26, P<0.05]), but not between wild-type and heterozygous mice (first test, [$t_{(26)}$=1.10, NS]; second test, [$t_{(23)}$=0.01, NS]). (B) Number of attacks (means ± SEM) during the 3-min session. ANOVA: (first test, [$F_{(2,39)}$=7.39, P<0.01]; second test, [$F_{(2,37)}$=4.48, P<0.02]. T-tests: wild-type versus mutants (first test, [$t_{(24)}$=3.19, P<0.01]; second test, [$t_{(22)}$=2.32, P<0.05]), heterozygotes versus mutants (first test, [$t_{(28)}$=2.16, P<0.05]; second test, [$t_{(26)}$=2.44, P<0.05]), wild-type versus heterozygotes (first test, [$t_{(26)}$=1.72, NS]; second test, [$t_{(25)}$=0.99, NS]).*

KEY: *=P<0.05; **=P<0.01.

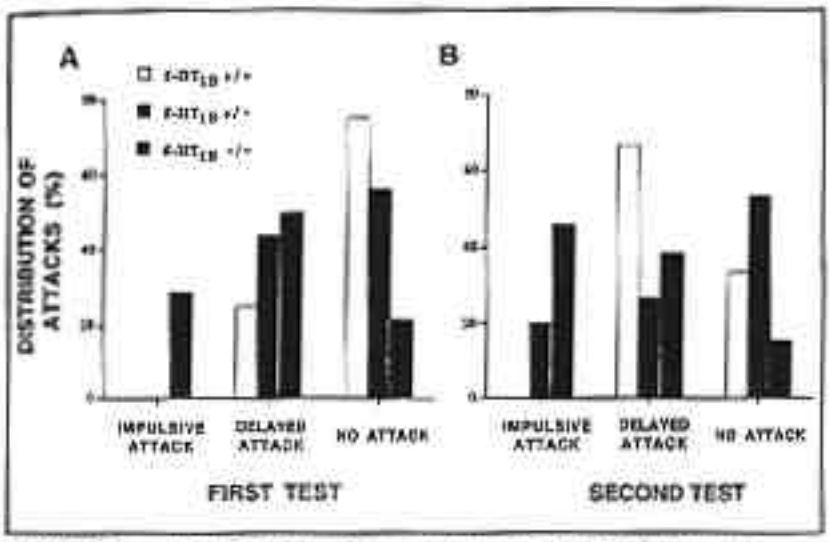

FIGURE 6. *Resident-intruder aggression test: distribution of attacks. Attacks were categorized as impulsive attacks (attacks within less than 10 sec), delayed attacks (attacks displayed between 10 and 180 sec), and no attacks during the 3-min test session. (A) correspond to the first test and (B) to the second test. The values presented here derive from the experiment described in figure 5.*

not attack during the 3-minute test. In the second test, the percentage of mutants displaying impulsive attacks was even higher (46percent), while still no wild-type animals performed such short latency attacks (figure6B). These results indicate that the 5-HT1B-minus mice are more aggressive than their wild-type or heterozygous littermates.

DISCUSSION

The authors have generated, by homologous recombination, mice lacking the 5-HT1B receptor. Autoradiographic data demonstrated the absence of 5-HT1B receptors in the homozygous mutants. Such mice develop and live apparently normally. Preliminary histological analyses of their central nervous systems did not reveal any obvious defect (not shown). A number of behaviors were analyzed that were thought to be modulated by activation of 5-HT1B receptors, such as locomotion, anxiety, and aggression (Fernandez-Guasti et al. 1992;

Green et al. 1984; Griebel et al. 1990; Kennett et al. 1987; Koe et al. 1992; Oberlander et al. 1986, 1987; Olivier et al. 1987; Pellow et al. 1987). Surprisingly, in the authors' test conditions, no differences were detected in the levels of basal locomotor activity or anxiety in the mutant mice. However, the hyperlocomotor effect of the 5-HT1 agonist RU 24969 was totally absent in the mutants, demonstrating that this effect is mediated by 5-HT1B receptors. A decrease in locomotor activity in the 5-HT1B-minus mice might therefore have been expected. The absence of such a motor effect in these experimental conditions suggests either that compensatory mechanisms occurred during development or, alternatively, that in normal "baseline" conditions the 5-HT1B receptor is not activated. Preliminary results indicated that the levels of the 5-HT1A and 5-HT1Da receptors were not altered in the mutants (not shown). The levels of 5-HT and catecho-lamines as well as the levels of aminergic receptors that are involved in motor control and which might have compensated for the absence of the 5-HT1B receptor are currently being analyzed. An alternative possibility, that 5-HT1B receptors are activated in response to environmental changes such as stressful situations, is appealing in light of the results obtained in the aggression test. When the mutants are group housed they do not appear to be more aggressive than grouped wild-type mice. However, after a month of isolation and in the presence of an intruder, the mutants are significantly more aggressive than the wild-type mice. In male mice, isolation and the presence of a conspecific male intruder have been shown to increase aggressive behavior (Lagerspetz and Lagerspetz 1971). The authors' results indicate that 5-HT1B-minus mice are more responsive to the isolation and the stress or fear generated by the intruder and suggest that 5-HT1B receptors might be activated in stressful situations such as those encountered in this test.

The increased aggressiveness of 5-HT1B-minus mice is in good agreement with the fact that a family of 5-HT1B agonists termed serenics have anti-aggressive properties (Olivier et al. 1987). These compounds were shown to decrease aggressive behavior in several animal models including isolation-induced aggression in mice (Olivier et al. 1989), resident-intruder aggression in rats (Flannelly et al. 1985; Mos et al. 1992), and maternal aggression in rats (Mos et al. 1992). The authors' results suggest that the 5-HT1B receptor is at least in part responsible for the antiaggressive properties of the serenics, but do not rule out a participation of other receptors with a high affinity for these compounds such as the 5-HT1A receptor. The effects of the serenics in the 5-HT1B-minus mice are currently being tested in order

to determine whether 5-HT1B receptors are the preferential target of these drugs.

Several studies have revealed an association between aggressive behavior and a reduction in the activity of the serotonergic system. In rodents and primates, aggressiveness is increased after inhibition of 5-HT synthesis (Higley et al. 1992; Vergnes et al. 1986) or destruction of serotonergic neurons (Molina et al. 1987). Mouse strains that display increased aggressiveness have low brain 5-HT levels (Maas 1962). In humans, impulsive aggressive behaviors have been associated with a deficit in central serotonin (Coccaro 1989). Cerebrospinal fluid (CSF) concen-trations of the 5-HT metabolite 5-hydroxyindole acetic acid (5-HIAA) is reduced in the brain of violent offenders (Brown et al. 1979), arsonists (Virkkunen et al. 1987), and people who committed violent suicide (Coccaro 1989; Mann et al. 1989). Interestingly, in one recent study, impulsive violent offenders had low CSF 5-HIAA levels, while offenders who premeditated their acts had high CSF 5-HIAA levels (Virkkunen et al. 1994). Similarly, in studies of suicide victims, only those who committed violent suicides exhibited low CSF 5-HIAA levels (Olivier et al. 1987; Pellow et al. 1987). These findings suggest a link between low serotonin levels and a lack of impulse control. The aggressive behavior displayed by the 5-HT1B-minus mice might be considered impulsive, since the mutants attacked much faster than the wild-type mice. In light of these results it is tempting to speculate that low serotonergic activity would result in a decreased activation of 5-HT1B receptors, which might trigger aggressive behavior.

The 5-HT1B receptor is localized both presynaptically on serotonergic terminals, where it inhibits the release of 5-HT, and postsynaptically on other nerve endings, where it might inhibit the release of various neuro-transmitters (for a review see Bruinvels et al. 1993). The antiaggressive effects of serenics are most likely mediated by postsynaptic receptors since they are not affected by lesions of serotonergic neurons (Sijbesma et al. 1991). Such postsynaptic receptors might be localized in the central gray, a brain structure involved in defensive behavior and response to fear (Fanselow 1991) and containing moderate densities of 5-HT1B receptors (Boschert et al. 1993; Bruinvels et al. 1993). Activation of the 5-HT1B receptor might be a component of the adaptation to fearful stimuli. Interestingly, several behavioral responses elicited by fear such as those observed in a flight situation (increased locomotion and decreased aggressiveness, sexual activity, and food intake) are also induced by 5-HT1B agonists. These behaviors as well as the level of

stress hormones are currently being analyzed in order to determine whether adaptation to stress or fear is altered in the absence of the 5-HT1B receptor.

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Mechanisms Regulating Proenkephalin Gene Expression: Contributions of Transgenic Models

Steven E. Hyman and David Borsook

INTRODUCTION

Transgenic mouse models can contribute significantly to substance abuse research in several ways. In this monograph, the use of gene knockout mice is discussed separately (Hen, this volume). With the caveat that developmental anomalies and gene compensation may distort physiology or mask an interesting phenotype, gene knockout mice are currently one of the most powerful tools available to study the role of particular proteins in neural function. Transgenic mice may also be engineered to express dominant negative mutations of certain proteins; one possible advantage of this approach, where possible, is that gene inactivation can be targeted to a particular brain region. Transgenes can be inserted in a normal or a gene knockout background to study the effects of a mutated gene or of overexpression or ectopic expression of a particular gene. Additionally, transgenic strategies can be used to deliver a foreign protein to a site of interest.

In the research to be described here, a transgenic strategy to study gene regulation in the brain was used. The authors have followed up on previous studies of the human proenkephalin promoter that had utilized transformed cell lines in order to facilitate studies of transcriptional regulation by physiologically relevant stimuli in neuronal cell types of interest.

PROENKEPHALIN GENE REGULATION

The proenkephalin gene encodes the precursor of the endogenous opioid peptides met- and leu-enkephalin. Enkephalin peptides appear to play roles in a variety of physiological processes including descending analgesia, regulation of hormone release, the stress response, and brain reward. Expression of the proenkephalin gene is highly regulated by synaptic activity, cyclic adenosine monophosphate (cAMP), and protein kinase C (PKC), with the

predominant effect on synthesis being transcriptional. For example, in bovine adrenal chromaffin cells in primary culture, proenke-phalin messenger ribonucleic acid (mRNA) levels have been shown to be increased by nicotinic stimulation (Eiden et al. 1984), cAMP (Schwartz et al. 1984; Kley et al. 1987), K+- or veratridine-induced depolarization, calcium ionophores (Kley 1988; Kley et al. 1987), and PKC (Kley 1988). In C6-glioma cells, proenkephalin mRNA levels have been shown to be increased by ß-adrenergic receptor stimulation, which increases intra-cellular cAMP (Yoshikawa and Sabol 1986). In the brain, chronic treatment with dopamine type 2 (D2) receptor antagonists, including haloperidol, increases enkephalin peptides and proenkephalin mRNA in rat striatum (Romano et al. 1987; Sabol et al. 1983; Sivam et al. 1986; Tang et al. 1983). Lesions of the nigrostriatal dopamine pathway, which block transmission via all dopamine receptor types, also induce proenkephalin mRNA (Gerfen et al. 1990, 1991; Jiang et al. 1990; Normand et al. 1988; Voorn et al. 1987; Young et al. 1986). Other neural stimuli that induce proenkephalin mRNA include splanchnic nerve stimulation in the adrenal gland (Fischer-Colbrie et al. 1988; Kanamatsu et al. 1986), electrical stimulation and seizures in the hippocampus (Hong et al. 1980; Morris et al. 1988; White and Gall 1987; Xie et al. 1989; Yoshikawa et al. 1985), nociceptive stimuli in the dorsal horn (Draisci and Iadorola 1989), and stressors such as hypertonic saline or naloxone-precipitated opiate withdrawal in the hypothalamus (Lightman and Young 1987).

The authors' group and others have studied the mechanisms by which the human proenkephalin gene is regulated by cAMP and Ca2+ using muta-genesis and transfection into transformed cell lines. It was found that an enhancer comprised of three elements acting in combination conferred second messenger activation upon the gene. Within this enhancer, the deoxyribonucleic acid (DNA) regulatory element that was shown to be absolutely required for regulation of the proenkephalin gene by cAMP, depolarization, and Ca2+ is the proenkephalin cAMP response element 2 (CRE-2) element that contains the sequence TGCGTCA (Comb et al. 1988; Hyman et al. 1988, 1989; Nguyen et al. 1990). This element can bind both activator protein 1 (AP-1) and CRE binding protein (CREB) in vitro (Comb et al. 1988; Kobierski et al. 1991; Konradi et al. 1993; Sonnenberg et al. 1989). To complicate matters, the proenkephalin promoter can also be activated by both AP-1 proteins (Kobierski et al. 1991; Sonnenberg et al. 1989) and by CREB (Huggenvik et al. 1991) in cotransfection studies. Both in vitro binding studies (whether by electro-phoretic mobility shift or by footprinting) and cotransfection

studies provide information as to possible transcription factor-target interactions, but they cannot determine what actually happens in particular cell types in vivo.

Because gene regulation may differ markedly between transformed cell lines and neurons, two approaches have been taken. The authors have begun to study binding of transcription factors to key regulatory elements in extracts made from neuronal cell types, rather than using purified proteins from cell lines, and study of promoter function by transfection into neurons in primary culture and by use of transgenic mice has begun. The importance of transgenic models is underscored by several issues. The proenkephalin gene is expressed in a highly complex pattern in the brain, but appropriate cell culture models for the multiple neuronal cell types expressing the gene are lacking. Moreover, transformed cell lines cannot model the connectivity, neurotransmitter receptors, networks of intracellular signal transduction pathways, or the precise mixtures of transcription factors that characterize the different types of enkepha-linergic neurons in the brain. The use of transgenic animals permits investigation of whether the same regulatory elements that appear to be utilized in cell culture models are also utilized in vivo. The use of a beta-galactosidase reporter gene in transgenic models has the additional advantage of simplifying the approach to colocalization of the target gene of interest with putative regulatory proteins because the histochemical reaction used to detect beta-galactosidase activity is highly compatible with immunohistochemistry (Borsook et al. 1994b). A final advantage is that there is no endogenous beta-galactosidase activity in eukaryotic cells, permitting very sensitive detection of proenkephalin gene expression in this transgenic model.

EXPRESSION AND REGULATION OF AN ENKEPHALIN-BETA-GALACTOSIDASE FUSION GENE IN TRANSGENIC MICE

The three initial transgenic lines that were made contained a fusion construct with 3 kilobases (kb) of human proenkephalin gene 5' flanking sequence, the first exon and intron of the human proenkephalin gene, the E.coli lac-Z transcription unit fused to the second proenkephalin exon, and l.2kb of human proenkephalin 3' flanking sequences (Borsook et al. 1992). All of the independent lines of mice, ENK l.l, l.2, and l.3, expressed the fusion gene to some degree. None of the mice had apparent morpho-logic or behavioral abnormalities. No ectopic expression of the gene product was observed when the beta-galactosidase expression was compared to the

endogenous proenkephalin mRNA by in situ hybridization (Borsook et al. 1992, 1994a). However, none of the lines had complete expression of the gene, suggesting that there might be strong integration effects or that some additional regulatory sequences were missing. All three of the lines had good fidelity of expression in the reproductive system (Borsook et al. 1992), and all had some degree of expression in the brain. The ENK l.2 line had expression only in regions corresponding to the periaqueductal gray matter but nowhere else in the brain, and was not further analyzed. ENK l.l and l.3 had much fuller brain expression including the hypothalamus (Borsook et al. 1994a). None of the lines, however, had the expected expression of the transgene in the striatum.

Because proenkephalin gene expression was known to be highly regulated by pharmacologic and physiologic stimuli in the hypothalamus (Lightman and Young 1987), and because in the ENK l.l and l.3 lines basal expression of the transgene in the hypothalamus recapitulated the pattern of expression of the endogenous gene, a detailed analysis of regulation of the transgene within the hypothalamus was undertaken. All further analyses described here were performed with the ENK l.l line.

REGULATION OF THE TRANSGENE IN THE HYPOTHALAMUS

The paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus are major sites of integration of the stress response. The proenkephalin precursor and enkephalin peptides are found at high levels within these nuclei, where they are thought to play an important role in the control of release of hypothalamic stress hormones including cortico-tropin releasing factor (CRF). It has previously been shown in rat models that certain acute and chronic stressors produce significant induction of proenkephalin gene expression within these nuclei. For example, intra-peritoneal (IP) injection of hypertonic saline and naloxone-precipitated opiate withdrawal have been shown to produce rapid and significant inductions of proenkephalin mRNA in the rat PVN (Lightman and Young 1987). Multiple IP daily injections of hypertonic saline have been shown to induce proenkephalin mRNA in the rat PVN even more strongly (Young and Lightman 1992).

Basal expression of the transgene appears as small blue puncta within cells, which may represent inclusion bodies; with induction, neurons fill out with the blue beta-galactosidase reaction product (Borsook et

al. 1994a). By comparing histochemistry for the beta-galactosidase reaction product with in situ hybridization for the endogenous proenkephalin mRNA, the authors' group found that in the ENK 1.1 line of transgenic mice, basal expression of the transgene and induction by hypertonic saline injection (figure 1) and naloxone-precipitated opiate withdrawal closely paralleled expression of the mouse endogenous proenkephalin gene and the regulation previously reported for the rat (Borsook et al. 1994a, 1994c; Lightman and Young 1987). The transgene was also induced in the PVN by other stressors, such as hypovolemia, cold swim, and lipopolysaccharide injection. Since the endogenous gene and the transgene contain similar genomic regulatory sequences but express entirely different mRNAs, the observation that they are induced in parallel is consistent with the hypothesis that stress activation of proenkephalin gene expression in the hypothalamus occurs at the transcriptional level.

CREB INTERACTS WITH THE PROENKEPHALIN GENE IN THE HYPOTHALAMUS

As described above, both AP-1 proteins and CREB can interact with the proenkephalin gene in vitro. However, in cell extracts from rat striatum (Konradi et al. 1993) and mouse hypothalamus (Borsook et al. 1994b), the authors detected CREB, but not Fos binding to the enkephalin CRE-2 element (TGCGTCA) using electrophoretic mobility shift assays with antibody supershifts. CREB binding is detected even under conditions such as acute stress, which give robust AP-1 binding to a consensus AP-1 site (TGACTCA). The CRE-2 site is contained within the regulatory sequences of the transgene, thus the authors performed combined beta-galactosidase histochemistry with immunocytochemistry for CREB to determine the localization of CREB within the PVN with respect to cells expressing the transgene. CREB protein was found in essentially all neurons within the PVN (Borsook et al. 1994b). Moreover, no differences in total CREB protein levels could be observed by immuno-histochemistry in uninjected, normal saline-injected, or hypertonic saline-injected mice, consistent with the idea that CREB is constitutively expressed in most known cell types and is activated by phosphorylation (Gonzalez and Montminy 1989). The authors then sought to determine whether hypertonic saline stress induced phosphorylation of CREB on Ser133 in transgene-expressing neurons. The authors therefore colocalized beta-galactosidase activity with phosphoCREB using an antiserum that specifically detects CREB phosphorylated on Ser133 (Ginty et al. 1993).

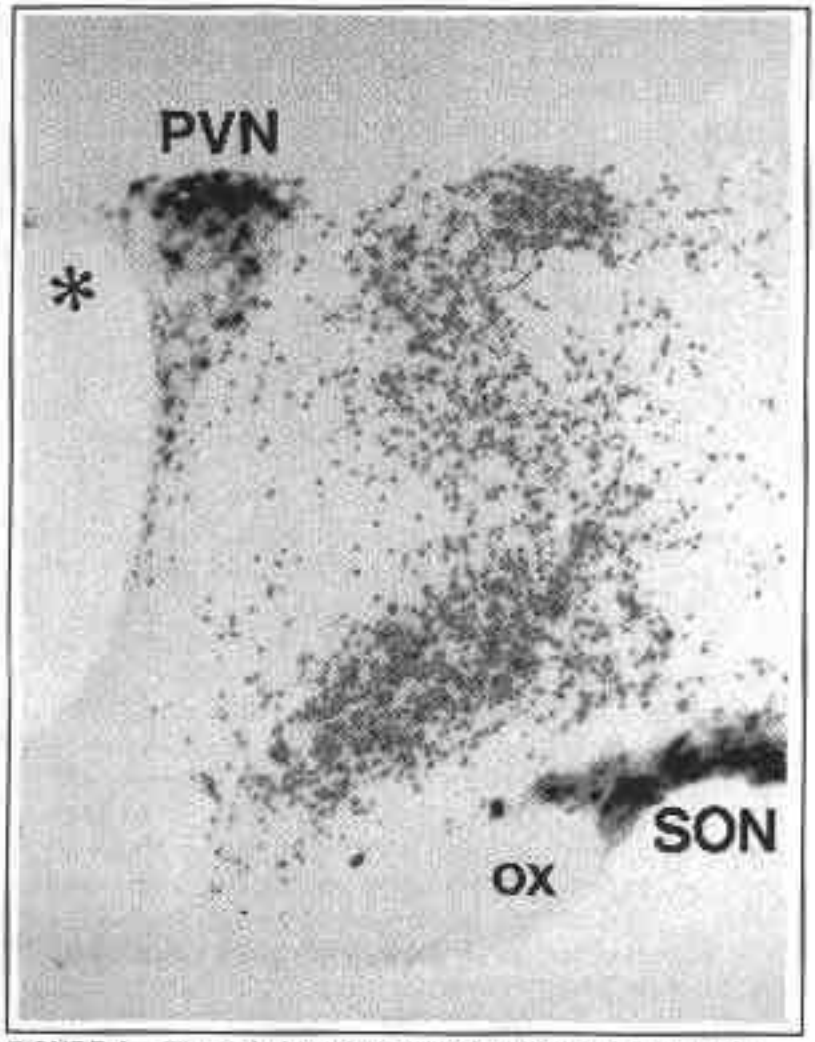

FIGURE 1. *The right hypothalamus of an ENK 1.1 transgenic mouse stained for beta-galactosidase activity after a hypertonic saline stress. The asterisk marks the third ventricle.*

KEY: PVN = paraventricular nucleus; SON = supraoptic nucleus; ox = optic chiasm.

Ten minutes after an injection of 0.15 molar (M) saline, only minimal staining for phosphoCREB was observed in the PVN, indistinguishable from an uninjected animal. Ten minutes after an injection of 1.5 M saline, intense phosphoCREB staining was seen throughout the PVN,

including all cells observed to express the transgene (Borsook et al. 1994b). Mutational analyses are planned to determine whether the CREB binding site is required for stress induction of the transgene within the PVN.

MODULATION OF STRESS-REGULATED PROENKEPHALIN GENE EXPRESSION BY OPIOIDS

In addition to facilitating studies of promoter function, the sensitivity of the transgene also permitted detection of novel forms of regulation of the proenkephalin gene within the hypothalamus. Since the regulatory sequences within the transgene are known, studies of the molecular mechanisms responsible for this regulation will be possible.

Two decades ago, researchers hypothesized that chronic morphine treatment might result in agonist-induced feedback inhibition of endogenous opioid biosynthesis and that this suppression of endogenous opioid peptides might contribute to aspects of opioid dependence and withdrawal (Hughes et al. 1975; Waterfield et al. 1976). Early studies designed to test this hypothesis found little change in steady-state brain enkephalin peptide levels. However, these studies did not address turnover rate or changes within restricted populations of cells. More recently, opioid agonists have been shown to decrease (Uhl et al. 1988) and opioid antagonists to increase (Tempel et al. 1992) expression of proenkephalin mRNA or enkephalin peptides in some cell types, suggesting a direct or indirect opioid receptor-mediated effect on endogenous opioid gene expression.

Using the authors' mouse model, it was found that chronic morphine administration dramatically suppresses transgene expression compared with animals that received either placebo pellets or no pellets. The chronic morphine regimen consisted of 8 milligram (mg) morphine pellets inserted subcutaneously (SC) for 3 days, followed by 25 mg pellets for 4 days. Control animals received either placebo pellets SC for 7 days or were unoperated. The suppression of transgene expression by chronic morphine administration was to almost unobservable levels, and was seen throughout the hypothalamus (Borsook et al. 1994c). Animals injected with morphine (10 mg/kg) or normal saline IP every 6 hours for 5 days displayed a level of suppression of transgene expression that was similar to the animals that received morphine pellets. There were no differences between the placebo pellet and the unoperated conditions.

Chronic (7 days) administration of naltrexone pellets (10 mg or 25 mg) SC had minimal effects on transgene expression (Borsook et al. 1994c). While there was some slight increase in the intensity of puncta, there was no filling of cells with the beta-galactosidase reaction product, the indicator of substantial induction of the transgene.

Surprisingly, administration of morphine (10 mg/kg IP) 4 hours prior to a hypertonic saline stressor (acute condition) or administration of a morphine pellet (8 mg) for 24 hours prior to a hypertonic saline stressor (subacute condition) produced a marked enhancement of the already substantial stress-induced increase in transgene expression within the PVN and SON (Borsook et al. 1994c). In addition, regions of the hypo-thalamus, in which transgene expression is not induced by hypertonic saline stress alone, showed marked induction of the transgene when stress followed morphine pretreatment. These areas include the preoptic region, the nucleus circularis, the lateral hypothalamus, the ventromedial nucleus, and the retrochiasmatic SON (Borsook et al. 1994c). Thus, acute or sub-acute morphine administration markedly sensitizes restricted populations of proenkephalin-expressing hypothalamic neurons to stress. Chronic opioid pretreatment (7 days) prior to hypertonic saline stress inhibits this response, suggesting that desensitization has occurred. Pretreatment with naltrexone inhibits stress induction of transgene expression below the expected level observed in mice receiving only hypertonic saline injections (Borsook et al. 1994c).

In this series of experiments, the authors' transgenic model was used to examine the effects of acute, subacute, and chronic opioid administration on stress regulation of proenkephalin gene expression within the hypo-thalamus. The advantages of the transgenic model are that it is more sensitive and more readily quantified than in situ hybridization and that genomic sequences responsible for the observed regulation are known to be contained within the transgene, making subsequent analysis of transcriptional mechanisms possible.

These results, which were confirmed by in situ hybridization for the endogenous mRNA (Borsook et al. 1994c), are consistent with the hypothesis that acute or subacute administration of morphine sensitizes enkephalinergic hypothalamic neurons to stress. The use of a morphine pellet in the subacute paradigm makes it unlikely that the superinduction is due to mild, behaviorally unobservable withdrawal acting along with the saline stress. Moreover, administration of

opioids chronically, using a paradigm that produced marked dependence (as demonstrated by the possibility of producing naloxone-precipitated withdrawal) not only suppressed basal expression, but also inhibited stress-induced expression of the transgene.

It was initially surprising that acute or subacute morphine pretreatment prior to a hypertonic saline stressor superinduces transgene expression. Morphine has principally inhibitory effects on target neurons mediated by G-protein inhibitory (Gi)-linked opiate receptors, especially mu receptors. As noted by Akil (this volume), there are relatively few mu receptors expressed on neurons in the PVN, although there are relatively high levels of kappa opioid receptors. These observations make it unlikely that the effects of morphine on PVN neurons are direct or cell autonomous, but the effects likely involve intermediary circuitry.

SUMMARY

This chapter gives an overview of the utility of one type of transgenic mouse model for substance abuse research, the use of transgenes in which genomic regulatory sequences of interest are coupled to a reporter gene. Using this mouse model, exploration of the mechanisms regulating expression of the proenkephalin gene in the mouse hypothalamus have begun. The resulting information supplements experiments on gene regulation performed in cell lines with information about regulation by pharmacologic and physiologic stimuli of interest within the brain. With special reference to mechanisms of opioid dependence, the particular model that has been examined appears to be a useful tool to investigate mechanistic aspects of the regulation of endogenous opioid genes by exogenous opioids.

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Human Opioid Receptors: Chromosomal Mapping and mRNA Localization

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INTRODUCTION

The opioid system modulates complex processes such as neuroendocrine function, analgesia, immunity, and cardiovascular regulation. A challenge in the field of neuroscience has been the molecular characterization of the opioid receptors. The recent isolation and sequencing of the genes and cDNAs encoding the opioid receptors permit studies of structure, function, and the cellular localization of these receptors. This chapter describes recent studies on the characterization and chromosomal mapping of the human opioid receptor genes and the localization of human opioid receptor messenger ribonucleic acid (mRNA) in the human brain.

MULTIPLE OPIOID RECEPTORS

Pharmacological studies using opioid peptides and alkaloid ligands have identified several classes of opioid receptor including the mu (μ), delta (d), and kappa (k) (Lord et al. 1977), (Martin et al. 1976). The first opioid receptor cloned was the mouse d receptor reported simultaneously by Evans and colleagues (1992) and Kieffer and colleagues (1992). This receptor was isolated by ligand screening of eukaryotic expression libraries. Subsequently the rat μ and d receptors were isolated by low stringency hybridization using probes derived from the d receptor cDNA (Chen et al. 1993; Fukuda et al. 1993). The mouse k receptor cDNA was isolated by polymerase chain reaction (PCR) using degenerate oligo-nucleotide primers to the structurally similar somatostatin receptor (Yasuda et al. 1993). Multiple similar cDNA clones for the μ, d, and k opioid receptors from several species have since been identified. Although subtypes for all three classes of opioid receptors have been proposed based on pharmacological studies, thus far only a single member from each class of opioid receptor has been cloned (Mattia et al. 1992; Pasternak 1986; Xu et al. 1991; Zukin et al. 1988). The fact that only one gene

locus was identified for each class of opioid receptors suggests that the subtypes identified by pharmacological studies may result from posttranslational, splicing modifications or differential interactions with associated proteins.

The cloning and sequencing of the opioid receptors also verified that these receptors belonged to the family of guanosine triphosphate binding protein (G-protein) receptors. G-protein receptors share some structural features such as seven alpha-helical hydrophobic domains that form the membrane spanning regions and methods of signal transduction that involve activation of specific G-proteins (Lameh et al. 1990; Strosberg 1991). It is the membrane-spanning regions and cytoplasmic loops that share the greatest sequence similarities between the classes of opioid receptors. The conservation of these regions within the family of opioid receptor genes may suggest overlapping functional and structural units of the opioid receptors (Douglass et al. 1984; Hershey et al. 1991; Traut 1988).

Several investigators have recently identified an additional G-protein receptor in rat (Bunzow et al. 1994; Chen et al. 1994; Fukuda et al. 1994; Wang et al. 1994a) and human brain (Keith et al. 1994; Mollereau et al. 1994) that has a high homology to the opioid receptor gene family. Although this receptor is not a μ, d, or k subtype, it shares significant homology to these opioid receptors at the amino acid level, particularly in the regions of the cytoplasmic loops and transmembrane domains. The abundant distribution of this receptor in structures of the hypothalamic-pitutary axis and cortical, spinal, and limbic nuclei suggests a role in neuroendrocrine function and the mediation of pain perception and analgesia (Bunzow et al. 1994; Keith et al. 1994; Wang et al. 1994a). Unfortunately, the lack of identification of the endogenous ligand for this receptor has delayed further characterization of the role of this relative of the opioid receptor family.

MOLECULAR CLONING AND GENE STRUCTURE

The genes for the human μ, d, and k opioid receptors have been isolated. The initial murine d opioid receptor cDNA was used by the authors to screen a human genomic phage library to identify clones containing the human genes. The authors subcloned μ, d, and k receptor fragments and sequence analysis of the three revealed the coding regions for the human opioid receptors. The human μ receptor gene shares 95 percent amino acid identity with the mouse

and rat μ receptors (Wang et al. 1994b). Three introns interrupt the μ receptor gene. The first two are located in the first and third intercellular domains. A third intron is located close to the carboxy tail of the receptor at amino acid position 397. Alternative splicing has been postulated at this site, which may result in a different carboxy terminus (Zimprich et al. 1994).

Analysis of the sequence of the human d gene fragment revealed 96-percent amino acid identity over 120 bases between the human genomic clone and the murine receptor cDNA (Selleri et al., in process). Knapp and coworkers (1994) also cloned a human d opioid receptor cDNA that encodes a 372 amino acid protein with 93 percent amino acid identity to the mouse d receptor. Partial sequence analysis by the authors of the human k gene showed a 100 percent DNA homology with the human sequence reported by Mansson and coworkers (1994). The introns in the murine k opioid receptor gene correspond to amino acid positions 86 and 184 (Yasuda et al. 1993). There appears to be conser-vation of these intronic positions in the human and rodent k, d, and μ opioid receptor genes (Mansson et al. 1994).

The opioid receptors, rhodopsin, the dopamine type 2 (D2)-like receptor, and the substance P receptors are a few among the many G-protein receptors reported to have introns within the coding region (Gingrich and Caron 1993; Hershey et al. 1991). In these receptors the intronic division within the coding region is similar, occurring at or near the membrane-spanning domain border. In each of the three opioid receptors the introns are found just beyond the first transmembrane spanning (TMS) region in the first intercellular loop and just beyond the fourth TMS region in the second cytoplasmic loop. The exception is the μ receptor, which also contains an additional intron near the carboxy terminus. The position of the introns in the human genes matches the position described for the rodent opioid receptors genes, which suggests stong evolutionary conservation of the intron/exon structure. The presence of introns and their precise location may be useful in defining functional units of the opioid receptors and in understanding the evolutionary divergence of the G-protein receptor genes.

Genetic regulation of opioid receptors is of great interest due to the role of the opioid system in syndromes believed to have heritable components such as pain response, metabolism of opiates, and addiction. Several groups of investigators are studying polymorphic markers for the opioid receptors. Two different types of

polymorphisms have been identified for the human μ receptor: a dinucleotide repeat and a restriction site polymorphism. PCR technology coupled with the identification of variable nucleotide repeats throughout the human genome has made it possible to rapidly type a particular allele. Only a fragment of the authors' clone of the μ receptor was found to contain a region containing a cytosine-adenosine (CA) dinucleotide repeat. PCR amplification of this region of DNA from 36 caucasian individuals reveals a significant degree of polymorphism (Selleri et al., in process). Wang and coworkers (1994b) also identified an Msp 1 polymorphism, which is under study to identify allelic variants associated with disorders of the opioid system.

CHROMOSOMAL LOCALIZATION OF OPIOID RECEPTORS

The chromosomal localization of the human μ, d, and k opioid receptor genes was determined by direct fluorescent in situ hybridization using the lambda phage DNA as probes to human metaphase chromosome preparations. Using this technique, the d receptor gene was mapped to chromosome 1p355-33, the k gene to chromosome 8q11.23-21, and the μ gene to chromosome 6q25-26 (Selleri et al., in process). Other investigators have also mapped the human opioid receptors to these locations (Befort et al. 1994; Wang et al. 1994b; Yasuda et al. 1994).

Each of the opioid receptors map to a single locus that apparently is not near receptors in which a mutation is suspected to cause any known human genetic disease. The chromosomal locations of the human μ, d, and k are in synteny with the reported position on mouse chromosomes (Selleri et al., in process). Southern analysis of murine opioid receptor genes in mouse neurogenetic mutants has not revealed gross alterations in the opioid receptor DNA (Befort et al. 1994; Kaufman et al. 1994). However, the information from polymorphic and chromosome markers will contribute to understanding the genetic regulation of the opioid receptors (Berrettini et al. 1994). Multiple familial and acquired diseases have been linked to abnormalities in G-protein receptors and the associated regulatory enzyme systems (Clapham 1993; Emala et al. 1994). Since the cloning of the opioid and other G-protein receptors has occurred quite recently, it is likely that additional diseases will soon be found that are linked to mutations or to regulatory alteration in these receptors.

LOCALIZATION OF OPIOID RECEPTOR mRNA

The field of opioid receptor biology has been advanced by anatomical studies of receptor distribution. Radiographic analysis with specific ligands revealed a unique neuroanatomical pattern for the μ, d, and k opioid receptors. With the cloning and isolation of the opioid receptors, direct analysis of the location of cells synthesizing opioid receptors can be made. A comparison of the correspondence between the binding data and the mRNA localization will provide insights into the trafficking and neural localization of these receptors.

The authors examined the distribution of the opioid receptor mRNA in the human prefrontal cortex and the striatum (Anton et al. 1994) and found that the patterns of distribution and expression level of the opioid receptor mRNA correlates well with the opioid receptor binding sites reported by autoradiography (Quirion and Pilapil 1991). In the prefrontal cortex, there is a high density of cells expressing μ opioid receptor mRNA found in cortical layers II and IV-V, and a moderate level of expression in layers I, III, and VI. Mu binding sites are the most selective for interaction with morphine and naloxone and they are also the most abundant and globally distributed opioid receptor in the human brain. The μ binding sites are concentrated in the superficial cortical laminae.

Delta opioid receptor mRNA was found in cells distributed homogeneously throughout all cortical laminae, although layers II-III and V-VI contained more densely labeled cells. Overall, the distribution of the d opioid receptor binding sites is similar to the μ receptor. In contrast, the k opioid receptor mRNA in the human prefrontal cortex is abundant in deep cortical laminae IV-VI. High levels of k opioid autoradiographic binding sites have also been reported within deeper cortical layers in this area, as well as other neocortical fields of human brain (Maurer et al. 1983; Pilapil et al. 1987; Quirion and Pilapil 1991). This localization in the deeper cortical layers has been postulated to induce the sedative properties of k agonists (Goodman and Snyder 1982).

The caudate-putamen is a central structure for the flow of information from the cortex to the ventral tegmental area and to the substantia nigra. Circuits involving these brain areas have been implicated in sensory-motor interaction, reward, and motivation. Moderate to high levels of hybridization signals were found in human caudate-putamen for μ, d, and k opioid receptor mRNAs (see table 1), although the density of μ opioid

TABLE 1. Opioid receptor mRNA distribution in human brain.

Region	Delta	Mu	Kappa
Prefrontal Cortex			
Layers			
I	0 to low	mod	0
II	mod	high	mod
III	high	low	mod
IV	mod	mod	high
V	high	high	high
VI	high	high	high
Striatum			
Caudate nucleus			
(interno-medial)	low to mod*	high*	mod to high**
Putamen	low to mod*	high*	high**

NOTE: 0, low, mod, and high indicate the relative density of cells expressing receptor mRNA.

KEY:0 = undetectable; mod = moderate; * = diffuse; ** = asymmetric cell clusters.

receptor was higher than for the d and k receptors. Cells containing µopioid receptor mRNA were homogeneously distributed throughout the human caudate-putamen. This homogeneous pattern of µ binding sites at the striatal level was also seen in human autoradiographic studies. These studies of the µ receptor highlight interesting species differences in the localization of opioid receptors. In the caudate-putamen, certain receptors and neurotransmitters preferentially bind the patch or matrix compart-ments. In the rat, guinea pig, and monkey the densest binding is observed for the µ receptor in the patches, and this neuroanatomical segregation is not seen in the human striatum (Herkenham and Pert 1981; Mansour et al. 1991).

Cells containing d and k opioid receptor mRNA were also found in the human caudate-putamen, though the levels of the d receptor were lower and more homogeneously distributed. A similar low density of d autographic binding sites was reported by Pilapil and coworkers (1987). Densely labeled k opioid receptor cells were grouped in clusters predomi-nantly in the caudate. These clusters of cells

expressing k opioid receptor mRNA suggests the patchlike striatal distribution. In support of these findings is the same striosomal compartmentalization of k opioid receptor binding sites in human caudate-putamen determined by the autoradio-graphic studies of Quirion and Pilapil (1991). To confirm the striosomal localization of k opioid receptor mRNA, additional histochemical studies are in progress using the combination acetylcholinesterase histochemistry and opioid receptor in situ hybridization in adjacent sections.

Prior to recent advances in the molecular characterization of the opioid receptors, the definition of classes of opioid receptors was made based on the order of receptor agonist and antagonist potency. The cloning of the opioid receptors has clearly identified a single gene for each of the human μ, d, and k opioid receptors. Conserved introns with the coding regions of these receptors may represent distinct functional domains. Conservation of the genomic structure suggests that there are overlapping functional and structural units, and perhaps evolution from a common ancestral gene. Conserved introns with the coding regions of these receptors may represent distinct functional domains. The question of subtypes remains, but the identification of an orphan opioid receptor promises interesting develop-ments on the horizon. The study of opioid receptor mRNA in the human establishes the foundation for further analysis of the precise distribution and mechanism of regulation of opioid receptor expression. Ideally, the analysis of the chromosomal localization will lead to the development of tools for the diagnosis and treatment of clinical syndromes associated with the opioid system.

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Molecular Biology of Opioid Receptors

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INTRODUCTION

Morphine and its alkaloid derivatives are used extensively in the treatment of pain (Gilman et al. 1990). These compounds are the most potent class of analgesics used clinically. The high potency and specificity of morphine suggest that it may bind to specific receptors in the nervous system to induce its biological effects. In the early 1970s, several groups of researchers identified specific opioid receptors in brain and peripheral tissues (Pert and Snyder 1973; Simon 1991). While these receptors were highly sensitive to morphine, morphine is not endoge-nously expressed in the body and therefore could not be the endogenous ligand for these receptors. This led to the search for the endogenous neurotransmitters at the opiate receptors and, in the mid-1970s, several groups identified the enkephalins (Hughes et al. 1975), dynorphins (Goldstein et al. 1979), and beta-endorphin (Bradbury et al. 1975) as endogenous peptide ligands for these receptors.

Both the enkephalins and dynorphins are neurotransmitters in the brain involved in pain perception, cognitive functions, affective behaviors, and locomotion, and they are involved in the central control of certain endocrine functions such as water balance (Herz 1993; Jaffe and Martin 1990; Simon 1991). Both peptides are widely distributed in the central nervous system (CNS) but localized to discrete neuronal pathways (Khachaturian et al. 1983). Beta-endorphin is expressed at much lower levels in the brain and only synthesized in a few neuronal pathways in the CNS, in particular in those originating from hypothalamic nuclei. As a result, the enkephalins and dynorphins are considered the predominant central opioid peptide transmitters.

The opioids induce their biological effects by interacting with three major classes of receptors: the delta, kappa, and mu receptors (Herz 1993). Delta opioid receptors express low affinity for dynorphin, but

high sensitivity to enkephalin, indicating that enkephalin may be an endogenous ligand for this receptor. In contrast, dynorphin A is very potent at kappa receptors, whereas these receptors have low affinity for enkephalin, suggesting that dynorphin A may be the endogenous transmitter for the kappa receptor. The mu opioid receptor expresses high affinity for enkephalin and the distribution of mu receptors and enkephalin messenger ribonucleic acid (mRNA) are highly correlated, suggesting that enkephalin may interact with mu receptors under physiological conditions (Delfs et al. 1994). Beta-endorphin binds potently to both the delta and mu receptor but has relatively lower affinity at kappa receptors, suggesting that in peripheral tissues, where endorphin is more abundant than enkephalins or dynorphin, beta-endorphin may be an endogenous ligand for the delta and mu receptors.

The pharmacological properties of the three opioid receptor classes are distinct and can be clearly differentiated (Goldstein and Naidu 1989; Herz 1993; Lutz and Pfister 1992; Portoghese 1993; Schiller 1993; Simon 1991). Delta opioid receptors have high affinity for the agonists deltorphin II, [D-penicillamine^{2}, D-penicillamine^{5}] enkephalin (DPDPE),[D-Ser^{2},O-Leu^{5}]-enkephaly-Thr (DSLET), and the antagonist naltrindole. These compounds essentially do not bind to the kappa or mu receptors. The kappa receptor has high affinity for the agonists U50,488 and U69,593 and the antagonist nor-binaltorphimine (nor-BNI). These agonists do not bind to the delta or mu receptor and the antagonist is much less potent at these other opioid receptors. Mu opioid receptors have high affinity for the peptide agonist [D-Ala^{2}-MePhe^{4}, Gly-ol^{5}] enkephalin (DAMGO), morphine and its derivatives, and the antagonists [D-Phe-Cys-Tyr-D-Trp-Orn-Pen-Thr-NH_2] (CTOP) and naloxonazine. DAMGO and the antagonists do not bind to delta or kappa receptors, and morphine and its derivatives are much less potent at the delta or kappa receptors. All three opioid receptors are sensitive to the antagonist naloxone, although to varying degrees. Kappa and mu receptors have high affinity for naloxone, while delta receptors have lower affinity for this antagonist.

The development of pharmacological agonists and antagonists that can distinguish the opioid receptors has facilitated investigations into their distinct functional roles. Such agents were necessary because most tissues express more than one opioid receptor type, which has made it difficult to study each receptor individually to reveal any unique biological actions. This problem has now also been overcome by the recent cloning of the three opioid receptor classes, since each

receptor can now be expressed in a separate cell line and their pharmacological, functional, and biochemical characteristics can be studied independent of the other receptors.

Cloning of the Opioid Receptors

The first opioid receptor to be cloned was the delta receptor. Two groups, Evans and colleagues (1992) and Kieffer and colleagues (1992), simultaneously identified the delta receptor from cDNA libraries derived from NG-108 neuroblastoma cells by expression cloning. Using the fact that opioid and somatostatin receptors have some similar pharmacological properties, Yasuda and associates (1993) cloned the kappa receptor as well as the delta receptor by screening a mouse brain cDNA library with probes selective for the cloned somatostatin receptors. The probes were directed against transmembrane spanning regions of the somatostatin receptors, which are conserved between the somatostatin and opioid receptors. Similarly, the rat kappa receptor cDNA has been cloned and its predicted amino acid sequence is almost identical to the mouse receptor (Meng et al. 1993; Minami et al. 1993). At approximately the same time as the initial cloning of the mouse kappa receptor, Chen and colleagues (1993) cloned the mu opioid receptor using probes against conserved regions of the delta opioid receptor to screen a rat brain cDNA library. This was possible because of the high degree of amino acid sequence similarity between the delta and mu opioid receptors, in particular in the transmembrane spanning regions, which is where the probes of Chen and associates (1993) were directed. Others (Thompson et al. 1993; Wang et al. 1993) have cloned the rat mu opioid receptor with similar if not identical amino acid sequences to those reported by Chen and colleagues (1993).

The predicted amino acid sequences of the three opioid receptors are shown in figure 1. They are approximately 65 percent identical with highest similarity in the transmembrane spanning regions and intracellular loops. Regions that diverge the most are the N- and C-termini and the extracellular loops. All three receptors have the aspartate-arginine-tyrosine (DRY) sequence and aspartates in the second and third transmembrane spanning regions that are conserved among guanosine triphosphate binding protein (G-protein)-linked receptors. Each receptor has multiple potential glycosylation sites in its N-terminal region. It is likely that each receptor has carbohydrate moieties associated with at least one of these sites since the size of the native receptors identified from biochemical studies is considerably larger than the size predicted

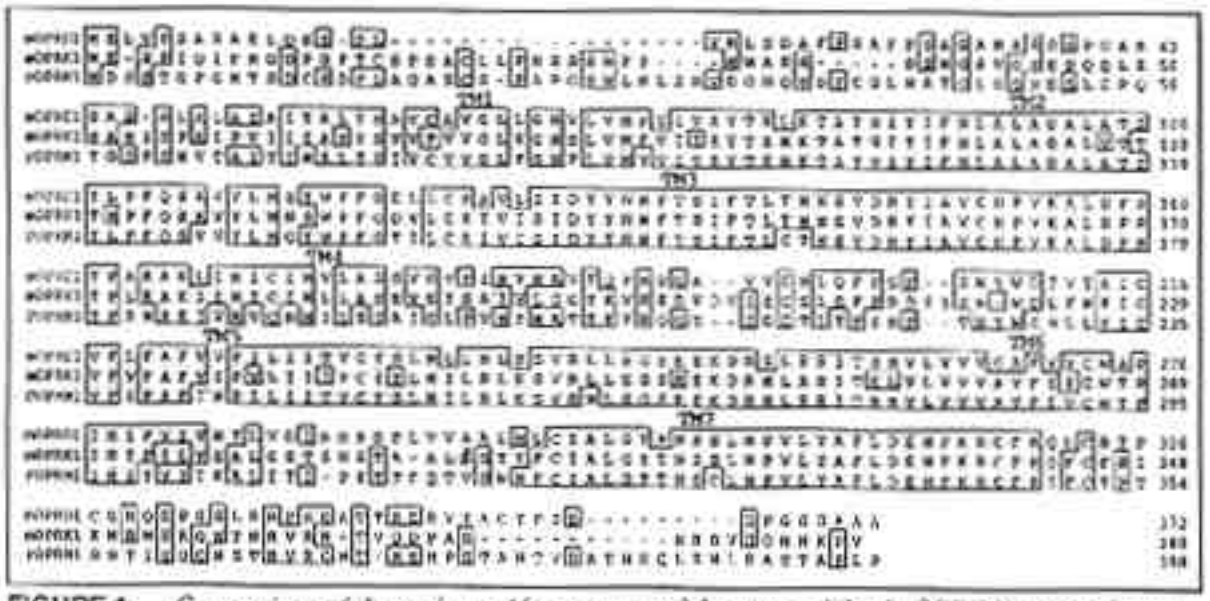

FIGURE 1. *Comparison of the amino acid sequences of the mouse delta (mOPRD1), mouse kappa (mOPRK1), and rat mu (rOPRM1) opioid receptors. The sequences are shown using the single-letter abbreviations for the amino acids. Residues identical in two or more of the receptors are boxed. Gaps introduced to generate this alignment are represented by dashes. The predicted transmembrane spanning regions are indicated by TM1 TM7.*

from the amino acid sequence of each cloned receptor. In fact, studies to covalently cross-link the cloned delta receptor expressed in CHO cells with ^{125}I-beta-endorphin reveals the size of the receptor as 55 to 60 kilodaltons (kDa), which is larger than the predicted size of 41 kDa (Raynor and Reisine, unpublished observations). Furthermore, the labeled receptor appears as a smear following SDS-PAGE and autoradiography, which is consistent with the glycoprotein nature of this receptor. Mutagenesis studies to knock out the glycosylation sites in these receptors may reveal the functional role of the carbohydrates on these receptors.

The three opioid receptors have high similarity to somatostatin receptors, particularly SSTR1, with approximately 40 percent amino acid sequence similarity (Reisine and Bell 1993; Yasuda et al. 1993). They have very low similarity with all other receptors. The similarity with somatostatin receptors is consistent with the pharmacological overlap of these receptors since some somatostatin analogs, including the clinically employed peptide SMS-201-995, which binds with moderate potency to mu opioid receptors (Maurer et al. 1982), and the extremely potent and selective mu antagonist CTOP, were developed using the structure of SMS-201-995 as its basis (Pelton et al. 1985).

PHARMACOLOGICAL AND FUNCTIONAL PROPERTIES OF THE CLONED OPIOID RECEPTORS

Following expression of the cloned receptors in COS-7, PC12, or CHO cells, the pharmacological and functional properties of these receptors were investigated (Kong et al. 1993*a*; Raynor et al. 1994*a*, 1994*b*; Reisine and Bell 1993; Yasuda et al. 1993). The pharmacological properties are described in table 1. The endogenous peptides, the enkephalins, potently bound to the cloned delta and mu receptors. Kappa receptors have very low affinity for these compounds. In contrast, dynorphin A potently bound to the kappa receptor, but it was less potent at the mu receptor and did not interact with the delta receptor. Both the delta and mu receptors have high affinity for beta-endorphin, whereas kappa receptors were relatively insensitive to this peptide. The binding of beta-endorphin to the receptors was dependent on the presence of the N-terminal tyrosine residue, consistent with the opioid nature of the receptors. The affinities of the cloned receptors for the endogenous peptide transmitters were similar to those reported for the native opioid receptors.

TABLE 1. Binding potencies (Ki-nM) of ligands for the cloned k, d, and µ opioid receptors.

	κ Receptor [3H]U-69,593	δ Receptor [3H]naltrindole	µ Receptor [3H]DAMGO
Nonselective Compounds			
Dynorphin A	0.5	>1,000	32
Leuenkephalin	>1,000	4.0	3.4
Metenkephalin	>1,000	1.7	0.65
ß-endorphin	52	1.0	1.0
des-Tyr1-ß endorphin	>1,000	>1,000	>1,000
(-)naloxone	2.3	17	0.93
(+)naloxone	>1,000	>1,000	>1,000
Levorphanol	6.5	5.0	0.086
Dextrorphan	>1,000	>1,000	>1,000
(±)bremazocine	0.089	2.3	0.75
Ethylketocyclazocine	0.40	101	3.1
Etorphine	0.13	1.4	0.23
Pentazocine	7.2	31	5.7
Diprenorphine	0.017	0.23	0.072
ß-FNA	2.8	48	0.33
Naltrexone	3.9	149	1.0
Nalbuphine	39	>1,000	11
Nalorphine	1.1	148	0.97

TABLE 1. Binding potencies (Ki-nM) of ligands for the cloned k, d, and µ opioid receptors (continued).

	κ Receptor [3H]U-69,593	δ Receptor [3H]naltrindole	µ Receptor [3H]DAMGO
Mu-Selective Compounds			
CTOP	>1,000	>1,000	0.18
Demorphin	>1,000	>1,000	0.33
Methadone	>1,000	>1,000	0.72
DAMGO	>1,000	>1,000	2.0
PLO17	>1,000	>1,000	30
Morphiceptin	>1,000	>1,000	56
Codeine	>1,000	>1,000	79
Fentanyl	255	>1,000	0.39
Sufentanil	75	50	0.15
Lofentanil	5.9	5.5	0.68
Naloxonazine	11	8.6	0.054
Morphine	538	>1,000	14

TABLE 1. Binding potencies (Ki-nM) of ligands for the cloned k, d, and µ opioid receptors (continued).

	κ Receptor [3H]U-69,593δ	Receptor [3H]naltrindole	µ Receptor [3H]DAMGO
Kappa-Selective Compounds			
NorBNI	0.027	65	2.2
Spiradoline	0.036	>1,000	21
U-50,488	0.12	>1,000	>1,000
U-69,593	0.59	>1,000	>1,000
ICI 204,488	0.71	>1,000	>1,000
Delta-Selective Compounds			
DPDPE	>1,000	14	>1,000
D-Ala2-deltorphin II	>1,000	3.3	>1,000
DSLET	>1,000	4.8	39
BW 3734	17	0.013	26
DADL	>1,000	0.74	16
SIOM	>1,000	1.7	33
Naltrindole	66	0.02	64
NTB	13	0.013	12
BNTX	55	0.66	18

Binding to the cloned receptors was stereospecific. The opioid antagonist (-) naloxone bound much more potently to the receptors than the (+)isomer. Similar selectivity was observed with the agonist levorphanol versus its inactive stereoisomer dextrorphan.

Kappa receptor selective agonists specifically bound to the cloned kappa receptor. Furthermore, the antagonist nor-BNI is one hundredfold selective for the kappa receptor. The pharmacological profile of the kappa receptor is consistent with it being a $kappa_1$ subtype defined previously in studies on the endogenously expressed kappa receptors (Clark et al. 1989).

Mu receptor selective agonists and antagonists specifically bound to the cloned mu receptor. Furthermore, many of the clinically used opioids, such as morphine, methadone, codeine, and compounds with high abuse potential such as fentanyl specifically bound to the cloned mu receptor. This finding suggests that the therapeutic as well as the side effects of physical dependence and respiratory depression induced by these compounds are due exclusively to their interaction with mu receptors.

A comparison of the potencies of a large series of opioids at binding to the cloned kappa and mu receptors with their potencies at binding to the endogenously expressed receptors reveals a very high correlation. This indicates that the ligand specificities of the cloned and endogenously expressed receptors are similar. This finding is of interest considering that the artificial cellular environment of the cell membrane where the cloned receptors are expressed would be expected to be quite different from the environment of the endogenously expressed receptor.

In contrast to the kappa and mu receptors, little similarity exists in the pharmacological profile of the cloned delta receptor compared to the endogenously expressed delta receptors. One clue that may explain this disparity is the much higher affinity of the cloned receptor for the antagonist NTB (the benzofuran analog of naltrindole) than the antagonist 7-benzylidenenaltrexone (BNTX). These compounds have been described as subtype selective, with BNTX selectively binding to the $delta_1$ receptor subtype and NTB selectively interacting with the $delta_2$ subtype (Portoghese 1993; Portoghese et al. 1993; Sofuglu et al. 1991*a*, 1991*b*). Furthermore, the $delta_2$ selective agonists DSLET and deltorphin II are more potent at binding to the cloned receptor than the $delta_1$ selective agonist DPDPE. These findings suggest that the cloned receptor may correspond to the $delta_2$ receptor subtype.

Since the radioligands that have been used to examine native delta receptors in brain do not distinguish the subtypes, the pharmacological profile of the endogenously expressed delta receptor may consist of a mixture of potencies at the two delta receptor subtypes, whereas the findings on the cloned receptor may represent the ligand selectivities of only one of the subtypes. If this is the case, the findings suggest that either one more delta receptor subtype remains to be cloned, or subtypes expressed endogenously in the nervous system and peripheral tissues are created by differential processing of a common gene product.

Following expression in cell lines, all three cloned opioid receptors have been found to couple to pertussis toxin-sensitive G-proteins and to mediate the inhibition of adenylyl cyclase activity by opioids (Chen et al. 1993; Kong et al. 1993*a*; Reisine and Bell 1993; Yasuda et al. 1993). Furthermore, the cloned kappa receptor has been reported to couple to a Ca^{++} channel and can mediate agonist inhibition of an N-type Ca^{++} current (Tallent et al. 1993). Kappa receptors in the brain have been reported to couple to N-type Ca^{++} currents in neurons (Gross and MacDonald 1987; North 1993; Weisskopf et al. 1993), and this interaction may be important for the ability of kappa agonists and dynorphin to presynap-tically inhibit neurotransmitter release. All three opioid receptors have been reported to couple to Ca^{++} and K^{+} channels and can mediate the modulation of ionic conductance through these channels via pertussis toxin-sensitive G-proteins (North 1993).

Neurotransmitter receptors have been proposed to couple to G-proteins via their intracellular loops (Dohlman et al. 1988). The intracellular loops of the opioid receptors are very similar, suggesting that they may couple to similar G-proteins, consistent with the similar effector systems they regulate. However, the C-termini of the receptors differ significantly in amino acid sequence, and for some neurotransmitter receptors the C-terminus has been shown to be critical for G-protein and effector system coupling. This has been most clearly shown in the case of the prostaglandin receptors (Breyer et al. 1994; Namba et al. 1993) and one of the somatostatin receptors, SSTR2 (Reisine et al. 1994), in which alternative splicing creates two receptors with divergent C-termini and differing abilities to couple to adenylyl cyclase. Direct studies to identify the G-proteins and effector systems associated with each cloned opioid receptor will be critical in establishing the cellular mechanism of action of opioids acting via each receptor type.

Structure-Function Analysis of the Cloned Opioid Receptors

The cloning of the opioid receptors has now allowed for analysis of the structural elements in the receptors involved in their functioning. Many G-protein-linked receptors can be regulated by sodium ions (Horstman et al. 1990; Kong et al. 1993*b*; Kosterlitz et al. 1988; Limbird et al. 1982; Minuth and Jakobs 1986; Neve et al. 1990; Pert and Snyder 1974). In particular, receptors that mediate agonist inhibition of cyclic adenosine monophosphate (cAMP) formation can have their affinity for agonists reduced in the presence of sodium ions. Sodium ions were first reported to reduce the affinity of opioid receptors for agonists (Kosterlitz et al. 1988; Pert and Snyder 1974). To investigate the mechanisms by which Na^+ regulates agonist binding to $alpha_2$-adrenergic receptors, Horstman and colleagues (1990) mutated an aspartate in the second transmembrane spanning region of the cloned $alpha_2$-adrenergic 2_a receptor to an asparagine and found that Na^+ regulation of agonist binding was abolished. Similar results were obtained with a mutation of the conserved aspartate in the second transmembrane spanning region of the somatostatin receptor subtype SSTR2 (Kong et al. 1993*b*). For both of these receptors few changes in ligand binding properties were reported. These studies indicated that a conserved aspartate in the second transmembrane spanning region of several G-protein-linked receptors was necessary for Na^+ regulation of agonist binding.

To investigate the mechanisms by which Na^+ modulates opioid receptor properties, Kong and associates (1993*a*) mutated aspartate 95 in the cloned mouse delta opioid receptor to an asparagine (see figure 2), and like the adrenergic and somatostatin receptors found that Na^+ regulation of agonist binding was lost, indicating that this residue was necessary for the modulatory actions of Na^+ on the delta receptor. However, in contrast to the results reported with the other receptors, the ligand binding properties of the mutant delta receptor were also altered. Whereas antagonist binding to the mutant and wild-type receptors was similar, selective delta receptor agonists had greatly diminished potencies at binding to the mutant receptor. This finding suggests that selective agonists and antagonists bind differently to the delta receptor, possibly by interacting with distinct ligand binding domains. Furthermore, non-selective agonists that are able to potently bind to other opioid receptors besides the delta receptor bound with similar affinities to the wild-type and mutant delta receptor. This finding indicates that selective and nonselective agonists bind differently to the delta opioid receptor. The findings of these mutagenesis studies indicate that aspartate 95 is

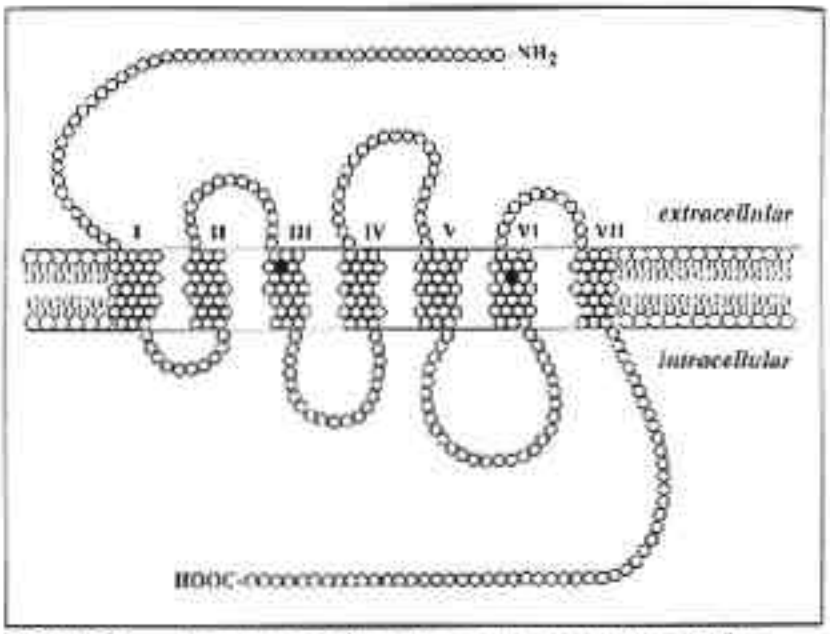

FIGURE 2. *Aspartates in the delta opioid receptor involved in ligand binding. The predicted amino acid sequence of the mouse delta opioid receptor is presented. The darkened circles represent aspartates that have been converted to asparagines by site-directed mutagenesis.*

necessary for Na^+ regulation of agonist binding to the cloned delta receptor. Furthermore, this amino acid residue appears critical for the high affinity binding of selective agonists to the delta receptor, but is not essential for the interaction of any other ligands with this receptor.

To further evaluate residues in the delta opioid receptor involved in ligand binding, Livingston and associates (submitted) mutated aspartate 128 in transmembrane spanning region 3 to an asparagine in the cloned mouse delta receptor (see figure 2) and tested the effects of this mutation on ligand binding. Previous studies on the beta-adrenergic receptor had shown that mutation of the aspartate 113 in the third transmembrane spanning region to an asparagine abolished high affinity antagonist binding to the receptor and modestly diminished agonist binding (Strader et al. 1987). It has been proposed that the aspartate provides a counterion to the cation of beta-adrenergic ligands and serves to stabilize binding through electrostatic interactions. Mutation of aspartate 128 of the delta receptor to an asparagine abolished high affinity agonist binding to the

receptor but did not alter antagonist binding. This finding differs from that reported on the beta-adrenergic receptor and indicates that agonists and antagonists bind differently to the adrenergic and delta receptors.

Both aspartate 95 and 128 of the delta receptor are necessary for high affinity binding of selective agonists. In contrast, only aspartate 128 is critical for nonselective agonist binding to the delta receptor. These findings indicate that different types of agonists at the delta receptor bind in fundamentally distinct manners. The aspartate 128 may serve as the counterion to the cation provided by charged nitrogens in opioid agonists. However, aspartate 95 contributes to the selectivity of recognition of specific delta agonists.

Interestingly, neither mutation of aspartate 95 nor 128 affected antagonist binding to the delta receptor. Antagonists also have positively charged nitrogens necessary for high affinity binding to opioid receptors. The lack of effect of mutation of the aspartates on antagonist binding suggests that some other residues within the delta receptor provide the negative counterion for antagonist binding. This finding supports the hypothesis that agonists and antagonists bind to the delta receptor via distinct molecular mechanisms, possibly to distinct ligand binding domains.

To more directly identify the ligand binding domains of the kappa and delta opioid receptors, Kong and associates (1994) have generated chimeric kappa/delta opioid receptor by exchanging the N-termini of each receptor and tested the mutant receptors for agonist and antagonist binding (see figure 3). Comparison of the predicted amino acid sequences of the kappa and delta opioid receptors reveals that the N-terminus is the region of each receptor that differs the most between each receptor and therefore is likely to contribute to variations in the ligand selectivity. A $kappa_{1-78}/delta_{70-372}$ bound delta selective agonists and antagonists in a similar manner as the wild-type delta receptor. Similar results were obtained with a truncated delta receptor lacking the N-terminal 70 residues. These findings indicate that the N-terminus of the delta receptor is not needed for ligand binding. It is likely that either the second and/or third extracellular loops of the delta receptor contain the ligand binding domains since these are the only other extracellular domains besides the N-terminus that differ in amino acid sequence between the delta receptor and the other opioid receptors. Mutagenesis studies to exchange these regions between the opioid receptor should help to test this hypothesis.

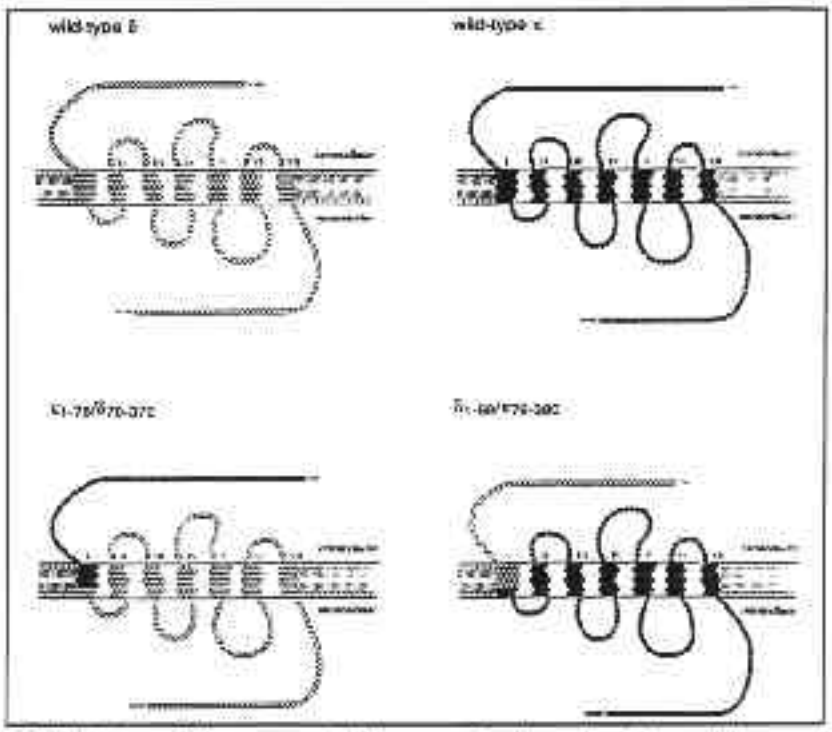

FIGURE 3. *Schematic of wild-type and chimeric delta and kappa opioid receptors.*

In contrast to the results with the mutated delta opioid receptor, the results of mutagenesis studies on the kappa receptor reveal that agonists and antagonists bind to this receptor in a manner fundamentally different from that found with any other neurotransmitter receptor (see figure 3). Kappa selective antagonists bound potently to the $kappa_{1-78}/delta_{70-372}$ chimera but kappa agonists did not. Conversely, kappa selective agonists interacted with a $delta_{1-69}/kappa_{79-380}$ chimera and a truncated kappa receptor lacking the N-terminal 78 amino acid residues. Kappa antagonists did not bind potently to these latter mutant receptors. These findings indicate that selective agonists and antagonists bind to clearly separable recognition sites in the kappa receptor with antagonists interacting with sites within the N-terminus and agonists binding to more C-terminal regions probably in either the second and/or third extracellular loops. The separation of the agonist and antagonist binding domains of the kappa receptor suggests that the N-terminus must be able to fold upon the receptor to allow the separate recognition sites to be in close proximity to allow for competi-tion of agonist and antagonist

binding. The identification of the specific residues in the kappa receptor involved in agonist and antagonist binding may facilitate the further development of therapeutically useful opioids. This will be particularly important in the case of the kappa receptor since kappa agonists have minimal abuse potential and do not cause respiratory depression, two major side effects of the use of mu receptor selective agonists. In contrast, kappa agonists are effective analgesics and useful diuretic agents.

Molecular Mechanisms of Tolerance Development to Opioids

A major limitation to the clinical use of opioids is the development of tolerance (Koob and Bloom 1992; Koob et al. 1992). With tolerance, increasing concentrations of an opioid are needed to maintain a constant therapeutic effect. All three opioid receptor types undergo tolerance development (Koob et al. 1992). The molecular basis of opioid tolerance is not established, although it has been suggested that tolerance development is related to opioid receptor desensitization (Childers 1991; Loh and Smith 1990; Nestler 1993). To investigate the molecular mechanisms of tolerance to kappa agonists, Raynor and colleagues (1994) expressed the cloned kappa receptor in mouse kidney fibroblast (COS) cells, treated the cells for increasing times with various kappa agonists, and showed that the kappa receptors desensitized. The desensitization was characterized by a reduced affinity of the receptor for agonists, an uncoupling of the receptor from G-proteins, and a diminished ability of the receptor to mediate agonist inhibition of adenylyl cyclase activity. The desensitization was reversible, blocked by opioid antagonists and induced in a stereoselective manner by agonists. Furthermore, the enzyme beta-adrenergic receptor kinase (BARK) (Benovic et al. 1989) appears to be involved in kappa receptor desensitization. This was suggested by experiments in which a BARK dominant negative mutant was cotransfected into COS cells with the kappa receptor cDNA; in those cells treatment with agonists did not cause receptor desensitization. The BARK dominant negative mutant has been shown to block the activity of wild-type BARK but can not catalyze the phosphorylation of substrates due to a single point mutation in its catalytic domain (Kim et al. 1993). These studies suggest that kappa agonists may stimulate BARK activity to catalyze the phosphorylation of the kappa receptor, thus causing an uncoupling of the receptor from G-proteins and effector systems. The intracellular domains of the kappa receptor have multiple potential phosphorylation sites that could act as substrates for BARK, and mutagenesis studies may reveal which one

of those sites is phosphorylated during desensitization and is critical for the desensitization of the kappa receptor.

Interestingly, the regulation of the three cloned opioid receptors by agonists differs considerably. Agonist pretreatment of COS cells expressing the cloned delta receptor results in rapid desensitization and downregulation of the receptor within an hour of treatment. This down-regulation of the delta receptor is not blocked by a BARK dominant negative mutant suggesting that different cellular mechanisms are involved in the regulation of the delta and kappa receptors. Furthermore, after 4 hours of agonist pretreatment, kappa receptors desensitize but do not downregulate, indicating that the sensitivity of the kappa and delta receptors to downregulation differs. This finding is of particular importance since the only intracellular regions of the two receptors that differ in amino acid sequence and that could represent domains of the receptors responsible for variations in agonist regulation are the C-termini. Mutagenesis studies to exchange the C-termini of the two receptors could test the hypothesis that these regions are involved in the regulation of opioid receptor sensitivity following agonist treatments and are critical for the development of tolerance of these receptors.

Prolonged treatment (4 to 16 hours) of COS cells expressing the cloned mu receptor did not desensitize nor downregulate this receptor. Since all three receptors were expressed in the same COS cell line, differences in sensi-tivity to agonist treatment cannot be explained by variations in cellular environment. Instead, these findings suggest that the three receptors are regulated differently at a cellular level. As a result, the cellular basis of tolerance to the three opioid receptors may be different. Tolerance to delta selective agonists may involve the desensitization and downregulation of the delta receptor. Tolerance to kappa selective agonists may involve the slow desensitization of the receptor via a BARK mediated mechanism. Tolerance to mu selective agonists such as morphine could involve postreceptor events such as changes in intermediate early gene expression or other cellular events. The fundamentally different adaptive responses of the opioid receptors to prolonged agonist treatment may reveal strategies to overcome tolerance development, which is a major limiting factor in the continuous use of opioids in the treatment of chronic pain.

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Mu Opioid Receptors: Cellular Action and Tolerance Development

Anton Mestek, Yan Chen, and Lei Yu

INTRODUCTION

Opioids are some of the most effective pain-relieving drugs used in the clinical management of pain (Gilman et al. 1990). In addition to their analgesic effect, opioid peptides and alkaloids also affect a number of physiological functions including hormone secretion, neurotransmitter release, feeding, gastrointestinal motility, and respiratory activity (Pasternak 1988). Extensive physiological, behavioral, and pharma-cological studies have defined at least three major types of opioid receptors designated mu, kappa, and delta (Corbett et al. 1993; Goldstein 1987; Wood and Iyengar 1988). Although there is substantial overlap in their tissue distribution and pharmacological profiles, each opioid receptor type maintains a unique pattern of expression while displaying characteristic binding affinities for various subtype-selective ligands. The delta receptors that bind the enkephalin peptides are expressed most predominantly in the basal ganglia, striatum, and cerebral cortex (Mansour et al. 1988; Wood 1988). Although delta receptors have been implicated in spinal analgesia (Porreca et al. 1984; Yaksh 1981), recently it has been suggested that specific delta receptor subtypes may also be involved in supraspinal analgesia (Pasternak 1993). The kappa receptors are most highly expressed in cortex, striatum, and hypothalamus (Mansour et al. 1987), with various subtypes identified by autoradiography using subtype-selective ligands (Nock et al. 1988; Unterwald et al. 1991). With the development of more highly subtype-selective ligands, these receptors have been shown to mediate both spinal and supraspinal analgesia (Pasternak 1993).

The mu receptor represents the third major class of opioid binding sites. Named after morphine, the mu opioid receptor is the physiological target of such potent analgesics as morphine and fentanyl, as well as the endogenous opioid peptides, ß-endorphin, enkephalins, and dynorphins (Wood and Iyengar 1988). Opioid drugs with high abuse liability such as morphine, methadone, and fentanyl all bind the mu receptor with high affinity. In addition, heroin (diacetylmorphine), a semisynthetic derivative of morphine, crosses the blood-brain barrier much more readily than morphine due

to its increased hydrophobicity. Once in the brain, heroin is rapidly hydrolyzed to morphine, which acts at the mu opioid receptor and results in a euphoric effect, thus conferring the reinforcing properties of the drug and contributing to the development of addiction. Because of the high affinity of these opioid narcotics at the mu receptor, it is considered the main cellular mediator in the development of tolerance (Loh et al. 1988) and opioid addiction (Di Chiara and North 1992).

Analgesia and the development of opioid tolerance, dependence, and addiction have been the subject of extensive studies (Collin and Cesselin 1991). Several schemes, including receptor-mediated modulation of membrane conductance, have been proposed for the acute and chronic actions of opioids in the central nervous system (Johnson and Fleming 1989). One scheme involves protein phosphorylation by various kinases as a possible way to regulate opioid-induced cellular processes. The molecular mechanism of such regulation, however, has not been clearly delineated.

MOLECULAR CLONING OF MU OPIOID RECEPTORS

To begin exploring the molecular basis for opioid tolerance, the authors sought to clone the cDNAs encoding mu opioid receptors. Using a strategy of low-stringency hybridization for isolating opioid receptors related to the mouse delta opioid receptor (Evans et al. 1992; Kieffer et al. 1992), the authors first reported the molecular cloning of a mu opioid receptor from the rat brain (Chen et al. 1993a), and at the same time, isolated cDNAs encoding a kappa opioid receptor (Chen et al. 1993b) and a novel member of the opioid receptor gene family (Chen et al. 1994). Using the rat mu opioid receptor as a hybridization probe, a cDNA for the human mu opioid receptor has also been isolated (Mestek et al. 1995). The human and rat mu opioid receptors are very homologous, with 94percent similarity at the amino acid sequence level (figure 1). The mu opioid receptors are also closely related to the delta and kappa opioid receptors, with 62percent and 58 percent similarities to the rodent delta (Evans et al. 1992; Fukuda et al. 1993; Kieffer et al. 1992) and kappa (Chen et al. 1993b; Liet al. 1993; Meng et al. 1993; Minami et al. 1993; Yasuda et al. 1993) opioid receptors, respectively. Figure 1 displays the alignment of the human mu opioid

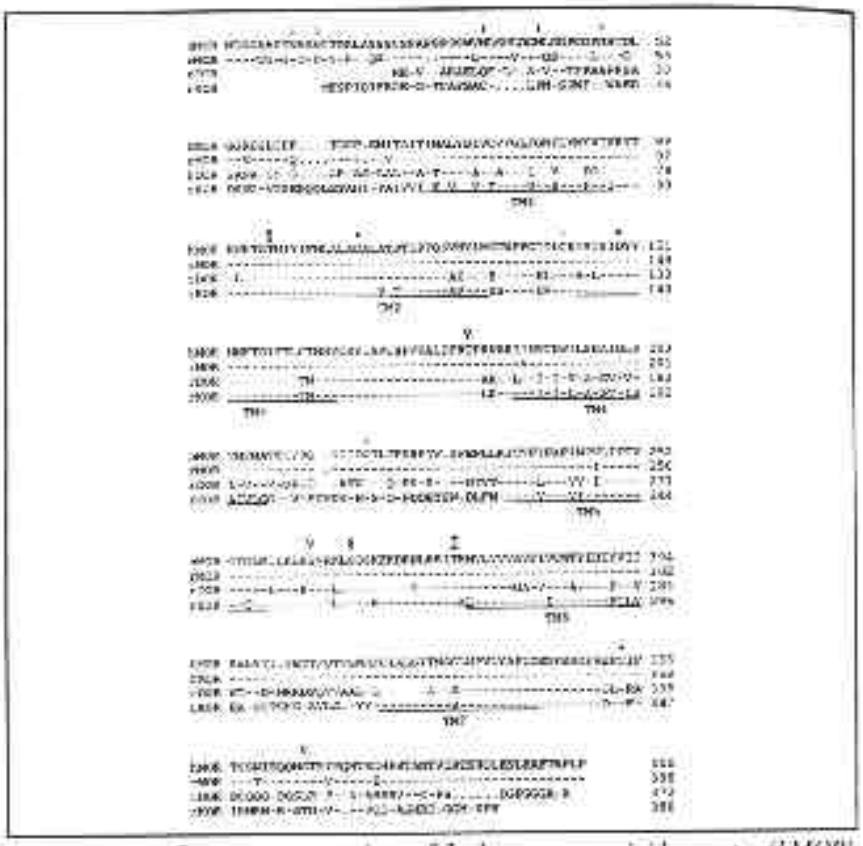

FIGURE 1. *Sequence comparison of the human mu opioid receptor (hMOR) and the rodent mu, kappa, and delta opioid receptors (rMOR, rDOR, and rKOR). Seven putative transmembrane domains are underlined and numbered TM1 through TM7.*

KEY:
- (-) = amino acids identical to those in human mu opioid receptor;
- (.) = gaps introduced for sequence alignment;
- (‡) = putative N-linked glycosylation sites;
- (§) = potential site for phosphorylation by multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase II);
- (♦) = conserved aspartic acid residues proposed to interact with the amine group of ligands;
- (=) = conserved cysteine residues that may form a disulfide bond;
- (▽) = potential sites for phosphorylation by protein kinase C (PKC);
- (⧖) = putative phosphorylation site for PKC or CaM kinase II;
- (⁀) = potential phosphorylation site for CaM kinase II or cAMP-dependent kinase A;
- (.) = potential palmitoylation site.

receptor sequence against the rodent mu, kappa, and delta opioid receptor sequences. The regions of greatest divergence among the different types of opioid receptors include the N-and C-termini, the fourth and sixth trans-membrane domains, and the second and third extracellular loops. Several potential sites for posttranslational modification are present (figure 1). The N-terminus contains five potential N-linked glycosylation sites that remain conserved between the human and rat mu opioid receptors. Aspartic acid residues that occur in other guanosine triphosphate binding protein (G-protein)-coupled receptors and have been shown to interact with the protonated amine group of various ligands (Dohlman etal. 1991) are also present in the putative transmembrane (TM) domains TM2 and TM3 of the mu receptor, and two conserved cysteine residues believed to be involved in disulfide bonding (Dixon et al. 1988) occur in the first and second extra-cellular loops. There are also potential phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC), as well as the multifunctional calcium/calmodulin-dependent protein kinase (type II Ca2+/calmodulin-dependent kinase, CaM kinase). One of these sites, conserved among all opioid receptor types, occurs in the third intracellular loop. This region between TM5 and TM6 is often referred to as the G-protein loop because of its importance in G-protein coupling (Dohlman et al. 1991). The conser-vation of this site suggests that phosphorylation may play a role in modula-ting signal transduction of all the opioid receptors. There is also a cysteine residue in the C-terminus that is conserved among many G-protein-coupled receptors and that may serve as a target for palmitoylation (Collins et al. 1991).

To characterize the pharmacological profile of the mu opioid receptors, cDNA fragments containing the protein coding region of each of the mu opioid receptors were subcloned into a mammalian expression vector containing the human cytomegalovirus promoter. Using these plasmids, the mu opioid receptor was either transiently expressed in COS-7 cells or stably expressed in Chinese hamster ovary (CHO) cells. Membranes from these cells were prepared and saturation binding was performed using either [3H]diprenorphine, a nonselective opioid antagonist with high affinity for all three types of opioid receptors, or [3H] [D-Ala2-Me Phe4, Gly-ol5]-enkephalin (DAMGO), a mu-selective agonist. The mu opioid receptors display high affinity binding to both [3H]diprenorphine and [3H]DAMGO, with dissociation constant (Kd) values in the nano-molar (nM) range. Also, various unlabeled opioid ligands were used in competitive displacement experiments. Several therapeutic opioid ligands, including morphine, methadone, and fentanyl, all displayed nM affinities at the mu opioid receptors, indicating that these highly potent analgesic drugs are mu-selective. In addition to these exogenous opioids,

all three types of the endogenous opioid peptides, ß-endorphin, Met- and Leu-enkephalins, and dynorphin A (1-17) displayed nM affinities at the mu receptors, suggesting that the mu receptors may be activated by any of these endogenous opioid peptides at physiological concentrations.

The high affinity of ß-endorphin at the mu receptors is expected, since it has long been thought that ß-endorphin is the endogenous ligand for the mu opioid receptor. Using the mu, delta, and kappa opioid receptor clones isolated from rodents, binding studies performed in parallel have demonstrated that enkephalins are also potent ligands at both the mu and the delta opioid receptors (Raynor et al. 1994). The nM affinity of dynorphin A (1-17) at the mu receptor is, however, somewhat unexpected, since dynorphin A has the highest affinity at the kappa opioid receptor (Goldstein 1987) and is thus considered a kappa agonist (Chavkin et al. 1982). However, studies have shown that while dynorphin A binds the kappa receptors with subnanomolar affinity, it does display reasonably good binding at the mu opioid receptors, with affinity values in the nM range (Goldstein 1987; Pasternak 1993). Thus, the possibility exists that the mu receptor may interact with dynorphin A under physiological conditions. Detailed studies of dynorphin A binding in the human brain are very limited. There are some reports, however, using membranes isolated from rat and bovine brain (Pasternak 1993). Using guinea pig brain membranes, it has been shown that dynorphin A (1-17) can displace radiolabeled PL-17, a mu-selective ligand, with a 5nM affinity (Kawasaki et al. 1990). In another study, dynorphin A (1-17) was found to displace radiolabeled DAMGO with 3 nM affinity (Vaughn and Taylor 1989). The authors' results further support the notion that the mu opioid receptor may serve as a natural target for dynorphin A.

CELLULAR FUNCTION OF MU OPIOID RECEPTORS

The mu opioid receptor is known to exert two types of inhibitory effects on a cell—reduction of the intracellular level of cyclic adenosine monophosphate (cAMP) and inhibition of neuronal firing. Using the cloned mu opioid receptors, the authors have begun to study the molecular mechanisms for both of these processes.

The mu opioid receptors contain seven transmembrane hydrophobic domains, a structural motif most commonly found in G-protein-coupled receptors (Chen et al. 1993a). This suggests that the mu opioid receptors may couple to heterotrimeric G-proteins to mediate intracellular signal transduction. Previous studies using cell lines that constitutively express endogenous opioid receptors also suggest that opioid receptors are

coupled to G-proteins (Carter and Medzihradsky 1993b; Childers 1991). Upon activation of a G-protein, the alpha subunit of the G-protein dissociates and displays an increase in its intrinsic GTPase activity. To test whether the cloned mu opioid receptor couples to G-proteins, GTPase activities from cell membranes expressing the mu receptor were measured in the presence and absence of mu-selective ligands. Treatment of CHO cells stably expressing the mu receptor with the mu-selective agonist DAMGO elevated the GTPase activity by 33percent. This stimulation was blocked by the opioid antagonist naloxone. In nontransfected parental CHO cells, on the other hand, GTPase activity was not affected by DAMGO treatment. Because an increase in the low affinity GTPase activity is indicative of G-protein activation, these results suggest that the mu opioid receptor is functionally coupled to the G-proteins in these mammalian cells.

Since it has been reported that activation of the mu opioid receptor exerts an inhibitory effect on adenylyl cyclase activity (Adams et al. 1991; Childers 1991; Cox 1993; Frey and Kebabian 1984; Sharma et al. 1975a), the authors next sought to examine the effect of mu opioid receptor activation on the intracellular levels of cAMP. In nontransfected COS-7 cells, treatment with the mu-selective ligands had no significant effects, while DAMGO stimulation of cells expressing the mu opioid receptor reduced the intracellular cAMP levels significantly. This effect on adenylyl cyclase activity was blocked by the mu-selective antagonist naloxonazine, indicating that the inhibitory effect was mediated through the activation of the mu opioid receptor and that the receptor was functionally coupled to the inhibition of adenylyl cyclase activity.

Studies in cell lines expressing endogenous opioid receptors demonstrated that chronic treatment with opioid agonists may modulate the coupling between opioid receptors and adenylyl cyclase (Carter and Medzihradsky 1993a; Puttfarcken et al. 1988; Sharma et al. 1975b). To test the effect of chronic morphine exposure on the intracellular levels of cAMP in CHO cells expressing the mu opioid receptor, cAMP levels were assayed by activation of the mu receptor with DAMGO before and after morphine treatment. Before chronic morphine treatment, the forskolin-stimulated cAMP level in morphine-naive cells was reduced to 70 percent relative to controls upon treatment with 1 micromlar (µM) DAMGO. After the cells were exposed to morphine for 24 hours, DAMGO-induced inhibition of cAMP levels was enhanced, resulting in a decrease of cAMP levels to 40percent relative to controls. This result suggests that morphine treatment may enhance the effectiveness of the mu receptor-mediated inhibition of adenylyl cyclase activity. When the forskolin-stimulated cAMP levels in naive cells were measured in the presence of

different concentrations of DAMGO, the intracellular cAMP levels were reduced by DAMGO in a dose-dependent fashion, with a 50percent effective concentration (EC50) value of about 30 nM. The effect of DAMGO was clearly the result of the mu receptor activation, since treatment of the cells with 1 µM DAMGO in the presence of 10 µM naloxone completely abrogated the inhibitory effect of DAMGO on cAMP accumulation. In contrast, DAMGO had no effect on the intracellular cAMP levels in non-transfected parental cells. Chronic treatment with morphine for 24hours enhanced the extent of the DAMGO-induced inhibition of adenylyl cyclase activity, but did not change the EC50 value of the DAMGO-induced inhibition. To test whether the number of receptors was changed upon chronic morphine treatment, CHO cells expressing the cloned mu opioid receptors were chronically treated with 1 µM of morphine for 24 hours. Using [3H]DAMGO and whole-cell binding to determine the number of the total surface receptors, it was observed that there was no appreciable change in the number of surface receptors, nor was there any noticeable change in the affinity for DAMGO after chronic morphine treatment. These results suggest that, unlike the changes in adenylyl cyclase activities upon morphine treatment, receptor downregulation does not occur after chronic morphine treatment in these CHO cells.

The EC50 value calculated from the dose-response curve observed with DAMGO after chronic morphine treatment was about 30 nM, compared to the 2 nM 50 percent inhibitory concentration (IC50) value derived from the competition binding studies. The difference between the functional potency and the binding affinity values may be due to the ratio of mu receptors on the cell and the G-protein or effector molecules to which they couple, but other possibilities may also exist. It should be noted that the efficacy for adenylyl cyclase inhibition by the mu opioid receptor in the CHO cells is similar to that in SK-N-SH and 7315c cells (Frey and Kebabian 1984; Yu et al. 1990). The relative efficacy of mu opioid receptors to inhibit adenylyl cyclase activity seems to be lower than that of other G-protein-coupled receptors that are linked to the inhibition of adenylyl cyclase, such as the a2-adrenergic receptor, serotonin 1A receptor, and dopamine receptors (Fraser et al. 1989; Raymond et al. 1992; Sokoloff et al. 1992). The differences in the ability of each receptor type to inhibit adenylyl cyclase suggests that different G-proteins may be involved in coupling distinct receptors to a common effector molecule. Since more than one adenylyl cyclase has been identified in the cell (Iyengar 1993), an alternative possibility may be that different subtypes of adenylyl cyclases couple differently to the various membrane receptors.

The protocol the authors used for studying the effect of chronic morphine treatment is similar to that used by many investigators (Puttfarcken and Cox 1989). First, cells are chronically exposed to a moderate concen-tration of agonist (morphine). The cells are washed to remove agonist, and then are acutely stimulated with various concentrations of agonist. Because the presence of the agonist during the chronic exposure presents a continued inhibitory input, agonist washout before acute treatment is equivalent to disinhibition, and has been compared to the withdrawal paradigm following the establishment of opioid tolerance (Sharma et al. 1975b). In the authors' cell line, both the basal and forskolin-stimulated cAMP levels were elevated after chronic morphine treatment and withdrawal. This suggests that a compensatory mechanism involving increased adenylyl cyclase activity may be responsible for the higher levels of cAMP that have been observed upon removal of mu receptor-mediated inhibition (Puttfarcken and Cox 1989; Sharma et al. 1975b). It is interesting to note that chronic morphine exposure displays a differen-tial effect on cAMP levels. Basal cAMP levels were about 60percent greater after morphine exposure than that observed in naive cells, while the forskolin-stimulated cAMP levels were more than doubled with morphine treatment. It is tantalizing to speculate that this difference between basal and forskolin-stimulated cAMP levels during chronic morphine exposure reflects a differential sensitivity to acute mu receptor activation of two populations of adenylyl cyclase molecules—those present before morphine exposure and those synthesized during morphine pretreatment as the compensatory mechanism becomes engaged. It is conceivable that these two populations of adenylyl cyclases may have different compositions with regard to cyclase subtypes, since molecular cloning has shown that multiple adenylyl cyclases exist (Iyengar 1993).

Another phenomenon of morphine treatment is that the DAMGO-induced cAMP inhibition in the morphine-treated cells is more robust, which resulted in doubling the percent inhibition of cAMP from approximately 25percent in naive cells to almost 60 percent in morphine-treated cells. These results suggest that morphine exposure "sensitized" the system, such that acute mu receptor activation was more effective in inhibiting the adenylyl cyclase activity. This is in contrast to the studies with cell lines expressing endogenous mu receptors, in which chronic morphine treatment caused a decrease in the ability of the receptor to inhibit adenylyl cyclase (Puttfarcken and Cox 1989; Werling et al. 1989). The lack of receptor desensitization in CHO cells suggests that receptor modulation of adenylyl cyclase in various cell types occurs differently. This may reflect the uniqueness in the composition of endogenous G-proteins and/or adenylyl cyclases in CHO cells versus

other cell types. Also of interest is that chronic morphine treatment of the transfected CHO cells did not cause downregulation of surface receptors. In morphine-treated cells, neither the number of cell surface receptors nor the Kd value for DAMGO was significantly different from those observed with naive cells. This is in contrast to agonist-induced downregulation in ß2-adrenergic receptors (Collins et al. 1991). The increase in cAMP levels observed with chronic morphine treatment and the sensitization of the signal transduction pathways involving adenylyl cyclase suggest that this cell line could serve as a cellular model, allowing the study of both the molecular mechanisms that link the mu opioid receptor to its effector systems and the changes associated with morphine tolerance and dependence.

Activation of opioid receptors has been known to affect membrane permeability to potassium (North 1993). Stimulation of the mu opioid receptor hyperpolarizes cellular membranes by increasing the K+ conduc-tance through an inwardly rectifying channel (North et al. 1987; Wimpey and Chavkin 1991). The recent cloning of an inwardly rectifying K+ channel (Dascal et al. 1993; Kubo et al. 1993) was shown to be expressed in the brain, and the authors were interested in testing whether the mu opioid receptor coupled to this G-protein-activated K+ channel (Chen and Yu 1994; Mestek et al. 1995). Messenger ribonucleic acids (mRNAs) encoding both proteins were generated by in vitro transcription and injected into Xenopus oocytes. Coupling of the receptor to the K+ channel was assessed by a two-electrode voltage clamp. In the oocytes injected with either the mu receptor mRNA or the K+ channel mRNA alone, no membrane current was observed with the mu-receptor agonist DAMGO. This indicated that there were no endogenous currents in oocytes activated by DAMGO, and that either the mu receptor or the K+ channel alone is not sufficient to generate DAMGO-induced currents. However, coexpression of both proteins gave rise to membrane currents upon DAMGO stimulation (Chen and Yu 1994; Mestek et al. 1995). Exposure of the oocytes to 1 µM of DAMGO produced an inward membrane current that was completely blocked by the opioid receptor antagonist naloxone. This current was carried through the inwardly rectifying K+ channel, since the current varied in amplitude with the concentration of K+ in the extracellular solution and was blocked by 100µM Ba2+, a known K+ channel blocker. The current-voltage relationship of this K+ channel was characteristic of an inward rectifier. With progressive hyperpolarization, the magnitude of current increases. However, as the membrane is depolarized, current flow decreases until there is little to none at a membrane potential of 0 millivolts (mV). Thus, the mu opioid receptor is capable of coupling to the G-protein-activated K+ channel.

Prolonged exposure to opioids is known to produce tolerance in neurons (Di Chiara and North 1992). At the cellular level, tolerance development is observed as a diminished response to opioids (Johnson and Fleming 1989). Since opioid narcotics with abuse potential such as morphine, methadone, and fentanyl interact with the mu opioid receptor with high affinities, the authors were interested in examining whether the intracellular signaling by the mu opioid receptor displays tolerance. Using the receptor-K+ channel coupling as an assay, oocytes were subjected to repeated agonist stimu-lation to determine whether functional desensitization of the mu receptor-K+ channel coupling occurred. For this purpose, a protocol of repeated agonist application was used, and "desensitization" was operationally defined as a reduction in the second response compared to the first response. The oocyte was superfused with high-potassium (HK) solution while the DAMGO-induced responses were measured. The superfusate was then switched to frog Ringer's solution and the cell was subjected to the various treatments. After treatment, DAMGO-induced responsiveness was measured again in HK solution. Comparison between the maximal response before and after the treatment thus indicates how much desensi-tization has occurred following the first stimulation with DAMGO. Repeated stimulation of the mu receptor resulted in a moderate and consistent desensitization as observed by a reduction in the second response. The second response was reduced to 80 percent relative to the first response. These results suggest that when expressed in oocytes, the coupling between the mu opioid receptor and the G-protein-activated K+ channel undergoes a process of desensitization.

Previous studies had shown that activation of PKC was capable of attenuating opioid receptor activity in neuroblastoma cells (Louie et al. 1990) as well as affecting ion conductances using acutely dissociated neurons in culture (Doerner et al. 1988). The authors were interested in observing whether stimulation of PKC affects the coupling of the mu opioid receptor to the K+ channel. Using the protocol described above, oocytes were superfused for 10 to 15 minutes after the initial stimulation with phorbol 12-myristate 13-acetate (PMA), a PKC-activating phorbol ester. Oocytes were again stimulated with DAMGO. Comparison between the peak current response to DAMGO before and after PMA treatment indicated that PMA reduced the second response by 70percent relative to the first. Thus, activation of PKC augmented the desensitization of the mu opioid receptor-activated K+ current. To control for possible non-specific effects caused by application of a phorbol ester to the cellular membrane, oocytes were treated with 4a-phorbol, a phorbol ester that does not activate PKC (Blumberg et al.

1984). Recordings of peak current amplitude show that the 4a-phorbol does not augment desensitization beyond that observed with no treatment, as membrane currents before and after 4a-phorbol treatment exhibited moderate desensitization similar to that with no treatment. This demonstrated that the inactive form of the phorbol ester does not augment desensitization. Therefore, these results indicate that augmentation of the desensitization by PMA reflects the activation of PKC.

Physiologically, PKC is activated by diacylglycerol (DAG), a second messenger of the phosphatidylinositol pathway. This signaling pathway also generates another second messenger, inositol 1,4,5-trisphosphate (IP3), which triggers an increase in intracellular calcium and results in the activation of the multifunctional Ca2+/calmodulin-dependent protein kinase (type II Ca2+/calmodulin-dependent protein kinase, CaM kinase II) (Schulman and Hanson 1993). The authors were interested in testing whether CaM kinase II could modulate the mu opioid receptor-mediated K+ current. Using the protocol described above, oocytes were injected with the activated form of CaM kinase II between the first and second stimulations with DAMGO, and the effect on the receptor-induced K+ current was evaluated. CaM kinase II clearly enhanced the desensitization, causing a twofold decrease in the second response relative to uninjected oocytes. As a negative control, the same CaM kinase II solution was placed in a boiling water bath for 5 minutes and chilled on ice before injection into the oocytes. Boiled CaM kinase II did not augment desensitization beyond that observed with uninjected controls. The authors concluded that activated CaM kinase II can therefore augment the desensitization of the mu opioid receptor-activated K+-current.

Since the mu opioid receptor can modulate the activity of adenylyl cyclase, there was also interest in determining whether cAMP, the product of adenylyl cyclase, had any effect on the mu receptor-K+ channel coupling (Chen and Yu 1994). Treatment of the oocyte with 8-chlorophenylthio-cAMP (8-CPT-cAMP), a membrane-permeable cAMP analog that can diffuse into the cell and stimulate cAMP-dependent protein kinase (PKA), completely abolished the desensitization observed in untreated oocytes, resulting in the K+ current being similar in amplitude for both the first and the second stimulation with DAMGO. To determine whether the 8-CPT-cAMP effect on preventing desensitization was mediated by PKA, the catalytic subunit of PKA was injected into the oocytes after the first DAMGO stimulation. This resulted in the same effect as 8-CPT-cAMP incubation, indicating that the blockade of desensitization was indeed mediated through PKA.

DISCUSSION

A Molecular Mechanism for Mu Opioid Receptor Function and Its Acute Desensitization

Results from the authors' studies indicate that cloned mu opioid receptor cDNAs can be used to express functional mu opioid receptors that display the expected pharmacological profile. The cellular functions affected by the activation of the mu opioid receptor include the inhibition of adenylyl cyclase activity and the activation of a K+ channel. Various protein kinases have also been shown to modulate mu receptor-mediated cellular processes. Based on these results, the authors propose the following model for mu receptor function and its acute desensitization. This model, shown schematically in figure 2, is discussed below.

A major effect of the mu opioid receptor in brain is the decrease of neuronal membrane excitability. One of the mechanisms for this effect is an increase in K+ conductance, accomplished by the opening of an inward rectifier, resulting in outward K+ currents and hyperpolarization of the cell membrane (Chavkin 1988; North 1986, 1993). With the cloning of an inwardly rectifying K+ channel that can be activated by a number of neurotransmitter receptors (Dascal et al. 1993; Kubo et al. 1993), it became possible to examine whether the mu opioid receptor could also activate this channel. It has been shown that the mu opioid receptor from both rat (Chen and Yu 1994) and human (Mestek et al. 1995) can activate this channel, causing an increase in K+ conductance. The receptor-channel coupling is clearly mediated through heterotrimeric GTP-binding proteins (G-proteins), since a nonhydrolyzable GTP analog, GTP-gS, can enhance the mu receptor-activated K+ current and pertussis toxin treat-ment can decrease it (Chen and Yu 1994). Thus, the authors' data

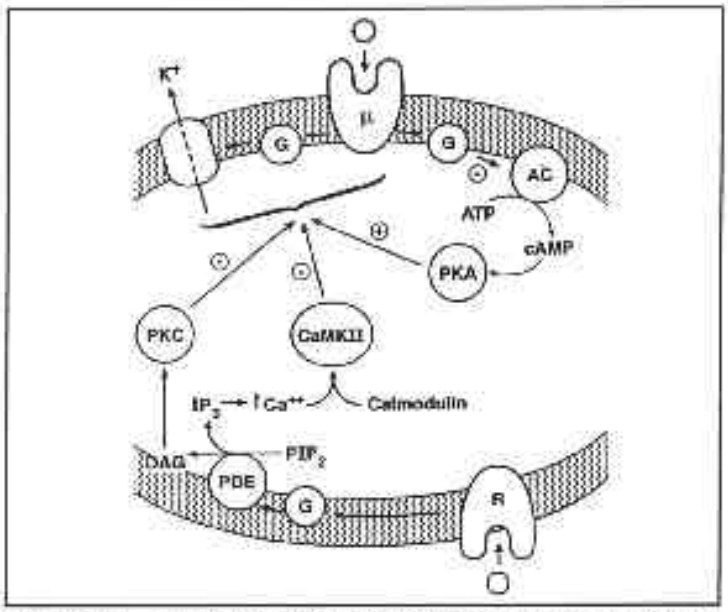

FIGURE 2. *A model depicting the molecular mechanism for mu opioid receptor function and its acute desensitization. The mu opioid receptor and another type of surface receptor in the cell membrane are shown as mu and R, respectively. Open circles represent ligands for the respective receptors. Potassium channel is shown with ionic efflux.*

KEY: G = G protein; AC = adenylyl cyclase; PKA = cAMP-dependent protein kinase; PKC = protein kinase C; CaMKII = multifunctional Ca^{2+}/calmodulin-dependent protein kinase; PDE = phosphodiesterase involved in PIP_2 hydrolysis. DAG = diacylglycerol; PIP_2 = phosphatidylinositol 4,5-bisphosphate; IP_3 = inositol 1,4,5-trisphosphate; ATP = adenosine triphosphate; cAMP = adenosine 3', 5'-cyclic monophosphate. Arrows pointing from second messenger molecules to proteins indicate a stimulatory influence. Arrows between proteins indicate either a stimulatory (+) or inhibitory (-) effect on interactions. Upward arrow adjacent to Ca^{++} indicates an increase in intracellular calcium.

SOURCE: Adapted from Mestek et al. 1995.

suggest that the mu receptor-channel coupling may be the basis for the mu receptor-mediated increase in K+ conductance.

Receptor-mediated signaling processes often display desensitization, operationally defined as a decrease in the cellular response to further agonist stimulation upon continuous or repeated exposure to agonist (Benovic et al. 1988). This may serve as a physiological mechanism to prevent overstimulation of the neuron. The mu opioid receptor is the physiological target of morphine and fentanyl, analgesics used in the clinical management of pain. Prolonged use of morphine and related opioids can lead to the development of tolerance, necessitating dosage increases to achieve the same degree of their initial physiological effect. At the cellular level, tolerance manifests itself as a desensitized responsiveness to repeated opioid applications, and it has been hypothesized that several of the intermediates in the mu receptor signaling pathway are involved (Nestler et al. 1993). In the neurons of the rat locus coeruleus, desensiti-zation was observed as a reduction in membrane hyperpolarization upon continued application of Met5-enkephalin. A decrease in K+ conductance was shown to be responsible for the observed effect (Harris and Williams 1991). In oocytes expressing the mu opioid receptor and the inwardly rectifying K+ channel, a protocol was used to evaluate desensitization by measuring the K+ currents evoked by sequential activation of the receptor with a mu-selective agonist. Comparison of the maximum K+ currents thus indicates the extent of desensitization between the first stimulus and second stimulus caused by activation of the receptor. Using this paradigm, desensitization occurred consistently, as observed by the reduction of the K+ current to 80 percent of the initial response evoked by receptor activation. Thus, desensitization of receptor-channel coupling appears to be a normal process when studied in oocytes, suggesting that such a phenomenon may exist as an adaptive process in neurons to modulate the responsiveness of the mu receptor-mediated increase in K+ conductance.

Desensitization of receptor-channel coupling may involve several mechanisms at the cellular level. For acute desensitization such as that studied here with a timescale of fewer than 30 minutes, de novo protein synthesis or receptor turnover are unlikely to account for the majority of the observed effects. Covalent modification through kinase-mediated phosphorylation, on the other hand, appears to play an essential role. The authors demonstrated that both PKC and CaM kinase II augment desensitization. Activation of PKC by the phorbol ester PMA and injection of the type II CaM kinase activated by autophosphorylation both resulted in augmentation of desensitization.

In contrast, treatment with the inactive 4a-phorbol ester or injection of boiled CaM kinase II did not augment desensitization beyond that observed in oocytes that had been untreated, suggesting that the augmentation effect is specific to the active form of these kinases. When activated individually, PKC and CaM kinase (Akasu and Tokimasa 1992; Shearman et al. 1989) have been shown to decrease K+ conductance in neurons, which supports the authors' observations in Xenopus oocytes. Furthermore, in rat locus coeruleus neurons containing mu opioid receptors, treatment with staurosporine, a nonselective kinase inhibitor that can inhibit both PKC (Tamaoki 1991) and CaM kinase II (Yanagihara et al. 1991), reduced desensitization of mu receptor-mediated hyperpolarization (Harris and Williams 1991). Processes that elevate the activity of these cellular kinases, therefore, may play an important role in regulating the extent of the mu receptor-K+ channel coupling.

Both PKC and CaM kinase II are cellular effectors of a G-protein-activated phosphodiesterase, phospholipase C (PLC). The activation of PLC causes the production of DAG and IP3, two intracellular second messenger molecules that represent a bifurcation in the signal transduction pathway (Berridge 1987). Whereas DAG activates PKC, IP3triggers Ca2+ release from intracellular stores. Since CaM kinase II is activated by physiological elevations in cytosolic Ca2+ levels (MacNicol and Schulman 1992a; Schulman and Hanson 1993), stimulation of receptors linked to PLC may cause activation of both PKC and CaM kinase (figure 2). Other neuro-transmitter receptors that belong to the family of G-protein-coupled receptors influence the steady-state levels of cAMP by either stimulating or inhibiting the activity of adenylyl cyclase, respectively, as is the case for the ß-adrenergic and opioid receptors. The widespread distribution of many G-protein-coupled receptors suggests that some may be found within similar structures of the brain. In fact, in situ hybridization has shown that messages encoding receptors that use either similar (Lester et al. 1993) or different (Weiner et al. 1990) signaling pathways do coexist within the same cell. The authors' data suggest that PLC-coupled receptors may augment the process of desensitization, which is observed as a reduction in receptor-channel coupling upon repeated stimulation of the mu opioid receptor (figure 2). Thus, it is plausible that signal transduction mechanisms may affect one another when the receptors to which they couple are present on the same neuron.

Since opioid receptor activation has been shown to affect cAMP levels, another important kinase in the regulation of opioid receptor activity is PKA. Opioid receptor activation inhibits adenylyl cyclase, resulting

in decreased levels of cAMP and a reduction in basal PKA activity. The authors previously demonstrated that an increased PKA activity, either by injection of the catalytic subunit of PKA into the cell or by exposing the cell to a membrane-permeable cAMP analog, eliminated desensitization of the mu receptor-K+ channel coupling (Chen and Yu 1994). Thus, agents that activate PKA would function antagonistically in relation to the mu opioid receptor-mediated channel activity. This presents an interesting control mechanism whereby the activity of the channel is subject to negative feedback inhibition modulated by the mu opioid receptor via a decrease in PKA activity (figure 2).

Phosphorylation by kinases is one of the most important mechanisms for the functional regulation of many cellular proteins including neuro-transmitter receptors and ion channels, with PKA and PKC being two of the most widely studied kinases (Krebs 1989; Shearman et al. 1989). Phosphorylation of ß2-adrenergic receptor by either PKA or PKC leads to its uncoupling from G-proteins, resulting in desensitization to further agonist stimulation (Lefkowitz and Caron 1988; Sibley and Lefkowitz 1985). In the case of voltage-dependent Ca2+ channels such as the endogenous oocyte Ca2+ channel, PKA- and PKC-mediated phosphory-lation is able to potentiate channel activity (Bourinet et al. 1992; Chen et al. 1993c). Cystic fibrosis transmembrane conductance regulator (CFTR), a Cl- channel associated with cystic fibrosis, is also regulated by cAMP through the PKA pathway (Anderson et al. 1991; Bear et al. 1991). The authors have attempted to investigate the role of each kinase in the mechanisms involved in acute desensitization. Figure2 depicts the individual effects of these kinases on opioid receptor-mediated K+ channel activity. Although precedents exist for their acting independently, these kinases are also subject to "cross-talk," which is the ability of one signal transduction mechanism to affect another (MacNicol and Schulman 1992b; Yamakawa et al. 1992). Taken together, the data suggest that an intricate network of modulation among receptors, G-protein effectors, and protein kinases may exist. It should be noted that although each element in the model has been demonstrated in oocytes and/or neurons, the complete scheme has not yet been confirmed with respect to the cross-modulation that may occur among multiple receptor types. With the cloning of the mu opioid receptors and the other proteins involved in cellular signaling, future efforts will undoubtedly further researchers' general understanding of the molecular mechanisms that underlie the regulation of receptor-mediated neuronal activity, with particular focus on the modulatory network involved with mu opioid receptor function.

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Cloning and Characterization of Multiple Opioid Receptors

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THE CLONING OF THE THREE MAJOR OPIOID RECEPTOR TYPES

Until recently, the study of opioid receptors, while greatly profiting from the rich array of synthetic peptides and alkaloids with high affinity and specificity, has been hampered by the absence of opioid receptor clones. Matters were changed, however, in December 1992, when two groups, Evans' and Kieffer's, independently cloned the mouse delta receptor from the NG-108 cell line (Evans et al. 1992; Kieffer et al. 1992). This work opened the door for rapid advances in this area. Several groups, including the authors, subsequently cloned the remaining two major types of opioid receptors from various rodent species (Chen et al. 1993; Fukuda et al. 1993; Kong et al. 1993; Meng et al. 1993; Minami et al. 1993; Thompson et al. 1993; Wang et al. 1993; Xie et al. 1994; Yasuda et al. 1993). The authors have independently cloned the three prototypical mu, delta, and kappa opioid receptors from rat brain, as well as the kappa receptor from guinea pig brain. The authors also have partial delta and mu receptor clones from guinea pig brain, which are currently being fully cloned and characterized (Fickel et al. 1994). In addition, in searching for other opioid receptor subtypes from rat and guinea pig brain, the authors have isolated several clones, also obtained by other laboratories (Mollereau et al. 1994), which appear to encode a protein closely related to the opioid family, but fails to bind a number of opiate alkaloids or opioid peptides. Thus, the question of the existence of opioid receptor subtypes at the molecular level remains to be fully elucidated.

SELECTIVITY OF THE CLONED RECEPTORS TOWARD THE ENDOGENOUS OPIOID LIGANDS

Since the endogenous opioid system is particularly rich in endogenous ligands, comprising three separate genes each giving rise to multiple active peptides, the issue of the exact relationship between

these endogenous ligands and the opioid receptors is of great importance. Previous studies in tissue homogenates led the authors to suspect that there is no one-to-one correspondence between any given precursor and any given receptor. Now that these receptors can be expressed indi-vidually, it is possible to carefully ascertain the selectivity profile of each of the endogenous ligands toward the individual receptors. The question of whether each precursor has at least one ligand that "sees" each of the receptors, or whether there is any exclusivity, can be addressed. For example, researchers already know that the mu receptor can interact with members of each of the families, but is kappa truly a "dynorphin" receptor, or can it be accessed by proenkephalin (proEnk) products? By comparing all three receptors and all the ligands side by side, a much more complete picture can be obtained than was had before. An equally important issue is that of efficacy at the receptors. While it may be assumed that all endogenous ligands are agonists, there is evidence that naturally occurring opioids, such as beta-endorphin1-27, can be antagonists in behavioral studies (Bals-Kubik et al. 1988; Tseng and Li 1986). This needs to be confirmed at the cellular level, and leads to the question of whether any of the other endogenous ligands can serve as antagonists or as partial agonists at one or more of the receptors.

A set of studies that is currently being performed in the authors' laboratory involves the evaluation of the selectivities of the endogenous opioid peptides for the cloned mu, delta, and kappa receptors. A series of proEnk, prodynorphin, and pro-opio-melanocortin peptides has been evaluated for affinity to the mu, delta, and kappa receptors that have been transiently transfected into COS-1 cells. Thus far, the data suggest that prodynorphin peptides have a high affinity for all three opioid receptor types, with approximately a tenfold selectivity for the kappa receptors. A surprising finding that is being further explored is that extended proEnk peptides such as Methione-Enkephalin-Arginine-Phenylalanine or Methione-Enkephalin-Arginine-Glycine-Leucine also have high affinity for kappa receptors in addition to mu and delta. Interestingly, Leu-enkephalin emerges as the most selective endogenous ligand, exhibiting a more than hundredfold preference for delta over kappa binding. On the other hand, the kappa receptor appears to have the widest range of selectivity vis-a-vis endogenous ligands, whereas mu and delta show relatively less discrimination (within one order of magnitude).

STRUCTURAL PROPERTIES OF THE OPIOID RECEPTORS—BINDING TO PEPTIDES VERSUS SMALL MOLECULES

The three opioid receptors cloned to date are all members of the guanosine triphosphate binding protein (G-protein)-coupled family of receptors containing seven transmembrane alpha helixes. While they exhibit significant homology to the somatostatin receptor family, they are particularly similar among themselves, exhibiting 61 percent level of identity at the amino acid level. The fact that opioid receptors belong to the seven transmembrane family of G-protein-coupled receptors tells a fair amount about their general topography based on analogy to bacterial rhodopsin, which has been visualized by electron microscopy (Findlay and Pappin 1986; Henderson et al. 1990). The seven alpha-helical transmembrane segments are thought to be arranged in a circular manner allowing the macromolecule to form a ligand binding cavity, and exposing three intracellular loops and the carboxy terminus to the cytoplasmic milieu and three extracellular loops and the amino terminus to the outside environment (Humblet and Mizadegen 1992). The intra-cellular loops, particularly the second and third loop, along with the carboxy terminal domain, are thought to be the site of interaction with G-proteins. The N-terminal domain and extracellular loops are in a position to play a role in receptor selectivity. It has been shown for several small neurotransmitter receptors that actual ligand binding takes place via specific interactions within the pocket formed by the trans-membrane domains. A great deal of this information derives from mutants and chimeras that have been constructed for many members of this superfamily, particularly the beta-adrenergic receptor (Dixon et al. 1987; Dohlman et al. 1987). The structure/function relationship of the dopamine receptors by site-directed mutagenesis has been studied in the authors' laboratory (Mansour et al. 1992), as well as by constructing chimeras between dopamine type 1 (D1) and dopamine type 2 (D2) (Meng et al. 1992).

In spite of the general model presented above, a great deal remains to be learned about the family of G-protein-coupled receptors in terms of actual three-dimensional arrangement, means of achieving ligand selectivity, molecular basis of drug efficacy and receptor-effector coupling, require-ments for selective G-protein interactions, and mechanisms of receptor regulation such as desensitization. For example, the homology of the mammalian receptors to rhodopsin, even in the transmembrane helixes, appears imperfect when examined in greater detail (Pardo et al. 1992). In addition, while they are discussed as a superfamily, recent studies have pointed to differences

as well as similarities between these receptors, especially between some inhibitory G-protein (Gi)-coupled receptors and the prototypical stimulatory G-protein (Gs)-coupled ß-adrenergic receptor (Dixon et al. 1987; Dohlman et al. 1987). Most importantly, while researchers' understanding of the interactions of small ligands with G-protein receptors is reasonable, understanding how peptide ligands bind and activate these structures is more limited.

Studying the opioid receptors at this structural level is of interest for several reasons. Such an analysis might help scientists understand the cellular basis of tolerance by describing mechanisms of receptor desensitization (e.g., phosphorylation), internalization, and down-regulation. Equally important, however, this level of analysis will allow researchers to understand, at the molecular level, the issue of receptor heterogeneity and of ligand selectivity in the context of the opioid system, which is unusually rich in endogenous ligands and in pharmacological probes. It is particularly advantageous that, in the opioid system, multiple peptides and multiple alkaloids interact with one family of receptors. A first-order question is, do they interact at the same sites (i.e., with the same critical residues) in the receptor? A related question is, do these receptors achieve selectivity toward peptide ligands in the same way that they achieve selectivity toward alkaloid ligands? The authors' working hypothesis is as follows: Both peptide and alkaloid agonists are likely to interact with the same region of the "binding pocket" of the receptor (though not necessarily in exactly the same way) to trigger a chain of allosteric events, which leads to a change in the interaction with G-proteins, thereby initiating the signal transduction cascade. However, it is possible that the selectivity between opioid peptides and opioid receptors is achieved through mechanisms quite distinct from those at play in alkaloid selectivity.

All known mammalian opioid peptides begin with the sequence Tyr-Gly-Gly-Phe- (YGGF), followed by Leu or Met. Clearly, this sequence, sometimes termed the "message," is critical to ensure interaction with the binding pocket of all opioid receptors, but the selectivity rests in the remaining sequence, the carboxy terminal extension beyond the penta-peptide (ranging from 2 to 26 residues in length), which provides the "address" (Schwyzer 1986). This carboxy terminal domain, at least when it is long, is likely to interact with the N-terminal domain or extracellular loops in a distinctive manner that may contribute to selectivity and that cannot be achieved by the much smaller alkaloids. The authors have proposed this model for kappa selectivity (Robinson and Berridge 1993) as the unique presence of

negative charges were noted on the second extra-cellular loop of the receptor, which the authors proposed to be interacting with the positive charge of the Arg-Arg residues in the kappa-selective prodynorphin products. The authors have already obtained some evidence in support of this model (see below). Interestingly, a similar model has been subsequently suggested for the thrombin receptor (Gerszten et al. 1994). This model for opioid selectivity does not exclude the possibility that opioid peptides, short or long, may also achieve discrimination by other means (e.g., by interacting with residues or sequences within the binding cavity).

The authors have embarked on a series of studies using the construction of chimeric receptors and the use of site-directed mutagenesis in order to examine the structural basis of receptor selectivity and specificity. In particular, the authors are very interested in the question of how this set of receptors achieves selectivity toward the family of endogenous ligands. A related question is whether peptides and opiate alkaloids bind to the receptor in the same way, or whether the complex structure of a peptide endows it with unique ways of interacting with the receptor protein.

Structure Function Analysis of the Receptors: The Chimera Approach

The authors have identified specific locations in each of the three opioid receptors that are good candidates for introducing mutations in order to make cassettes for the construction of chimeras. The mutations have been engineered into all three receptors, and the authors have constructed over 20 of the possible chimeras. Each receptor can now be divided into seven distinct domains, labeled "a" through "g" moving from the N- to the C-terminal domains. One type of study carried out to date has revolved around delta/kappa chimeric receptors constructed by using native restriction sites in these two receptors. Because both the rat kappa and delta receptors contain an Afl3 restriction site in the middle of transmembrane 3 (TM3) and a Bgl2 site at the beginning of TM5, the authors were able to construct six chimeras directly from the wild-type receptors. Two of the chimeric receptors did not show any binding to [3H]ethylketo. The arrangement of their DNA fragments appears to be correct as judged by several restriction enzyme digests. Their inability to bind is probably due to a disruption of some subtype-specific interactions among the different domains in a receptor. At this stage, it was not possible to localize these interactions because the domains involved were relatively large; this issue is being addressed with single-segment-exchanged chimeric receptors. However, the authors were able to

obtain complete binding profiles for the four remaining chimeras along with the wild-type kappa and delta receptors when they are labeled by 1 nano-molar (nM) [3H]ethylketocyclazocine (EKC). Multiple scatchard plot analyses were carried out using several classes of ligands, including nonselective ligands, highly selective kappa and delta ligands, and endogenous ligands, particularly members of the prodynorphin family. The following conclusions can be derived from these data:

1. The authors had hypothesized that high-affinity binding of the prodynorphin peptides to the kappa receptor was related to the presence of the highly negatively charged N-terminal and extra-cellular loop 2 in that receptor (Meng et al. 1993). The present results seem consistent with this hypothesis. A domain that includes extracellular loop 2 (negatively charged) appears to be particularly critical. When replaced by the delta sequence, the resulting chimeric receptor shows very low affinity for prodynorphin products while retaining excellent affinity for EKC, naloxone, or naltrexone. On the other hand, when these kappa domains are preserved but the regions C-terminal to them are replaced with delta fragments, the resulting receptor exhibits excellent kappa affinity. Thus, extracellular regions, particularly extracellular loop 2, may be critical for both the high affinity and high selectivity of DynA for the kappa receptor.

2. There is a high-affinity Tyr-Gly-Gly-Phe binding pocket in the delta receptor. This pocket is likely localized in the TM5-TM7 region in the delta receptor. This can be seen by comparing the binding of delta-selective ligands (peptides or alkaloids) to a chimeric receptor that contains domains TM5-TM7 of delta versus one that does not (i.e., delta receptor with kappa TM5-TM7). Whenever the C-terminal domain of delta is preserved, high delta affinity is maintained, but replacing it with its kappa equivalent abolishes this high-affinity delta binding.

3. Taken together, these results suggest that, in general, the delta recep-tor binds its selective ligands differently from the kappa receptor. Thus, the N-terminal half appears more critical for kappa binding and the C-terminal part for delta binding. This is revealed by the fact that a receptor with a kappa N-terminus and a delta C-terminus binds almost all ligands tested with an affinity comparable to wild type, whereas its mirror image (delta-N-terminus/kappa-C-terminus) binds all the specific ligands with low affinity but still binds the nonspecific ligands with good affinity, showing that the protein is being expressed and a generic opiate binding pocket is formed.

4. Several other observations can be made regarding the binding of alkaloids versus peptides, and of highly selective versus nonselective ligands. There are also some interesting exceptions to the general rules that the authors have tried to derive, suggesting that certain ligands with unusual structures have unique modes of interfacing with the receptor proteins.

Several mutants have been constructed. In the case of kappa, the authors examined the effect of simultaneously mutating the three negative charges on extracellular domain 2 to test the possible role of this region in interacting with the positive charges found in dynorphin. These mutations resulted in a small decrease in the affinity of Dyn A, Dyn B, and alpha neoendorphin by less than one order of magnitude. This finding suggests that while the negative charges may play a small role in the binding of prodynorphin products, other features of the extracellular loop may be even more critical for kappa selectivity. Binding of EKC and nor-binaltorphimine (nor-BNI) was not significantly altered by any manipulations of this region, supporting the notion that the extracellular loop may be particularly important in interaction with the peptides.

Structure Function Analysis of the Receptors: The Modeling Approach

The authors have used as a starting point the fact that all three cloned opioid receptors interact with the Tyr-Gly-Gly-Phe sequence. The most likely sites of interactions within this tetrapeptide are the NH3 + of the N-terminal Tyr, the OH group on the phenyl ring of this same Tyr1, possibly the NH group of Gly3, and the potential aromatic interactions with the phenyl ring of Tyr1 and Phe4. These sites are likely to bind to the receptors through hydrogen bonds, charge interactions, or hydro-phobic interactions. Therefore, it is presumed that there are comple-mentary sites on opioid receptors that interact with these active groups. As a working hypothesis, it can be assumed that these are among the amino acid residues that are conserved across the three opioid receptors. Some of these residues should be unique to the opioid receptor family, and distinctive from sites found on other members of the G-protein super-family. However, this latter criterion should not be used to exclude important residues such as the Asp in TM3, since this negatively charged residue is used by other receptors for ionic interactions and may also be recruited in opioid receptors to perform a similar function. Thus, the authors sought to

identify residues within the TM domains that are common to delta, mu, and kappa receptors and which, by their charge, their hydrogen bonding potential, or their potential for interaction with aromatic nuclei, may be involved in binding the opioid core sequence.

With these notions in mind, two models of the binding pocket of opioid ligands have been developed, one within the authors' group and the other in collaboration with Dr. Henry Mosberg and colleagues. The details of these models and their similarities and differences are beyond the scope of this paper. Suffice it to say that although independently derived, they share some common amino acids as key in the binding pocket, but they differ in their orientations of the ligands within the pocket. The advantage of having two working models is to force researchers to consider alternatives in interpreting empirical results. Mutation studies will first examine the residues chosen by both models as being important in the binding. Once so-called critical residues have been ascertained, focus will then shift toward testing orientation of ligand within the pocket. Testing has already begun on the proposed critical sites in the mu receptor. Preliminary results with several mutants created based on modeling are encouraging and show the usefulness of this modeling approach, which has led to specific residues of the receptors in domains not previously seen as important anchor points in the monoaminergic receptors.

Taken together, structural studies of the opioid receptors carried out to date, along with the findings of others in the field, strongly reinforce the view that these receptors are much more complex than previously anticipated, that multiple domains are used for multiple types of inter-actions, and that small molecules versus large peptides may interact very differently with these receptors. It is anticipated that continued efforts in this arena will lead to a better understanding not only of the opioid receptors but also of peptide receptors in general.

ANATOMICAL STUDIES

Regardless of their relative preferences at the pharmacological level, opioid signaling in a particular region or circuit depends primarily on the local opioid anatomy. For example, even if Leu-Enk exhibits a preference for the delta receptor, if no delta sites are found in the vicinity, but mu sites are, Leu-Enk may act as a mu agonist. When both mu and delta sites are present, then the difference in selectivity becomes a way in which to code the presence and concentration of

ligand in a more com-plex way than would be possible with one site. If this logic is extended to multiple endogenous ligands deriving from one or more precursor, and is interacting with multiple opioid receptors, it can be seen how this system changes a binary signal (receptor bound or unbound) to an analog communication mechanism. Thus, ideally, to describe a system under-lying a given behavior or function, it would be necessary to delineate the local complement of receptors and endogenous ligands with their range of affinities, selectivities, and efficacies.

The location of opioid receptors vis-a-vis their endogenous ligands has been the subject of much interest and discussion—reports of "mismatch" between the two (Herkenham and McLean 1986) have described several types of lack of concordance between the distribution of receptors and ligands. While this can be seen as the basis of nonsynaptic communi-cation, with diffusion of the ligands to distant sites, there are a number of alternative interpretations, including the fact that there is not a one-to-one correspondence between a given receptor and the selectivities of products deriving from a single opioid precursor (not even for kappa and proDyn, as shown above). Thus, ligand-receptor matching needs to include the relation of all opioid receptors to all endogenous ligands. Furthermore, the level of resolution possible with receptor autoradiography does not allow detailed anatomical studies possible with in situ hybridization (ISH) and immunocytochemistry (ICC) with specific antibodies directed at the individual receptors. The use of these approaches has only recently become possible, and these tools can now be used to readdress the ques-tion of the anatomical relationship between opioid peptides and receptors.

Anatomical procedures for identifying the receptor messenger ribonucleic acids (mRNAs) that encode the three classical opioid receptors have been developed. ISH studies demonstrate that cells expressing the mu, delta, and kappa receptor mRNAs are differentially distributed in the central nervous system (CNS) and spinal cord and correspond well to known receptor binding distributions defined by receptor autoradiography. Three separate studies were carried out comparing the individual receptor mRNAs to their respective binding sites using combined ISH and receptor autoradiographic techniques. In addition, a study was completed revealing the overall distribution of kappa receptors, in comparison to the expression of pro-dynorphin, using adjacent sections. This body of work has been published detailing the anatomy of the cells that express the opioid receptor genes in relation to various known anatomical and functional

characteristics of the endogenous opioid system in the brain (Mansour etal. 1993, 1994a, 1994b).

To the authors' anatomical armamentarium have recently been added antibodies that have been raised to nonhomologous regions of the mu and kappa receptors. The mu antibody is the best characterized with fairly complete immunohistochemical maps in both colchicine and non-colchicine-treated animals. The distribution of mu receptor protein corresponds well to mu receptor binding and mRNA expression with high levels of expression in such regions as the striatal patches, medial habenula, interpeduncular nucleus, and the dorsal horn of spinal cord. The importance of developing this and other opioid receptor antibodies lies in the higher cellular resolution that can be achieved and in the visualization of fibers and terminals, which is imperative in under-standing the anatomy of these receptors. Both immunofluoresence and diaminobenzidine (DAB)/nickel chloride visualization procedures have been developed, allowing for the direct co-localization with mRNA probes and antibodies to other molecules of interest.

The kappa antibody is in the latter stages of development. Specific immunohistochemical staining is observed in such regions as the nucleus accumbens, paraventricular hypothalamus, median eminence, substantia nigra (pars reticulata), and periaqueductal grey. The ideal immuno-histochemical conditions have presently, however, not been achieved, and further studies are in progress. The delta antibodies are in the early stages of development, and peptides are being produced in order to inoculate rabbits. These antibodies when fully characterized will be invaluable in studying the opioid receptor proteins anatomically and in regulatory studies.

SUMMARY

Over the course of 1 to 2 years, the field has moved swiftly to investigate the functional and structural properties of the newly cloned opioid receptors. Achieving a better understanding of these macromolecules is likely to have profound implications for drug design aimed at the production of better analgesic drugs, for a more fundamental under-standing of mechanisms of action of drugs of abuse, and for a more comprehensive knowledge base regarding the biology of opioid peptides in particular, and neuroactive peptides in general.

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Regulation of Acute and Chronic Opioid Receptor Functions by OBCAM, a Cell Adhesion-Like Molecule

Horace H. Loh and Andrew P. Smith

INTRODUCTION

Opioid receptors were one of the first class of cell surface receptors to be identified using in vitro binding assays with brain tissue, but for many years efforts to purify and clone them were unsuccessful because of their sensitivity to detergents, their heterogeneity, and the lack of a simple biochemical assay for their function (Loh and Smith 1990). Several laboratories, including the authors' own, reported purification of opioid-binding proteins (Cho et al. 1986; Gioannini et al. 1985; Maneckjee et al. 1985; Newman and Barnard 1984), but were unable to demonstrate unequivocally that they possessed all the pharmacological properties of in vivo receptors.

This situation has been dramatically altered by the recent successful cloning of the delta opioid receptor from NG108-15 cells, as reported at the end of 1992 by two independent laboratories (Evans et al. 1992; Kieffer et al. 1992). The two laboratories used a similar approach (expression cloning), preparing a cDNA library from NG108-15 cells, transfecting pools of this cDNA into mammalian cells, and then assaying the cells for binding of a radioactive opioid ligand. The cloned, expressed receptor showed typical binding properties expected of an opioid receptor, including high affinity, stereoselectivity, and preference for a particular class of opioids, namely delta; in addition, Evans and colleagues (1992) demonstrated that binding to the expressed receptor inhibited adenylyl cyclase, as is the case with native delta-opioid receptors on NG108-15 cells.

In NG108-15 cells (Koski and Klee 1981), as well as in at least some areas of the mammalian central nervous system (CNS) (Attali and Vogel 1989; Blume et al. 1979; Childers and Snyder 1978; Law et al. 1981), opioid receptors are coupled to guanosine triphosphate binding proteins (G-proteins), so it was no surprise that the predicted amino acid sequence of the cloned opioid receptor cDNA was homologous to other members of the G-protein-coupled receptor

superfamily (Dohlman et al. 1987); in particular, the sequence displayed seven characteristic hydrophobic regions that are presumed to span the cell membrane. The cloned delta opioid receptor (hereafter referred to as DOR) cDNA possesses several other features in common with other G-protein-coupled receptors, including aspartate residues in characteristic positions in transmembrane regions 2 and 3, which are thought to be involved in ligand binding and coupling to adenylyl cyclase; cysteine residues in extracellular loops 2 and 3, which may form a disulfide bridge between these regions; several putative glycosylation sites in the N-terminal region; and several putative phos-phorylation sites in the C-terminal region, which in other G-protein-coupled receptors are thought to be involved in regulation of the receptor during chronic agonist treatment (e.g., desensitization and downregulation). Among the other members of this superfamily, the somatostatin receptors are most closely related to the delta opioid receptor, exhibiting 35 to 40percent sequence homology, and this is consistent with the report that somatostatin ligands can bind to opioid receptors.

Whenever the cDNA for a receptor becomes available, it becomes of interest to use it as a probe to screen libraries in search of structurally similar sequences. In the case of the delta opioid receptor, this search has taken on particular importance, for it seemed possible that the other types of opioid receptors that have been defined pharmacologically, in parti-cular the mu receptor (the major mediator of opioid antinociception) and the kappa receptor, might have similar nucleotide sequences. This has proved to be the case. Chen and associates (1993) isolated from rat brain a cDNA that expressed receptor selective for mu ligands such as [D-Ala2-MePhe4, Gly-ol5]-enkephalin (DAMGO), and which was negatively coupled to adenylyl cyclase. Yasuda and colleagues (1993) also isolated a clone from rat brain that appears to express a receptor selective for kappa opioids. Both of these receptors exhibit about 60 percent amino acid homology with the delta opioid receptor as well as with each other. The greatest homology, as expected, is in the seven putative transmem-brane regions, while the least is in the N-terminal and C-terminal sequences, and in the second and third extracellular loops formed by amino acids between transmembrane regions 4 and 5 and 6 and 7.

Of particular surprise and interest, however, is the very strong sequence homology—about 90 percent—of the third cytoplasmic loop, between transmembrane regions 5 and 6, in all three of these opioid receptor sequences. In other members of the G-protein-

coupled superfamily, this region is thought to be critical in coupling to G-proteins, and consistent with the wide variety of G-proteins available for coupling to different receptors, the homology in this area is relatively low even between highly related receptors. For example, the somatostatin 1 and 2 receptors have about 40 percent sequence identity in this region, as do the ß1- and ß2-adrenergic receptors. The unexpectedly high homology in the case of opioid receptors suggests that these three receptors may couple to the same G-proteins. This raises the question of how these receptors are regulated so that they mediate distinct physiological responses.

Studies in the authors' laboratory have begun to address the question of opioid receptor regulation. To determine which G-proteins the receptors couple to, a technique involving labeling G-proteins with a stable guanosine triphosphate (GTP) analog has been employed. After stable transfection of mu or delta opioid receptor cDNA into Chinese hamster ovary (CHO) cells, the authors determined the ability of various opioid ligands to induce covalent labeling of the alpha subunit of G-proteins by the radioactive GTP analog [32P]-alpha-azidoanilido GTP. In CHO cells, four different Gas could be separated by SDS/urea gel electrophoresis and identified by appropriate antibodies: Go2a, Gi2a, Gi3a, and another, unidentified Goa. All four of these Gas were labeled by selective opioid ligands (mu or delta) in CHO cells transfected with mu or delta opioid receptor cDNA, though there was some preference demonstrated. In most cases, there was no correlation between the potency of a ligand to label a G-protein and its affinity for the expressed receptor or its potency to inhibit adenylyl cyclase.

The authors have also begun to identify the genetic elements that may be responsible for regulating the synthesis of opioid receptors. The sequence of the mu opioid receptor gene has been determined, and a number of sequences have been found that fit the consensus for certain regulatory elements. These include AP-1 and AP-2, which are regulated by cyclic adenosine monophosphate (AMP), and NF-GMb and NF-IL6, which are involved in the regulation of cytokine receptors. The existence of potential cyclic AMP (cAMP) regulatory elements is of interest, as opioid receptors in many systems inhibit cAMP synthesis. On the other hand, the discovery of potential immune regulatory sites is intriguing in light of accumulating evidence of links between opioids and the immune system.

Another possibility, however, is that opioid receptors may be regulated by some other molecule. For the past several years, the authors'

laboratory has characterized a protein, opioid binding cell adhesion molecule (OBCAM), that appears to play a regulatory role in opioid receptor function. OBCAM has homology to neural cell adhesion molecule, amalgam, and other cell adhesion molecules, possessing three immuno-globulin domains that are presumed to be oriented on the extracellular surface of the cell membrane. Since it has no putative cytoplasmic domain, it is difficult to see how it could behave as a complete signal transducing unit, yet it might still regulate opioid receptor function in some way. In this chapter, some of the evidence that OBCAM plays such a role will be discussed.

ROLE OF OBCAM IN OPIOID RECEPTOR BINDING AND ACUTE ACTION OF OPIOIDS

OBCAM was originally isolated on the basis of its ability to bind opioids, though it required the additional presence of acidic lipids (Hasegawa et al. 1987). As discussed above, both the lack of a putative cytoplasmic domain in OBCAM, as well as the demonstration that G-protein-coupled receptors bind opioids, make it unlikely that OBCAM directly binds opioids. Nevertheless, several studies in the authors' laboratory indicate that OBCAM may regulate opioid binding.

First, antibodies have been raised both to purified OBCAM and to peptides corresponding to portions of its predicted amino acid sequence, and have demonstrated that these antibodies inhibit opioid binding to the purified protein as well as to brain membranes (Roy et al. 1988a, 1988b). In addition, antibodies to OBCAM also block opioid antinociception when injected into the brain. These antibody data do not establish that OBCAM itself binds opioid ligands in situ, but they strongly suggest some kind of close association between OBCAM and the opioid receptor.

A second line of evidence indicating that OBCAM regulates opioid binding has come from studies using antisense cDNA. This technique has gained widespread use in recent years as a relatively easy and highly specific way of testing the role of a particular gene product in some function by selectively inhibiting expression of that gene. The authors have applied this technique to NG108-15 neuroblastoma x glioma hybrid cells, which contain a homogeneous population of delta opioid receptors coupled to adenylyl cyclase (Chang and Cuatrecasas 1979; Sharma et al. 1975a). By transfecting neuroblastoma x glioma NG108-15 cells with antisense cDNA to OBCAM, a stable cell line has been created (ST7-3) in which opioid binding is greatly reduced relative to that of cells transfected with OBCAM

sense (ST8-4), as well as nontransfected cells (Ann et al. 1992). The selectivity of this effect is suggested by the observation that binding of ligands to other cell surface receptors in ST7-3 cells was unaffected. Scatchard analysis of the binding indicated that most of the reduction was due to a decrease in receptor number, not affinity. Moreover, the remaining receptors could be further downregulated by chronic opioid agonist treatment of the cells. Thus, it appears that the OBCAM antisense has greatly reduced the number of opioid receptors on the cell without affecting their intrinsic response to acute or chronic opioid agonist treatment. This result again is consistent with an association between OBCAM and another molecule functioning as the receptor.

The antisense OBCAM cDNA has also been used to create a line of transgenic mice. The antisense was injected into mouse oocytes, which were implanted into pseudopregnant females; upon birth and maturation, the mice were selectively bred to create a stable line of transgenics. Founder as well as first- and second-generation transgenic mice showed a reduced response to the antinociceptive effect of morphine, as determined by the tailflick test, and also reduced sensitivity to acute tolerance to morphine. In the latter test, the animals were pretreated with a fixed dose of morphine, followed by determination of the morphine median analgesic dose (AD50) in the presence of a fixed dose of the antagonist naloxone. Normal mice show an increased AD50 in the presence of naloxone, and the higher the pretreatment dose, the higher the AD50. This increase was greatly reduced in the transgenic mice.

These studies all suggest that OBCAM plays a role in opioid receptor function, but do not really address the question of how it could do so. The authors have recently shown that coupling to G-proteins is altered in ST7-3 (OBCAM antisense-transfected) cells (Gavitrapong et al. 1993). To demonstrate this, the authors made use of the fact that cholera toxin (CTX) induces adenosine diphosphate (ADP)-ribosylation of G-proteins only in the presence of ligand, which promotes coupling between receptor and G-protein. Thus ribosylation can be used as a convenient assay of the degree of coupling induced by ligand for a specific type of receptor. In untransfected cells or cells transfected with OBCAM sense (ST8-4), CTX was shown to induce, in the presence of opioid agonist, ADP-ribosylation of Gi and Go on the basis of the reactivity of SDS gel bands with antibodies for these G-proteins. In the antisense (ST7-3) cells, in contrast, this labeling was greatly inhibited; as shown in table 1, the median effective dose (ED50) for DADLE to induce labeling was increased thirtyfold to fiftyfold.

TABLE 1. DADLE-induced, CTX-catalyzed ADP-ribosylation.

Membrane	ED50 (nM)
NG108-15	20
ST8-4 (sense)	30
ST7-3 (antisense)	-1,000

The reduced coupling between receptor and G-protein in ST7-3 cells was also manifested in reduced ability of opioid ligand to inhibit either basal or forskolin-stimulated adenylyl cyclase. The basal data are shown in figure 1a. While [2-D-Ala-5-D-Leu-] enkephalin (DADLE) at

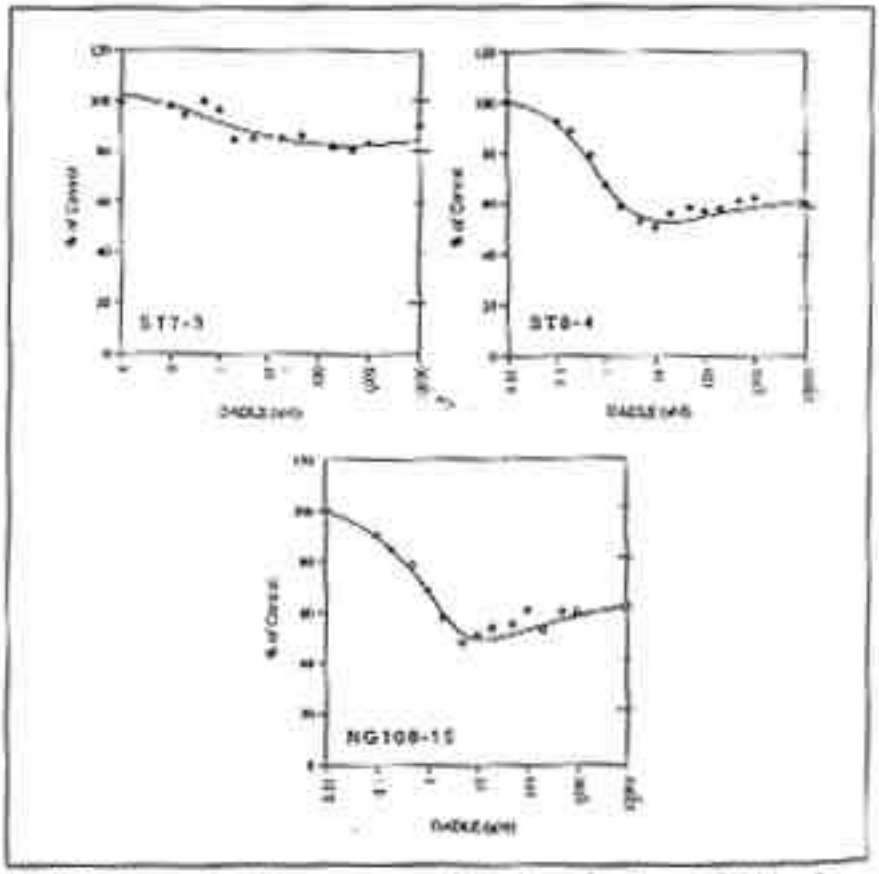

FIGURE 1a. *Opioid receptor coupled adenylyl cyclase inhibition in intact cells.*

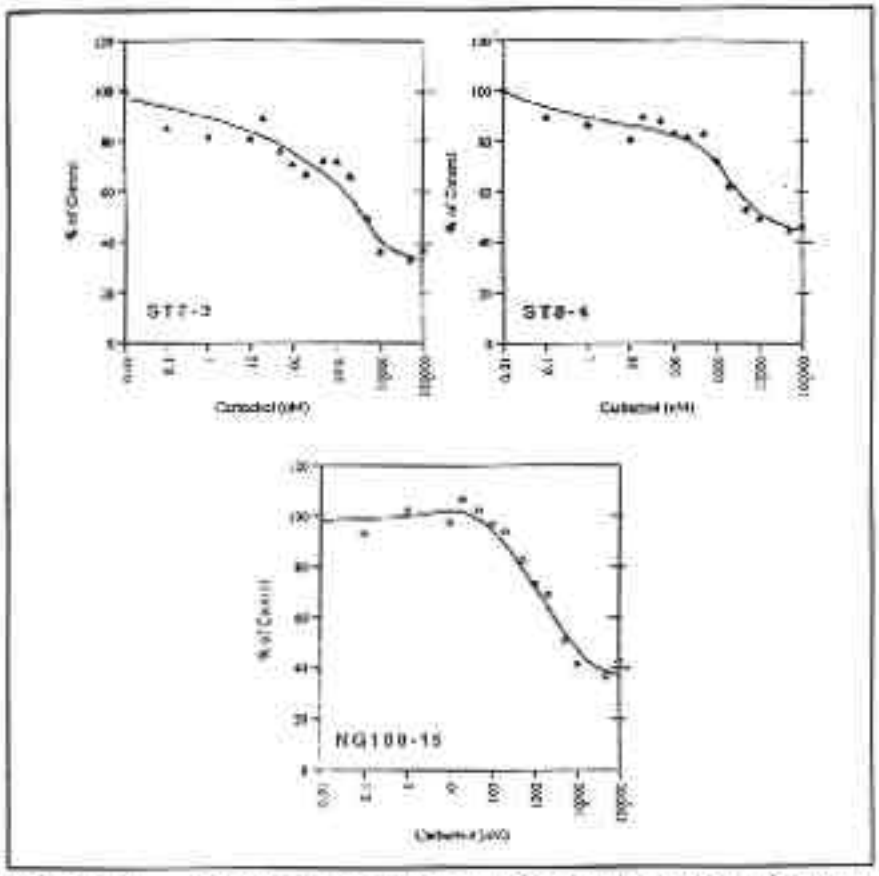

FIGURE 1b. *Muscarinic receptor coupled adenylyl cyclase inhibition in intact cells.*

concentrations of 1 to 10 nanomalors (nM) inhibited cyclase approximately 50percent in untransfected or sense-transfected cells, maximal inhibition was less than 20 percent in antisense-transfected cells, and even then required much higher DADLE concentrations. Furthermore, the antisense effect appeared to be specific to opioid receptors, as inhibition of adenylyl cyclase by adrenergic and muscarinic ligands was the same in ST7-3 cells as in ST8-4 or untransfected cells (figures 1b and 1c). Finally, the ability of DADLE to stimulate GTPase, a normal concomitant of coupling, was also inhibited in ST7-3 cells.

In summary, the presence of antisense OBCAM cDNA in NG108-15 cells has somehow interfered with coupling of opioid receptors to G-proteins, reducing the ability of the receptors to inhibit adenylyl

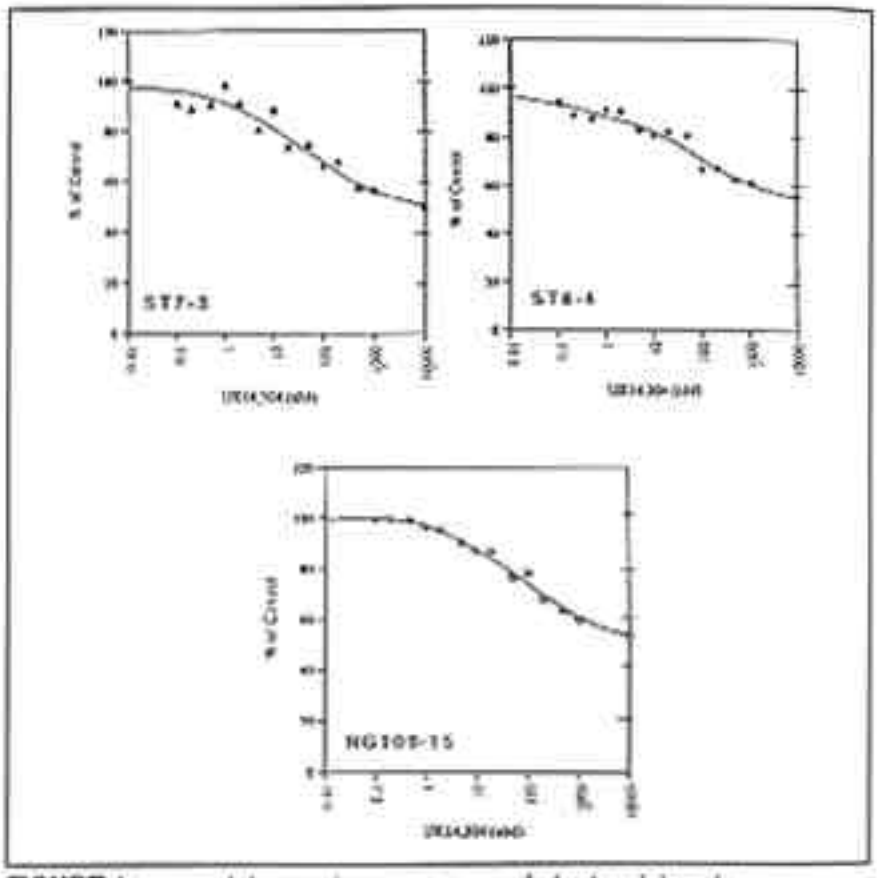

FIGURE 1c. *α_2-Adrenergic receptor coupled adenylyl cyclase inhibition in intact cells.*

cyclase. It is not clear how OBCAM, an extracellular molecule, could couple a seven transmembrane receptor to G-proteins, which are located on the cytoplasmic face of the cell membrane. One possibility is that OBCAM interaction with the receptor alters its conformation, so that those portions of the receptor that are located in the cell, and couple directly to G-proteins, are altered. Alternatively, the presence of OBCAM may be necessary to promote a lipid environment in which receptor and G-protein may couple.

ROLE OF OBCAM IN CHRONIC OPIOID EFFECTS

One of the signal features of opioid drugs is the ability to induce tolerance and dependence upon chronic administration to humans or animals. Tolerance may be defined as a state in which the dose of drug required to achieve a given effect is larger than normal. Dependence is a state in which regular doses of drug are required to prevent withdrawal symptoms.

The molecular basis of opioid tolerance and dependence is unknown, but a useful model system is provided by NG108-15 cells. When treated chronically with an appropriate opioid agonist such as DADLE, these cells become less responsive to opioids in a tolerant-like manner (Sharma et al. 1975b). Studies of the molecular basis of these effects have shown that several processes underlie this reduced sensitivity, including uncoupling of the opioid receptors from adenylyl cyclase, an effector for opioids in this system, and downregulation of the receptors, a process by which they are lost from the cell surface (Law et al. 1984).

Studies in the authors' laboratory have suggested that OBCAM plays a role in these processes also. The fate of OBCAM during chronic opioid treatment of NG108-15 cells has been studied by the use of fluorescent antibody labeling in conjunction with confocal microscopy. The antibody used was raised against a 12-amino-acid peptide (MN-3) corresponding to a portion of OBCAM in the third immunoglobulin domain. Initial studies demonstrated heavy fluorescence on the surface of NG108-15 cells treated with this antibody, which was blocked by pretreatment of the antibody with the peptide. In addition, cells transfected with OBCAM antisense cDNA (see above) showed much less immunofluorescence.

When NG108-15 cells were chronically treated with a delta opioid agonist such as DADLE, there was a significant reduction in the amount of antibody staining. The dose dependence and timecourse of this reduction closely paralleled the downregulation of opioid receptors; furthermore, the ability of a number of different agonists to induce downregulation of OBCAM-like immunoreactivity also paralleled their ability to downregulate opioid receptors. Thus delta agonists were the most effective, while mu and kappa opioids were much less so; the addition of an antagonist such as naloxone blocked the downregulation of OBCAM-like immunofluorescence. The specificity of this effect for opioid receptors was demonstrated by control experiments in which OBCAM-like immunoreactivity was

unchanged following chronic treatment of the cells with ligands for other types of receptors, such as muscarinic and adrenergic. Furthermore, chronic treatment with DADLE had no effect on the surface levels of neuronal cell adhesion molecule (N-CAM).

The recent cloning of the delta opioid receptor has made it possible to study the relationship of this receptor with OBCAM more directly, by transfection of cDNA into mammalian cells. With the collaboration of Dr.Ping Law, the authors have obtained CHO cells stably transfected with DOR cDNA. OBCAM expression, as measured by the appearance of M2 antibody fluorescence, is greatly increased in these cells, though interes-tingly, there is no correlation with increased opioid binding; that is, the level of immunofluorescence is about the same in the transfected CHO cells as in untransfected NG108-15 cells, though the level of opioid recep-tor binding is several times higher in the former (Ko and Loh, unpublished data). In addition, the authors have stably transfected neuro 2A cells with DOR, and OBCAM immunofluorescence does not correlate with opioid binding levels in different stable transfectants. Nevertheless, this work, together with the downregulation studies, indicates that manipu-lations that raise or lower the level of opioid receptors on the surface of NG108-15 cells result in a corresponding increase or decrease in OBCAM levels.

CONCLUSION

The cloning of mu, delta, and kappa opioid receptors has ended a two- decade search in pharmacology, but raised new questions concerning the mechanisms by which these receptors are regulated. The authors' studies with OBCAM suggest that this cell adhesion-like molecule may play a role in both the acute and chronic actions of opioids. Thus OBCAM seems to be necessary for normal coupling between opioid receptors and G-proteins, as well as undergoing downregulation in parallel to that of opioid receptors upon chronic opioid treatment. Further studies should clarify the role of OBCAM in opioid receptor function.

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Discrete Structural Domains and Cell-Specific Expression Determine Functional Selectivity of the Dopamine and Norepinephrine Transporters

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INTRODUCTION

The dopamine (DA) transporter (DAT) and norepinephrine (NE) transporter (NET) terminate catecholaminergic neurotransmission at synapses by high-affinity reuptake into presynaptic terminals and are the initial sites of action for a variety of drugs of abuse and therapeutic antidepressants. Recent cDNA cloning studies have demonstrated that NET and DAT are members of a family of sodium and chloride ion-dependent carriers, and that single cDNAs for NET and DAT effectively reconstitute many properties of the native transporters, including appro-priate pharmacology and ion dependence. Although catecholamine transporters have a high degree of sequence similarity, they are distin-guished by their monoamine substrate selectivities and their differential sensitivities to a wide spectrum of transport antagonists. DAT mediates uptake of DA, but it is an inefficient carrier of NE and other biogenic amines (Giros et al. 1991, 1992; Kilty et al. 1991; Shimada et al. 1991; Usdin et al. 1991), whereas NET transports both DA and NE (Pacholczyk et al. 1991). 1-methyl-4-phenylpyridinium (MPP+), the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is also a substrate of DAT. This compound causes a selective and irrever-sible loss of nigrostriatal DA neurons and provides the basis for an experimental model of Parkinson's disease (Boyson 1991; Snyder and D'Amato 1986).

Reuptake systems for monoamines are also sensitive to a wide range of uptake inhibitors (e.g., therapeutic drugs including antidepressants and drugs of abuse such as cocaine). NET exhibits marked sensitivity to many therapeutic antidepressants, including desipramine (Pacholczyk et al. 1991). Clinical and behavioral studies of tricyclic antidepressant drugs suggest that inhibition of NE reuptake correlates with antidepressant activity (Baldessarini 1985). In contrast, drugs

such as cocaine, amphetamine, and methylphenidate, which nonselectively inhibit DAT and NET, are poor antidepressants, despite the fact that they have stimulant and euphoric effects in some individuals (Baldessarini 1985).

The structural determinants responsible for the functional properties of NET and DAT, such as selectivity for substrates and antidepressants, shared recognition of cocaine and amphetamine, and conserved mechanistic features (e.g., sodium and chloride ion coupling and substrate translocation) remain to be elucidated. NET and DAT cDNAs predict protein sequences of 617 and 618 amino acids, and hydropathy analyses of the sequences indicate 12 hydrophobic regions proposed to represent membrane-spanning domains (Amara and Kuhar 1993). The two transporters are most similar in the putative transmembrane domains (TM), and least conserved in the NH2 and COOH termini thought to be oriented on the cytoplasmic face of the plasma membrane. A large extracellular domain between TM3 and TM4 shows a similar lack of conservation. In order to assign the kinetic and pharmacologic properties of NET and DAT to general structural domains, a series of recombinant chimeric transporter genes was generated and expressed in mammalian cells (Buck and Amara 1994). This approach has proven successful in the structural analysis of guanosine triphosphate binding protein (G-protein)-coupled receptors (Frielle et al. 1988; Kobilka et al. 1988), G-proteins (Masters et al. 1988), and ligand-gated ion channels (Elsele et al. 1993; Li et al. 1992). Unlike other conventional methods of mapping functional domains, such as analyses of site-directed or deletion mutants in which the function of interest is frequently destroyed, chimeras can provide an assayable phenotype that allows positive inferences to be drawn from functions associated with specific protein domains. Analyses of the functional properties of NET/DAT chimeric transporters have delineated structural domains that determine apparent transport affinity and translocation selectivity of catecholamines and MPP+ (Buck and Amara 1994), and sensitivity to desipramine and GBR12935 (1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)-piperazine).

Pharmacologic and Kinetic Selectivity of Wild-Type Catecholamine Transporters

NET and DAT have strikingly similar sequences, with 64 percent amino acid identity and nearly 85 percent sequence similarity considering conservative amino acid differences. Despite their structural similarities, these two transporters demonstrate

physiologically and clinically important differences in selectivity for substrates and for a variety of uptake inhibitors. The functional properties of NET and DAT were examined by expression in HeLa cells using a vaccinia virus-based transient expression system. These studies show that NET has high apparent affinity for both NE and DA transport kinetic constant (KT)=0.4±.1 micromolars (µM) and 0.2±0.1 µM, respectively), whereas DAT expresses lower apparent affinity for NE and DA uptake (KT=5.7±2.7 µM and 3.2±0.4 µM, respectively) (figure 1, see also table1). In these kinetic analyses, differences in substrate affinity for the kinetically relevant site(s) are reflected in the Michaelis constant (KT). However, this constant is also influenced by rate constants for a number of events that occur subsequent to substrate recognition (e.g., trans-location and dissociation). In addition to the apparent affinity constant for substrate transport (KT), a second kinetic parameter, the velocity of transport at steadystate (Vmax), can be used to assess the capacity of a carrier to catalyze translocation. Vmax reflects the turnover number of the carrier, which varies with different substrates. However, Vmax is also influenced by expression levels, and therefore cannot be compared between cells with different numbers of carriers at the surface. Thus, for each transporter, translocation efficacy is reported as a rank order Vmax in cells transfected and assayed in parallel for dopamine and norepinephrine transport (table 1). Differences in the rank order Vmax between trans-porters reflect the relative efficiency with which different substrates are translocated. For example, cells transfected with DAT efficiently translocate DA as compared to NE (rank Vmax 1:9 for NE and DA). In contrast, NET accumulates dopamine nearly as efficiently as NE (rank Vmax 2:1 for NE and DA). These data clearly show that KT and rank Vmax can vary independently (figure 1).

Chimeric Catecholamine Transporters

To determine which structural domains confer distinct functional properties of NET and DAT, a series of chimeric recombinant transporters from NET and DAT cDNAs was constructed using a novel in vivo method that generates chimeras which junction in regions of sequence similarity (Buck and Amara 1994) (figure2). Briefly, DAT/NET and NET/DAT chimeras were engineered by subcloning the coding regions of NET and DAT in tandem into a pB SKII- plasmid vector. The DAT/NET and NET/DAT constructs were linearized and used to transform bacteria. The chimeric constructs are formed from the

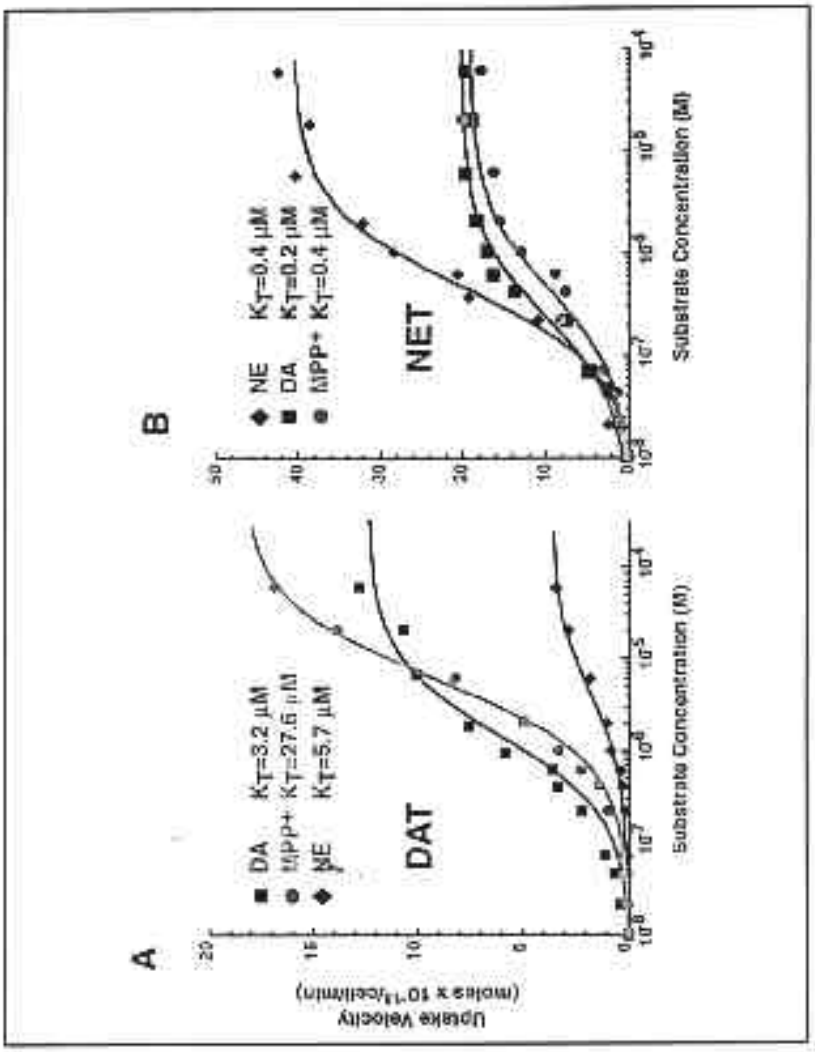

FIGURE 1. *Differences in substrate selectivity between NET and DAT. Panel A shows the uptake velocity of the DAT plotted as a function of substrate concentration for the substrates DA, NE, and MPP+. The DAT was expressed in HeLa cells as described and uptake of each of the substrates was measured in parallel assays done on the same transfected cells. The K_T for each substrate was obtained by nonlinear least-squares fits of substrate/velocity profiles using a data analysis program. Panel B shows the results of the same analysis for cells transfected with the NET.*

TABLE 1. Substrate selectivity of wild-type and chimera transporters.

KT (μM) (apparent affinity constant)			Vmax (uptake efficacy)	
Transporter	NE	DA	Relative (NE:DA)	Normalized (DA)
NET	0.4±0.1	0.2±0.1	2:1	1.6
ND 11	0.4±0.1	0.3±0.1	1:1	1.3
ND 10	0.9±0.4	0.6±0.3	1:1	1.1
ND 4	1.6±0.1	1.5±0.5	1:1	1.3
ND 3	1.7±0.5	0.8±0.2	1:1	0.6
ND 1	2.6±0.8	2.6±0.7	1:4	1.0
DAT	5.7±2.7	3.2±0.4	1:9	1.)
DN 10	2.4±0.8	2.2±0.8	1:20	1.8
DN 9	2.3±0.6	2.1±0.5	1:17	1.7
DN 3	2.0±0.6	1.1±0.4	1:5	0.8
DN2	2.1±0.3	1.8±0.2	4:5	0.9
DN1	0.4±0.1	0.2±0.1	1:2	0.7

NOTE: Kinetic analysis of substrate transport in HeLa cells expressing chimeric transporters or wild-type NET or DAT. Chimeras are identified as ND or DN, and numbered to reflect the transmembrane domain near which they junction. The kinetic constants, KT, and Vmax for each of the substrates were obtained by nonlinear least-squares fits of substrate/velocity profiles using data analysis program. The apparent affinity constant (KT) for transport of the substrates NE and DA is reported as the mean KT ± SEM as determined from three to six independent experiments performed in quadruplicate. For each transporter, translocation efficacy is reported as a rank order Vmax for DA and NE transport in cells transfected and assayed in parallel.

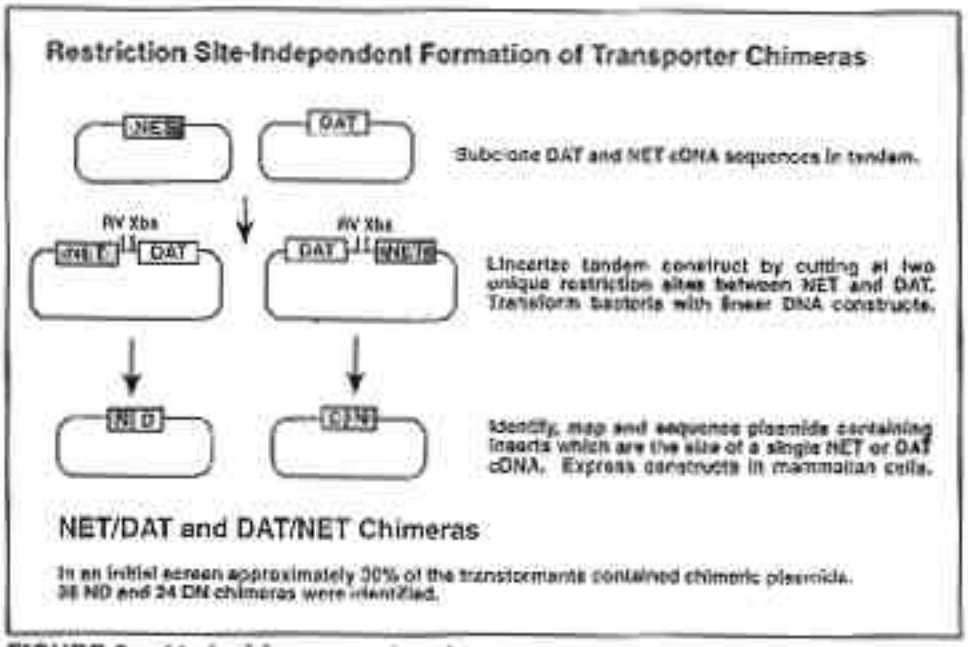

FIGURE 2. *Method for generating chimera transporters.*

linear DNA within the bacterial host in a process that probably involves endogenous exonuclease digestion of linear DNA, base pairing of homologous regions, and subsequent bacterial repair of the plasmid. The approach used favors generation of chimeras which junction at single sites in regions of conserved nucleic acid sequence, and typically resulted in functional chimeric transporters. Expression of the chimeric trans-porters in HeLa cells using a vaccinia virus/T7 polymerase-based expression system (Blakely et al. 1991) allowed an assessment of their apparent affinity constants (KT) and translocation efficacy (rank Vmax) for each catecholamine, and sensitivity to uptake antagonists such as DMI and GBR 12935. To provide an indication of the robust activity of these chimeras, the Vmax of each chimera was compared to the wild-type DAT activity observed in a parallel experiment (table1, normalized Vmax). Specific functional properties of NET and DAT were found to be correlated with particular protein sequence elements of the chimeric transporters, allowing differential properties of the two transporters to be assigned to specific domains. The structures of this series of chimeras, their apparent substrate affinity constants for uptake of NE and DA, and their selectivity for inhibition by desipramine are compared relative to wild-type NET and DAT in figure 3.

Transport is attenuated in all chimeras that junction in a region spanning TM5 through TM8 (including DN5, ND7, DN8, and ND8, figure 3c). Chimera DN5 demonstrated a dramatic reduction in transport (i.e., Vmax <5percent for NE uptake by NET) with no decrease in apparent substrate affinity relative to DN3 and DN9. However, surface [125I]RTI-55 binding to intact cells expressing these chimeric transporters was approximately 40 to 50 percent of binding to intact cells transfected with NET or 25 to 35percent of binding to cells expressing DAT. RTI-55 is a structural analog of cocaine with high affinity for DAT (Boja et al. 1991), which also recognizes NET although with lower affinity. These results suggest that this region spanning TM5 through TM8 may contain important elements of a catalytic domain involved in substrate translocation. However, in general the low transport capacity of chimeras junctioning between TM5 and TM8 limited detailed analysis of their transport kinetics, and may have restricted the ability to localize additional determinants influencing translocation efficacy and substrate KT which may also exist between TM5 and TM8. However, apparent substrate affinity and translocation efficacy were readily compared in chimeric transporters that junction in or before TM4 (e.g.,DN1, DN2, DN3, ND1, ND3, ND4) or after TM9 (e.g., DN9, DN10, ND10, and ND11) (figure 3b).

Discrete Domains Influence Catecholamine Substrate Selectivity

A goal of these studies was to identify structural domains that influence apparent affinity (KT) and catalytic efficacy (rank Vmax) of NET and DAT. The kinetics of catecholamine uptake by wild-type and chimeric trans-porters are summarized in table 1. The apparent substrate affinities of DN1 are consistent with those of NET, indicating that substitution of NET NH2-terminal sequences with that of DAT does not reduce apparent affinity relative to NET (table 1 and figure 3). DN3 has a fivefold higher KT for DA, and fivefold higher KT for NE than DN1 or NET (table1). These two chimeras define a domain that has a pronounced effect on substrate KT within a region spanning TM1 and TM3. The comple-mentary chimeras ND1 and ND3 further underscore the importance of this region in differentiating substrate KT values of NET and DAT. Furthermore, because ND1 has a modest two- to threefold lower apparent affinity than ND3 for both substrates, additional determinant(s) may reside near the amino-terminus of TM1. A region spanning TM10 and TM11 also contributes to differences in KT between NET and DAT for catecholamines.

DAT substrate selectivity for DA is largely due to the rate (Vmax) with which it transports DA relative to NE (see figure1). Chimeras DN3 and ND1 translocate DA more efficiently than NE (rank Vmax > 4:1 for DA and NE), indicating that amino-terminal determinants also influence relative Vmax. Thus, TM1 through TM3 play an important role in determining substrate KT, and TM2 through TM3 influence rank Vmax. DN9 and DN10 even more closely resemble DAT in their capacity to translocate DA relative to NE. These results suggest that some deter-minants that enhance the efficacy with which DA is transported relative to NE may fall within the central domain (TM5 through TM8) identified above as involved in catalyzing substrate translocation (see rank order Vmax in table 1).

Desipramine Specificity Involves Two Structural Domains

The NET and serotonin transporters are important initial targets for a number of tricyclic and other antidepressants used in the treatment of human depression. An important goal of the authors' studies was to identify the structural domain(s) involved in differential sensitivity of NET and DAT for antidepressants. Desipramine is one of the most potent tricyclic antidepressants in blocking NET, but is more than a

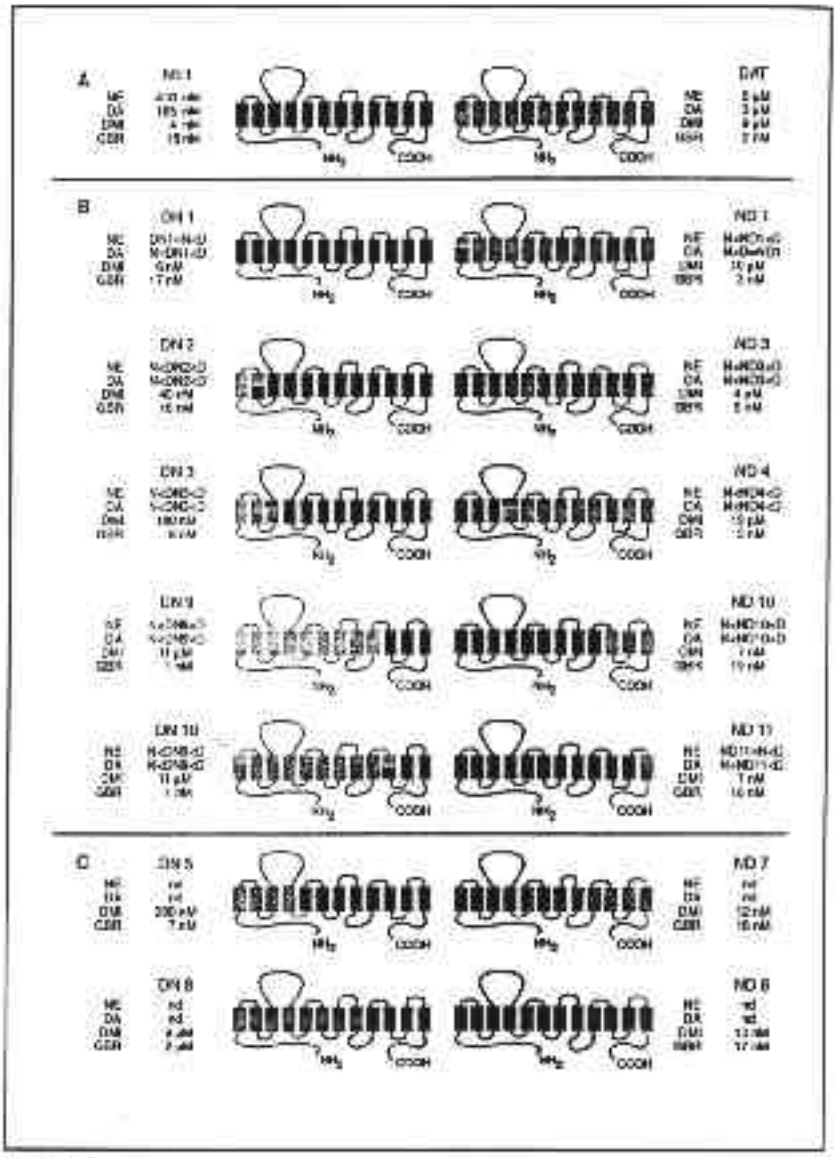

FIGURE 3.

FIGURE 3. *Wild-type and chimeric catecholamine transporters: summary of pharmacological selectivity for substrates and inhibitors. Panel A. Diagram of wild-type NET (in black) and DAT (in grey). Twelve hydrophobic domains are modeled as membrane-spanning domains. The NH_2 and COOH termini are presumed to lie in the cytoplasm (intracellular). Beside each transporter is a summary of its pharmacological characteristics. The following properties are summarized: the apparent affinity constant (K_m) for transport of the substrates NE and DA and the potency (K_i) with which desipramine (DMI) and GBR12935 inhibit [^{3}H]dopamine transport. DAT expresses lower apparent affinity for transport of the substrates DA, and NE than NET. The therapeutic antidepressant DMI demonstrates considerable selectivity for NET and GBR12935 is moderately selective for DAT. Panel B. Properties of functional chimeras. The functional characteristics of each chimera are summarized relative to the wild-type NET (N) and DAT (D). Chimeras are referred to as ND (i.e., NET/DAT) or DN (i.e., DAT/NET), and numbered to reflect the transmembrane domain nearest their junction. Uptake inhibition by desipramine and GBR 12935 was examined using [^{3}H]dopamine (10 nM). Uptake inhibition constants (K_i's) were determined using INPLOT. Data represent the mean $K_i \pm$ SEM determined from three independent experiments performed in triplicate. Panel C. Chimeras with reduced function. Apparent substrate affinities were not determined (nd) for several chimeras in which transport was significantly attenuated (e.g., DN5, ND7, DN8, and ND8), all of which function within a region spanning TM5 through TM8. For these chimeras apparent dopamine transport affinities were not readily determined. Thus, IC_{50} values determined using 200 nM substrate in the presence of increasing concentrations of unlabeled inhibitor are reported for these chimeras.*

163

thousandfold less potent as an inhibitor of DAT (figure 3a). Antagonism of DA uptake by the chimeric transporters identifies two domains of primary and secondary importance that are required for the effective blockade of catecholamine transport by desipramine (figure 3b).

The most influential determinants of desiramine selectivity for NET lie within a region spanning TM5 through TM7, delineated in part by ND7 and DN5, which differ seventeenfold in their sensitivity to desipramine. These two chimeras (as well as ND8, ND10, ND11, DN1, DN2, and DN3), each with nanomlar (nM) affinity for desipramine, share a region of sequence from NET extending from the amino-terminus of TM5 through TM7. In contrast, chimeric transporters that pharmacologically resemble DAT (e.g., despramine inhibition constant (Ki) = 9.1 to 10.0 μM for ND1, DN8, DN9, DN10) all possess sequence from the analogous region of DAT (figure 3). ND4 has lower sensitivity to desipramine than either DAT or NET, suggesting that this chimera may reflect a disruption in desiramine recognition rather than a shift toward DAT-like sensitivity. Several chimeric transporters (DN2, DN3, DN5, and ND3) displayed intermediate sensitivity for uptake antagonism by desipramine, and delineate a secondary domain spanning TM1 through TM3 that also influences desipramine recognition. Chimeras with NET sequence in the primary domain and DAT sequence in the secondary domain demonstrate intermediate selectivity for desipramine, which more closely resembles NET (e.g., Ki = 352 nM, 178 nM, and 189 nM for DN2, DN3, DN5) (figure 3). In contrast, a chimera with DAT sequence in the primary domain and NET sequence in the secondary domain displays an intermediate antidepressant sensitivity much more similar to DAT (e.g.,ND3 with Ki= 2.7 μM). Chimera DN1 displays specificity for desipramine consistent with NET, indicating the cytoplasmic NH2-terminus does not contribute to this secondary domain. Thus, secondary deter-minants for desipramine potency lie within a region spanning TM1 through TM3, and may overlap with the amino-terminal domain described above, which influences both KT and rank Vmax for the catecholamines DA and NE. In addition, the domain that has the greatest impact on desipramine Ki is within a region spanning TM5 through TM7, and may overlap the domain thought to be involved in catalyzing substrate translocation.

GBR 12935 Inhibition of Dopamine Uptake

GBR 12935, a potent uptake antagonist, is moderately selective for DAT relative to NET (Anderson 1987). Chimeric transporters (e.g.,

ND4 and ND7 with Ki values of 3±1 nM and 18±5 nM, respectively) delineate a domain spanning TM4 through TM7, which confers modest selectivity for GBR 12935 (figure 3). This domain may overlap with a region extending from TM5 through TM8, which contributes to selective translocation of DA and MPP+ by DAT. This domain may also overlap in part with a homologous region of NET, which is largely responsible for desipramine selectivity. Interestingly, antagonism of DA uptake by GBR 12935 is not impaired in ND4 relative to other chimeric trans-porters, as was inhibition by desipramine. These results suggest that ND4 may dissociate the inhibitory actions of GBR 12935 and desipramine.

Distribution of the Dopamine and Norepinephrine Transporters in the Central Nervous System

The distribution of the transporters in different neuronal populations in large part determines which substrates they are likely to encounter and which pathways they are most likely to influence. The two carriers show a distinct and nonoverlapping distribution. NE-containing cell bodies in the locus coeruleus and lateral tegmentum of the brainstem express NET messenger ribonucleic acid (mRNA), whereas DAT mRNA is expressed in dopaminergic cell groups of the mesencephalon, and the A12 and A13 dopaminergic cell groups of the diencephalon (Lorang et al. 1994). The cell type-specific expression of catecholamine transporters suggests that DAT and NET gene expression may be closely linked to cellular mechanisms that specify transmitter phenotype. Thus, the role of the transporters in DAT and NET influence catecholaminergic neurotrans-mission and appear to be determined by their distribution in the brain, as well as differences in their pharmacologic and kinetic properties.

Studies combining in situ hybridization of DAT and NET with immuno-histochemical detection of enzymes for catecholamine synthesis indicate that these two transporters are expressed only in catecholaminergic neurons. NET mRNA labeling is found in regions of the medulla oblon-gata and pons known to contain noradrenergic cell bodies (corresponding to the A4, A5, A6, and A7 cell groups) and neurons in the lateral teg-mentum and nucleus of the solitary tract that correspond to the locations of A1 and A2 catecholaminergic neurons. The locus coeruleus A6 cell group contains the greatest density of heavily labeled NET mRNA-containing neurons. No evidence for NET mRNA in tyrosine hydroxylase (TH)-positive dopaminergic neurons was observed even though NET has a higher affinity for DA than NE (Buck and Amara 1994; Pacholcyzk et al. 1991). Despite

the possibility that NET could serve as the transporter for epinephrine, no NET mRNA was detected in phenylethanolamine-N-methyl transferase (PNMT)-immunoreactive neurons.

Combined immunohistochemical/in situ hybridization analysis (see figure4) of NET mRNA in catecholaminergic neurons revealed that an overwhelming majority of dopamine ß-hydroxylase (DBH)-immuno-reactive cells in the A1-A7 cell groups also contain NET mRNA. Noradrenergic cells do not appear to express DAT mRNA, which is restricted to dopaminergic cells of the diencephalon and mesencephalon of the central nervous system (CNS). Northern blots show a single DAT

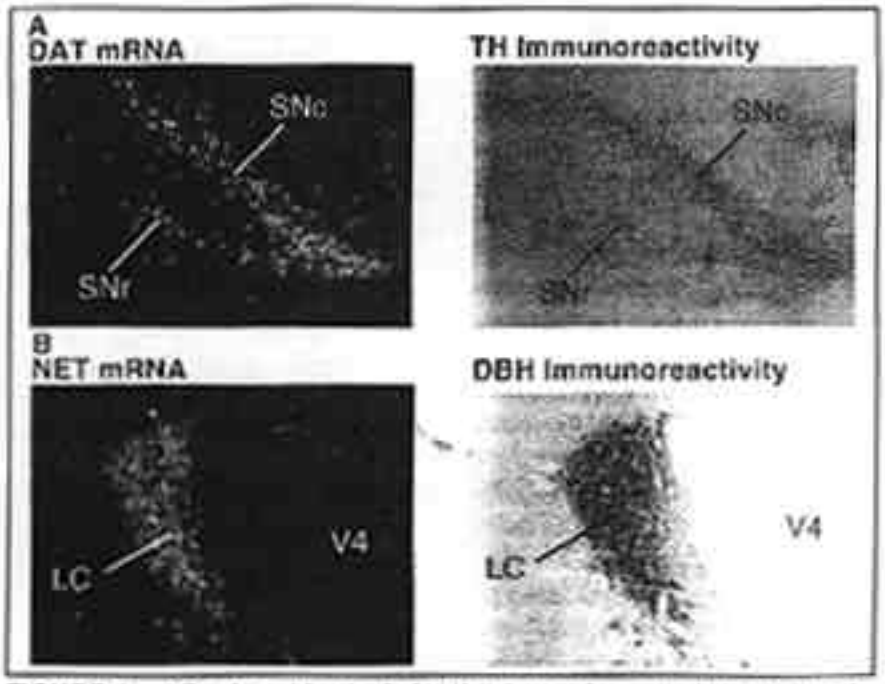

FIGURE 4. *Combined in situ hybridization and immunohistochemical analysis of the expression of the norepinephrine and dopamine transporters. Panel A. Darkfield and brightfield photomicrographs (4X) of the same field showing DAT mRNA containing neurons (left) and TH immunoreactivity (right) in the ventral tegmental area and substantia nigra (SN) of the rat. Panel B. Darkfield and brightfield photomicrographs (6.6X) of the same field showing NET mRNA containing neurons (left) and DBH immunoreactivity (right) in the locus coeruleus.*

transcript in neurons that comprise both the mesostriatal and mesocorti-colimbic dopaminergic pathways. Thus, neurons that send projections to the ventral and dorsal striatum appear to express the same DAT mRNA as do neurons that project to limbic regions, isocortex, and the spinal cord. The cells showing the most intense in situ hybridization to DAT mRNA are found in the substantia nigra and the ventral tegmental area, which corres-pond to the A8, A9, and A10 mesencephalic dopaminergic cell groups. Immunohistochemical analysis confirms that greater than 90percent of the TH-immunoreactive cells of the zona compacta and zona lateralis of the substantia nigra, and throughout the ventral tegmental area, are also intensely labeled for DAT mRNA. In the diencephalon, intense hybridi-zation with DAT mRNA is found in the zona incerta in cells presumably belonging to the A13 cell group, and moderate levels of labeling are found over neurons in the A12 cell group in the arcuate nucleus. Interestingly, TH-positive dopaminergic neurons in the preoptic area, periventricular nuclei, and the posterior hypothalamus do not appear to contain DAT mRNA, suggesting that DA release from terminals does not undergo rapid inactivation by reuptake into presynaptic cells. Thus, despite marked similarities in their primary structures and shared pharmacological properties, the nonoverlapping and differential expression patterns of DAT and NET suggest that these transporters function independently in distinct neuronal pathways. Moreover, the possibility that the expression or activity of these transporters is differentially regulated in these pathways provides a possible basis for understanding how pharmacological agents that act on both transporters exert such diverse effects on catecholaminergic neurotransmission.

DISCUSSION

In order to establish a potentially general approach to study the superfamily of sodium and chloride ion-dependent carriers, the authors constructed and expressed a series of functional recombinant chimeric transporters in which similar sequence domains and distinct functional properties of catecholamine transporters are exchanged (Buck and Amara 1994). These studies examine the structural domains responsible for differential selectivity of NET and DAT for a variety of substrates (e.g.,-DA, NE, MPP+) and uptake inhibitors (e.g.,GBR 12935 and desipramine), and are the first step in identifying the specific structural or regulatory determinants responsible for the distinct functional properties of NET and DAT. Antagonist recognition and substrate translocation are likely to involve some shared determinants of NET and DAT. However, studies

employing chimeric catecholamine transporters focus on structural features unique to NET and DAT that contribute to differences in their apparent substrate affinities, translocation efficacies, and antagonist selectivities. These data are summarized in the model shown in figure 5.

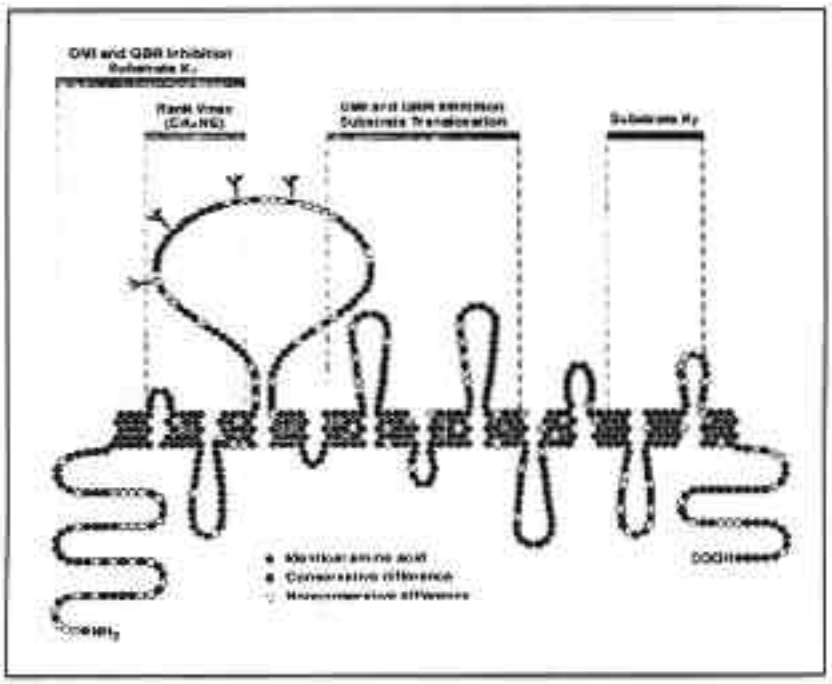

FIGURE 5. *Summary of structural domains influencing catecholamine transporter kinetics and pharmacology. Structural domains identified as having a major (1°) or secondary (2°) role in determining different kinetic properties and pharmacological selectivity of NET and DAT are illustrated in this schematic representation of a catecholamine transporter. Domains which influence apparent substrate affinity (K_m), inhibition by GBR 12935 and desipramine (K_i) and translocation capacity (rank V_{max}) are identified. Conservative and nonconservative amino acid differences between NET and DAT are indicated.*

Structural Determinants of Antidepressant and Catecholamine Selectivity

Structure activity studies indicate that a protonated amine group is a critical feature of both transported substrates and a number of therapeutic antidepressants recognized by monoamine transporters (De Paulis et al. 1978; Koe 1976; Maxwell and White 1978; Maxwell et al. 1969, 1976). The terminal ammonium of monoamines and antidepressants may associate with a negatively charged residue of the transporters, whereas planar aromatic moieties of these compounds may associate with an analogous surface by hydrophobic and/or van der Waals bonding. Current studies delineate determinants within a region spanning TM1 through TM3 which have a pronounced effect on apparent substrate affinity. Additional determinants within a region spanning TM2 and TM3 may be involved in selective translocation of DA relative to NE by DAT. A homologous region of NET may influence selective antagonism by desipramine. Within these domains, negatively charged aspartate (D) and glutamate (E) residues at positions NETD64/DATD68, NETD75/DATD79, and NETE113/DATE117 are conserved in monoamine carriers and may recognize the terminal ammonium of substrate catecholamines and tricyclic antidepressants. NETD75 and DATD79 are of particular interest because they are uniquely conserved in monoamine carriers. Mutation of aspartate residue DATD79 to glutamate or to neutral alanine or glycine residues has been reported to dramatically impair DA uptake (Vmax values were < 7 percent to 24 percent of wild-type values), and results in a threefold to sixfold decrease in the apparent affinity for DA uptake (Kitayama et al. 1992). Results obtained from studies of catecholamine transporter chimeras indicate that amino acids in close proximity to these conserved acidic residues are important in determining both the different apparent affinity and differential translocation efficacy of DAT and NET and differential sensitivity to antidepressants (figure 5). Within TM1, a single amino acid difference (valine for isoleucine) between NET and DAT is observed. In TM2, three conservative differences and a nonconservation substitution (NETT99 versus DATL103) are found, while the sequence between TM2 and TM3 is somewhat less conserved. These results underscore the possible importance of conservative amino acid substitutions, which have been shown to be critical for agonist pharmacology in other membrane proteins (Schmieden et al. 1992).

Studies of catecholamine binding to mutant adrenergic receptors indicate that serine and aspartate residues within the hydrophic

regions are also important in catecholamine recognition (Chung et al. 1988; Fraser et al. 1988; Strader et al. 1988, 1989; Wang et al. 1991). Through hydrogen bonds, serine residues may possibly interact with meta- and parahydroxyl moieties of the phenyl ring of the substrate catecholamines. In DAT, replacement of the serine residues at positions DATS356 and DATS359 in TM7 by alanine or glycine has recently been reported to cause reductions in DA and MPP+ uptake (Kitayama et al. 1992). These serine residues are conserved in NET, suggesting that these amino acids are not directly responsible for differences in the transport properties of the two carriers. However, DATS358 is not conserved in NET (which contains alanine). These serine residues fall within a region extending from TM5 to TM7, which in the current studies appear to be involved in translocation selectivity, and may also play a more basic role in the mechanism of substrate translocation. DATS358 or one of several other nonconserved residues within this domain may therefore be important in determining greater translocation efficacy for DA than NE by DAT. This domain does not appear to influence apparent substrate affinity, suggesting that substrate recognition is not altered. Thus, it appears that this central domain is primarily responsible for events occurring subsequent to substrate recognition. Interestingly, it has been suggested that a substrate dissociation or recycling processes following substrate recognition may be rate limiting in transport kinetics (Friedrich and Bonisch 1986; Schomig and Bonisch 1988; Zimanyi et al. 1989).

Overlapping Domains for Antagonist Affinity and Substrate Translocation

Structure-function analysis of the chimeric transporters indicates that overlapping domains may influence transporter affinity for desipramine (a potent NET antagonist) and GBR 12935 (a selective DAT inhibitor), and translocation efficacy (Vmax) (summarized in figure 5). Chimeras junctioning between TM5 and TM8 also demonstrate attenuated transport (i.e., the capacity of these chimeras to transport DA is < 5 percent that of NET), suggesting that they may define a structural domain involved in substrate translocation. Despite the low transport efficacy of these chimeras, the potency of various inhibitors was readily assessed. Primary determinants of desipramine affinity are found within a region spanning TM5 through TM7 delineated in part by DN5 and ND7. Similarly, determinants of substrate translocation selectivity lie within a region extending from TM3 through TM9.

An overlapping region spanning TM5 through TM8 may also define a domain critical for substrate translocation. These results imply that this domain may influence transport by positioning determinants involved in substrate translocation and antagonist recognition. To date, no infor-mation is available on the positioning of transmembrane domains relative to each other, or if the transporter is multimeric. In spite of these potential limitations, all of the chimeras reported here are functional to a significant degree, indicating that the secondary and tertiary structures of NET and DAT are largely maintained. Thus, structure-function studies of chimeric transporters can provide evidence that some domains are more important for certain functions than for others, although they may not distinguish between direct effects on residues interacting with the substrates and indirect effects modifying transporter conformation. As more information becomes available on the higher order structure of members of the family of sodium-dependent transporters, precise inter-actions between residues and domains identified in these studies and their role in transport will become apparent.

SUMMARY

The successful generation and functional expression of a series of recombinant chimeric transporters, in which distinct functional properties of NET and DAT are exchanged, have allowed the assignment of a number of important functional properties of MPP+ and antidepressant-sensitive catecholamine transporters to specific domains within their primary structure. These studies are the first comprehensive structure-function analysis of members of the rapidly growing superfamily of Na+/Cl- carriers using chimeric transporters. This represents the first step in identifying the specific structural or regulatory determinants that differentiate NET and DAT. An appreciation of the potentially distinct sites for substrate recognition, translocation, and transport inhibition of NET and DAT may facilitate the development of more selective drugs for the treatment of stimulant addiction, human depression, and other affective disorders.

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Drug Interactions with Vesicular Amine Transport

Doris Peter, Juan Jimenez, Yongjian Liu, Andrew Merickel, David Krantz, and Robert H. Edwards

INTRODUCTION

A large body of evidence has implicated monoamine neurotransmitters in a range of behavioral phenomena including mood. Reserpine depletes monoamines and induces a syndrome resembling depression (Frize 1954), giving rise to the monoamine hypothesis of affective disorders. In addition, many drugs used to treat depression act by inhibiting the reuptake of norepinephrine and serotonin from the synapse (Axelrod et al. 1961; Iversen 1976). The reinforcing properties of cocaine derive from its action to inhibit the reuptake of dopamine. Antipsychotic drugs also interfere with signaling by dopamine by interacting with dopamine receptors. Thus, extensive pharmacologic observations have implicated monoamines in psychiatric disease and drug abuse. In each case, the mechanism of drug action has revealed important features of normal signaling by monoamines, such as the role of specific receptors and transport proteins. In the case of psychostimulants, however, the specific mechanism of action remains unclear. In contrast to cocaine, which blocks the reuptake of dopamine from the synapse, amphetamines induce the release of monoamine stored within the presynaptic cell, apparently through a mechanism fundamentally different from the quantal release of classic synaptic transmission.

Classical synaptic transmission involves the regulated release of neurotransmitter in response to neural activity. Although regulated release can result from the reversal of electrogenic plasma membrane neurotransmitter transporters by depolarization (Attwell et al. 1993; Schwartz 1987), the vast majority of regulated release in the nervous system results from the regulated fusion of vesicles with the plasma membrane. Thus, synaptic transmission usually requires the storage of neurotransmitters in specialized secretory vesicles.

Neural peptides and classical transmitters differ in the mode by which they enter secretory vesicles specialized for regulated release. In the case of neural peptides and hormones, protein precursors translocate

into the lumen of the endoplasmic reticulum during translation. The proteins then sort into large, dense core vesicles (or secretory granules in endocrine cells) and undergo proteolytic processing and other modifications before regulated release. In the case of classical transmitters, however, packaging of both newly synthesized transmitter and transmitter accumulated by plasma membrane reuptake occurs in the cytoplasm. Thus, storage in secretory vesicles depends on specific transport from the cytoplasm into the vesicle.

Classical studies have identified four distinct vesicular transport activities for monoamines: acetylcholine, glutamate, and the inhibitory transmitters gamma-aminobutyric acid (GABA) and glycine. The availability of bovine adrenal chromaffin granules with easily detectable transport activity for monoamines has enabled characterization of the bioenergetic basis for this class of neurotransmitter transport. In contrast to the plasma mem-brane transporters that use the Na gradient across the plasma membrane to remove transmitter from the synapse and so terminate its action, the vesicular transporter expressed by chromaffin granules uses the proton electrochemical gradient produced by a vacuolar H+-ATPase to drive transport (Johnson 1988; Kanner and Schuldiner 1987). Specifically, the transporter exchanges two protons in the lumen of the vesicle for one monoamine in the cytoplasm (Knoth et al. 1981). Since the H+-ATPase usually generates a ΔpH of approximately two (log units) in secretory granules, this active transport system can produce concentration gradients of up to 104 inside the vesicle relative to outside. Other vesicular neuro-transmitter transporters appear to use a similar mechanism. Like the vesicular amine transporter, the transporter for acetylcholine also uses predominantly the ΔpH component of the proton electrochemical gradient (Anderson et al. 1982), whereas the vesicular glutamate transporter uses mainly the electrical component of the gradient (Δy) (Carlson et al. 1989; Maycox et al. 1988) and the GABA transporter uses both ΔpH and Δy (Hell et al. 1990). Interestingly, the bioenergetics of vesicular amine transport appear to have an important role in the action of psychostimulants such as amphetamines.

Amphetamines appear to induce the release of stored monoamines by interfering with plasma membrane transport and vesicular amine storage rather than by inducing vesicle fusion with the plasma membrane. Inhibitors of plasma membrane amine transport block the effects of amphetamines, suggesting that reversal of the transporter mediates the release of monoamines from the cytoplasm into the synapse (Rudnick and Wall 1992a, 1992b, 1992c; Sulzer et al. 1993),

possibly by exchange of extracellular amphetamine for cytoplasmic monoamine. The lipophilic properties of amphetamines may enable them to diffuse rapidly back out of the cell after uptake, providing for virtually unlimited exchange and net efflux of the transmitter. Alternatively, protracted uptake of the amphetamine due to rapid diffusion back across the plasma membrane may eventually run down the Na+ gradient that drives transport, allowing monoamines to equilibrate across the plasma membrane in accord with their concentration and potentially accounting for the associated neural toxicity. In the case of either model, however, emptying the cytoplasmic pool of monoamine into the synapse would have little physiological effect unless the normally low cytoplasmic concentrations are increased by efflux from the storage vesicles.

The mechanism by which amphetamines induce the release of stored monoamines into the cytoplasm remains uncertain. Amphetamines may exchange for monoamines in the lumen of the vesicle. Recent obser-vations, however, suggest that amphetamines act as weak bases to disrupt the pH gradient across the vesicle membrane (Schuldiner et al. 1993b; Sulzer and Rayport 1990). Previous work has shown that in the presence of ΔpH, chromaffin granules retain loaded monoamines for up to an hour (Maron et al. 1983). In the absence of ΔpH, previously loaded mono-amines rapidly leak from the vesicle, suggesting that ΔpH prevents reversal of the transporter. Even without weak bases such as the amphetamines, efflux could occur under conditions of energy failure that deplete the ATP in nerve terminals and allow ΔpH to dissipate. The studies describing efflux did not, however, clearly indicate a role for the transporter in efflux. To understand these and other questions about the role of vesicular neurotransmitter transport in synaptic transmission, neuropsychiatric disease, and drug abuse, the authors have isolated cDNA clones for several vesicular neurotrans-mitter transporters and developed biochemical assays to characterize their functional properties, including their interaction with psychostimulants.

VESICULAR AMINE TRANSPORT CONFERS RESISTANCE TO MPP+

The potent neurotoxin N-methyl-4-phenyltetrahydropyridine (MPTP) produces a syndrome with remarkable clinical and pathological similarity to idiopathic Parkinson's disease (PD) (Langston et al. 1983). As with PD, MPTP produces clinical bradykinesia and rigidity. Pathologically, MPTP also results in the relatively selective

degeneration of dopaminergic neurons in the substantia nigra. In addition, the MPTP syndrome responds to L-dopa, with the eventual development of typical disabling dyskinesias.

The strong resemblance between MPTP toxicity and idiopathic PD has suggested that the study of MPTP toxicity will reveal mechanisms that also participate in the pathogenesis of PD. As a neutral lipophilic compound, MPTP easily penetrates the blood-brain barrier. Monoamine oxidase B, presumably expressed by glia, then converts MPTP to the active metabolite N-methyl-4-phenylpyridinium (MPP+) (Heikkila et al. 1984; Langston et al. 1984; Markey et al. 1984). Plasma membrane amine transporters recognize and accumulate MPP+ within monoamine cell groups, accounting for the selectivity of degeneration (Javitch et al. 1985). Inside the cell, MPP+ enters mitochondria and inhibits respiration, apparently at the level of complex I in the respiratory chain (Krueger et al. 1990).

A growing body of evidence supports the relevance of MPTP toxicity for idiopathic PD. The drug selegiline hydrochloride prevents the MPTP syndrome by inhibiting monoamine oxidase B and also appears to slow the rate of progression in PD (Parkinson Study Group 1989). Further, defects in complex I of the respiratory chain appear in PD as well as MPTP toxicity (Mizuno et al. 1989; Ozawa et al. 1990; Parker et al. 1989; Shoffner et al. 1991). However, particular features of the MPTP syndrome are still not understood, and these features may play an important role in idiopathic PD.

Adrenal chromaffin cells express a plasma membrane norepinephrine transporter and accumulate large amounts of MPP+ after systemic injection of MPTP, but, in contrast to dopaminergic neurons in the substantia nigra, these cells do not degenerate (Johannessen et al. 1985; Reinhard et al. 1987). Similarly, rat pheochromocytoma PC12 cells (derived from the adrenal medulla) accumulate MPP+ through a plasma membrane amine transporter but show toxicity only to extremely high concentrations (Denton and Howard 1987; Snyder et al. 1986). Although inhibition of plasma membrane amine transport blocks MPP+ toxicity entirely in PC12 cells, the Chinese hamster ovary (CHO) fibroblast cell line lacks plasma membrane amine transport activity and shows more sensitivity to MPP+ than do PC12 cells. Thus, this potent neurotoxin appears to have more toxicity for a fibroblast cell line than for a neural cell line.

To understand the basis of resistance to MPP+, the authors transferred DNA sequences from the relatively MPP+-resistant PC12 cells into the

relatively MPP+-sensitive CHO fibroblasts and selected the transformants in MPP+. After selection for several weeks, one colony of resistant cells appeared. To dissect the mechanism of their resistance to MPP+, the authors first determined whether the toxin affected respiration (Liu et al. 1992a). In contrast to wild-type CHO cells, which show the rapid inhibition of respiration by MPP+, the resistant cells showed no inhibition, indicating that resistance did not derive from a compensatory mechanism but rather from a primary failure of toxin action. The authors also determined that the resistant cells showed wild-type sensitivity to the other complex I inhibitor rotenone, indicating specificity of the resistance mechanism for MPP+. The authors then found that reserpine completely abolished resistance to the toxin and did not affect the sensitivity of wild-type cells. This suggested that sequences encoding vesicular amine transport had transferred from PC12 cells to CHO fibroblasts. It was presumed that the transporter protects against the toxin by sequestering it in vesicles, away from its primary site of action in mitochondria. To confirm this mechanism of resistance, CHO cells were loaded with large amounts of exogenous dopamine and the intrinsic fluorescence of this transmitter was used to determine its intracellular localization. Whereas wild-type CHO cells showed diffuse staining, MPP+-resistant cells showed a striking, particulate pattern that reverted to wild type in the presence of reserpine, strongly supporting the hypothesis that vesicular amine transport protected the resistant cells by sequestering the toxin in vesicles.

To isolate the sequences responsible for resistance to MPP+, the authors used plasmid rescue (Liu et al. 1992b). Retransfection of the rescued plasmids led to the eventual isolation of a single clone that conferred resistance to MPP+. This clone also conferred vesicular amine transport as determined by dopamine-loaded fluorescence, even in transfected cells not selected in MPP+. Also developed was a quantitative assay for vesicular amine transport using membrane vesicles from the transfected cells. Briefly, the cells are disrupted at narrow clearance (10 micromolars (μm)), the debris sedimented by centrifugation at low speed, and the supernatant incubated in the presence of tritiated monoamine for varying intervals, then rapidly diluted, filtered, and the bound radioactivity measured. As expected, transport activity depends on the presence of ΔpH generated by the vacuolar H+-ATPase; it also shows an affinity for monoamine substrates in the low micromolar range and inhibition by low nanomolar concentrations of reserpine. Surprisingly, tetrabenazine inhibited transport only at high concentrations, but cocaine and tricyclic antidepressant did not inhibit the activity at all. Thus, the

cDNA conferred virtually all of the physiological and pharmacological characteristics expected for the vesicular amine transporter.

The sequence of the cDNA conferring MPP+ resistance and vesicular amine transport predicted a novel protein with 12 transmembrane domains (Liu et al. 1992b). Although many transport proteins are predicted to have 12 transmembrane domains, the primary amino acid sequence of the vesicular amine transporter showed no similarity to the plasma membrane neurotransmitter transporters, or other mammalian transporters and thus appeared to define a novel mammalian gene family that is now known to include the vesicular transporters for other neurotransmitters such as acetylcholine (Erickson et al. 1994; Roghani et al. 1994; Varoqui et al. 1994) (figure 1). However, the first six transmembrane domains of the vesicular amine transporter show weak but definite homology to a class of bacterial antibiotic resistance proteins (Liu et al. 1992b). Interestingly, these proteins transport antibiotics out of bacteria, a phenomenon topo-logically equivalent to the transport of MPP+ into vesicles. Further, the bacterial transporters also act by proton exchange (Kaneko et al. 1985). In the case of the bacterial multidrug resistance transporter, reserpine inhibits its activity (Neyfakh et al. 1991). Thus, the vesicular amine transporter shows functional as well as structural similarity to these bacterial proteins. The relationship suggests that vesicular neurotransmitter transport evolved from these ancient detoxifying systems. Together with cloning of the vesicular amine transporter by selection in MPP+, the relationship raises the possibility that vesicular transport plays two roles in the nervous system: one in packaging transmitter for regulated release and the other in neural protection. However, MPTP or another exogenous toxin has not been identified in idiopathic PD (Tanner and Langston 1990).

Vesicular transport may protect against the normal transmitter dopamine itself. Monoamines and dopamine in particular oxidize very easily, producing free radicals that injure neural cells by a mechanism that does not involve interaction with a specific receptor (Cohen 1990; Michel and Hefti 1990; Rosenberg 1988). Vesicular amine transport would clearly protect against this form of endogenous toxicity as well as against the toxicity of MPP+. Thus, a defect in vesicular amine transport could contribute to the pathogenesis of idiopathic PD. The toxicity associated with amphetamines may also result from the efflux of monoamine stores into the cytoplasm. Indeed, recent imaging studies of primary dopaminergic neuronal cultures show localization of free-radical injury induced by amphetamines to the sites of vesicle accumulation (Cubells et al. 1994).

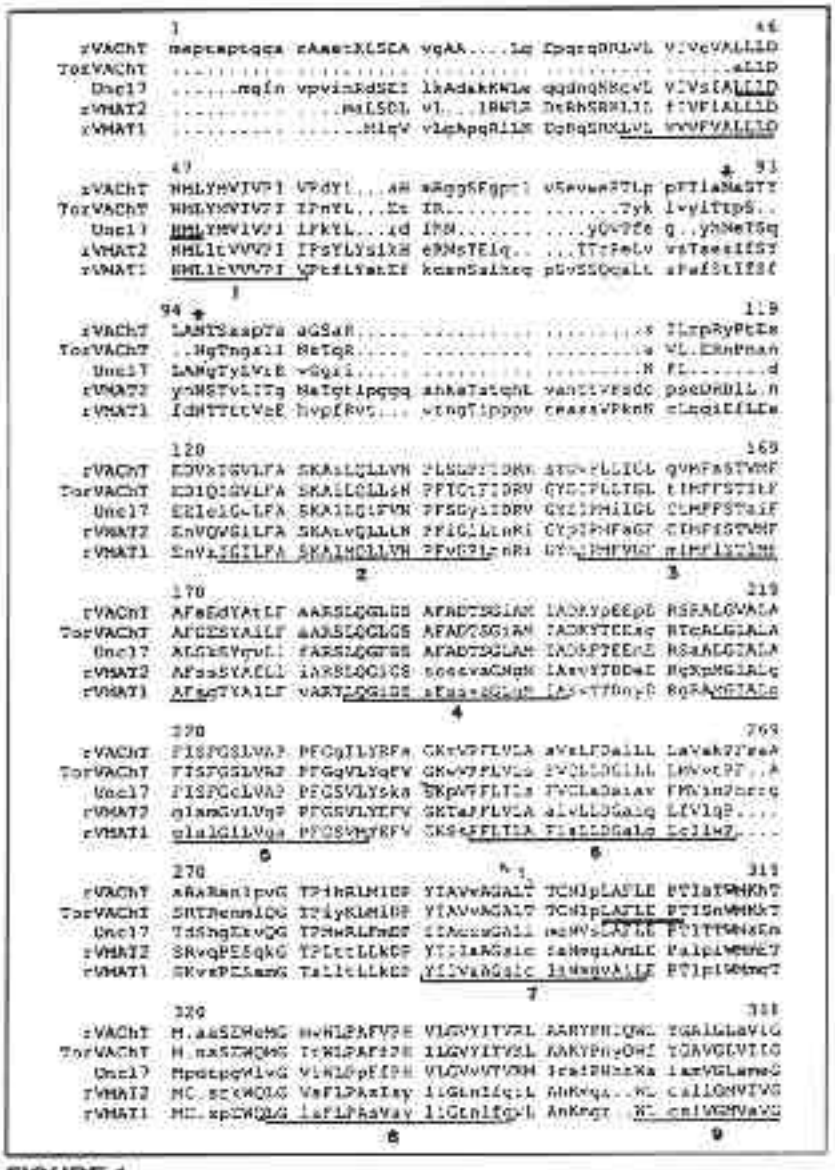

FIGURE 1.

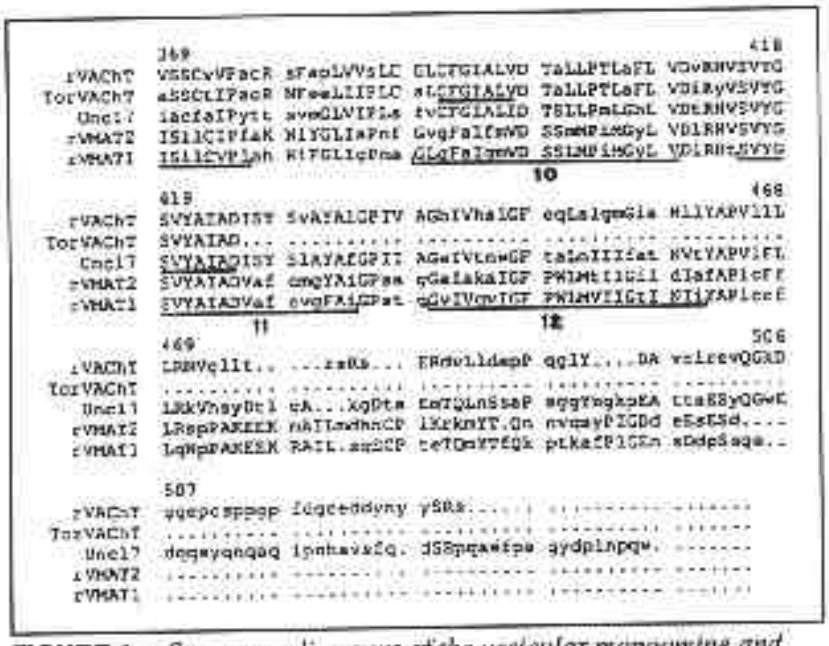

FIGURE 1. *Sequence alignment of the vesicular monoamine and putative vesicular acetylcholine transporters. rVMAT-1 and rVMAT-2 indicate the rat chromaffin granule amine transporter and rat synaptic vesicle amine transporter, respectively. Unc17, TorVAChT and rVAChT denote the putative vesicular acetylcholine transporters from* C. elegans, T. californica, *and rat. Upper case letters denote the consensus and lower case the divergent residues. The underlined sequences in unc17 indicate the regions used to design degenerate oligonucleotide primers for PCR amplification of TorVAChT. The underlined residues in TorVAChT indicate the regions used to design degenerate oligonucleotide primers for PCR amplification of rVAChT. Brackets mark the predicted transmembrane domains, asterisks the potential sites for N-linked glycosylation in rVAChT. The twelfth transmembrane domain and the C-terminus contain a leucine-rich region. The numbers refer to amino acid residues of rVAChT.*

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TWO DISTINCT GENES ENCODE VESICULAR AMINE TRANSPORT

Classical studies have shown that monoamine cell populations in the central nervous system (CNS) express vesicular amine transport activity that is inhibited by reserpine and tetrabenazine. However, Northern blot analysis of polyA+ ribonucleic acid (RNA) from different tissues including the brain showed expression of sequences encoding the chromaffin granule amine transporter (CGAT or VMAT-1) in only the adrenal gland. Although low levels of expression or expression by a small proportion of cells could account for the failure to detect a signal, the authors pursued the alternative possibility that the brain expresses a distinct vesicular amine transporter. Screening a brainstem cDNA library with the CGAT cDNA as probe under moderately reduced stringency revealed a second distinct but closely related transporter, originally termed the synaptic vesicle amine transporter (SVAT or VMAT-2) (Liu et al. 1992b) (figure 2). Northern analysis showed expression of this sequence in the brainstem but not the adrenal gland. In situ hybridization also showed expression by the expected dopaminergic cell groups in the substantia nigra and ventral tegmental area, the noradrenergic neurons of the locus coeruleus, and serotonergic neurons of the dorsal raphe, consistent with the previously demonstrated recognition of multiple monoamine transmitters with similar affinity by the transporter from bovine chromaffin granules. Thus, chromaffin cells and central neurons express distinct but highly related vesicular monoamine transporters.

Surprisingly, an amine transporter purified from bovine chromaffin granules corresponds more closely to the central rat transporter than to the adrenal transporter (Howell et al. 1994). Purification of proteins labeled by 3H-reserpine had in fact yielded two proteins that differ in isoelectric point (Stern-Bach et al. 1990), with the sequence of one corresponding to VMAT-2 (Howell et al. 1994). Additional study has further shown that the bovine and rat adrenal glands contain both transporters, although the rat adrenal contains an overwhelming preponderance of the expected VMAT-1 transporter (Peter, et al., in press). Further, the purified bovine transporter showed high sensitivity to inhibition by tetrabenazine (Howell et al. 1994), whereas rat VMAT-1 did not (Liu et al. 1992b). To under-stand the basis for these differences in pharmacology as well as to identify other differences that may play a role in synaptic transmission and possibly protect against neural degeneration, the authors have characterized the functional properties of cloned VMAT-1 and VMAT-2 (Peter et al. 1994).

Using expression in a heterologous system, it has been determined that VMAT-1 and VMAT-2 differ in their physiological properties as well as in their pharmacology (Peter et al. 1994). Both VMAT-1 and VMAT-2 protect against MPP+ toxicity after expression in CHO cells, and these stably transformed cell lines have been used to determine their functional characteristics. VMAT-2 has an approximately twofold to threefold higher affinity for most monoamine substrates than VMAT-1 (table 1). Both transporters have the highest affinity for serotonin, followed by dopamine, then norepinephrine and epinephrine. However, the transporters differ dramatically in their affinity for histamine, with low micromolar concentrations inhibiting transport of 3H-serotonin by VMAT-2 but two orders of magnitude more required to inhibit VMAT-1.

To further compare the physiologic and pharmacologic properties of VMAT-1 and VMAT-2, the authors have investigated the interaction of the transporters with the antihypertensive drug reserpine. Using membranes prepared from stably transfected CHO cell lines, it has been found that reserpine inhibits transport of amines by both VMAT-1 and VMAT-2 with high potency and binds to VMAT-1 with two distinct affinities (Peter et al. 1994; Schuldiner et al. 1993a). Previous work had shown that monoamines inhibit reserpine binding with an affinity similar to their apparent affinity as substrates for transport, indicating that reserpine binds at or near the site of substrate recognition. In addition, the imposition of ΔpH accelerates the rate of reserpine binding, suggesting that reserpine can detect conformational changes in the protein that occur during the transport cycle (Rudnick et al. 1990; Scherman and Henry 1984; Weaver and Deupree 1982). Both of these observations have been confirmed for VMAT-1 expressed in CHO cells (Schuldiner et al. 1993a). Binding to reserpine also enables quantitation of the transporter and hence calculation of the turnover number for VMAT-1 (~10/min) and VMAT-2 400/min at saturating substrate concentrations and 29°C (Peter et al. 1994). Thus, the transporter expressed by dopaminergic cells susceptible to MPTP (VMAT-2) has a higher apparent affinity for substrates and a faster turnover than the transporter expressed by more resistant cells in the adrenal medulla (VMAT-1) and so cannot account for the differential vulnerability seen in MPTP toxicity and PD. Rather, the results suggest that vesicular amine transport acts to protect against endogenous or exogenous toxins in both cell types but does not suffice to protect neurons in the substantia nigra.

In contrast to the apparent similarity of their interaction with reserpine, VMAT-1 and VMAT-2 differ in their interaction with another inhibitor of transport, tetrabenazine. As noted above, high concentrations of tetra-

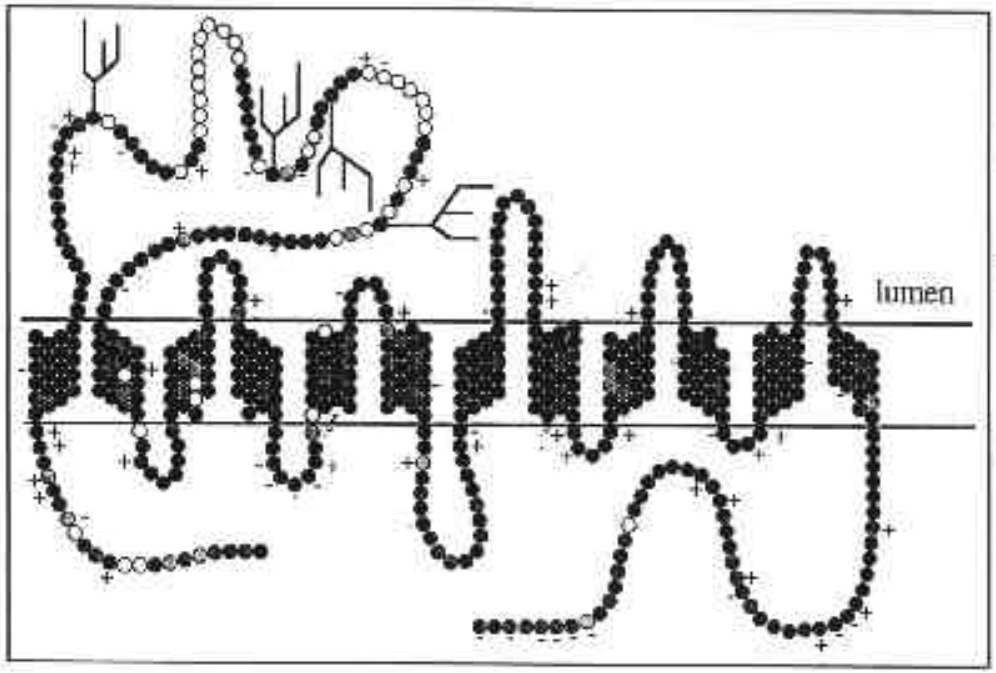

FIGURE 2.

Predicted structures of VMAT-1 and VMAT-2. The lumen of the vesicle is above, the cytoplasm below. Basic and acidic amino acid residues are indicated by a plus and minus, respectively. Branched structures in the first lumenal loop indicate potential N-linked carbohydrates. The residues in black are identical between the two vesicular amine transporters, the white residues divergent. The lightly stippled residues are conservative differences between VMAT 1 and VMAT 2. The darkly stippled residues are residues of VMAT-2 that diverge from VMAT-1 but show conservation to human VMAT-2.

SOURCE: Reproduced from Liu et al. 1992*b* with permission of Cell Press.

TABLE 1. Apparent affinity of VMAT-1 and VMAT-2 for monoamine substrates and MPP+. Standard transport assay (Liu et al. 1992b) was performed at 29oC for 2 minutes using 3H-serotonin. The values indicate the Km for serotonin and the Kis for other compounds.

Substrate	VMAT-1	VMAT-2
	μM	μM
Serotonin	0.85 ± 0.23	0.19 ± 0.04
Dopamine	1.56 ± 0.35	0.32 ± 0.04
Epinephrine	1.86 ± 0.11	0.47 ± 0.05
Norepinephrine	2.5 ± 0.4	0.33 ± 0.06
Histamine	436 ± 36	3.06 ± 1.0
MPP+	2.8 ± 0.6	1.6 ± 0.45

SOURCE: Reproduced from Peter et al. 1994 with the permission of the American Society for Biochemistry and Molecular Biology.

benazine are required to inhibit transport by VMAT-1, whereas the purified bovine chromaffin granule transporter shows sensitivity to low nanomolar amounts. Consistent with the sequence similarity to the purified bovine transporter, VMAT-2 also shows sensitivity to nanomolar concentrations of tetrabenazine (figure 3) (Peter et al. 1994). The differential inhibition of VMAT-1 and VMAT-2 by tetrabenazine, although not anticipated, does account for several classic pharmacological observations. In contrast to reserpine, which depletes both peripheral and central monoamine stores, tetrabenazine depletes predominantly central stores and so causes less hypotension (Carlsson 1965). Differences in the turnover of monoamine stores in the adrenal gland and the brain had been invoked to explain this differential effect (Scherman and Boschi 1988). However, differential inhibition of VMAT-1 and VMAT-2 by tetrabenazine now seems far more likely to account for the observations.

In further contrast to reserpine, classical studies have shown that only large amounts of monoamine transmitters displace 3H-dihydrotetrabenazine from the bovine transporter (Scherman and Henry 1984) and ΔpH does not

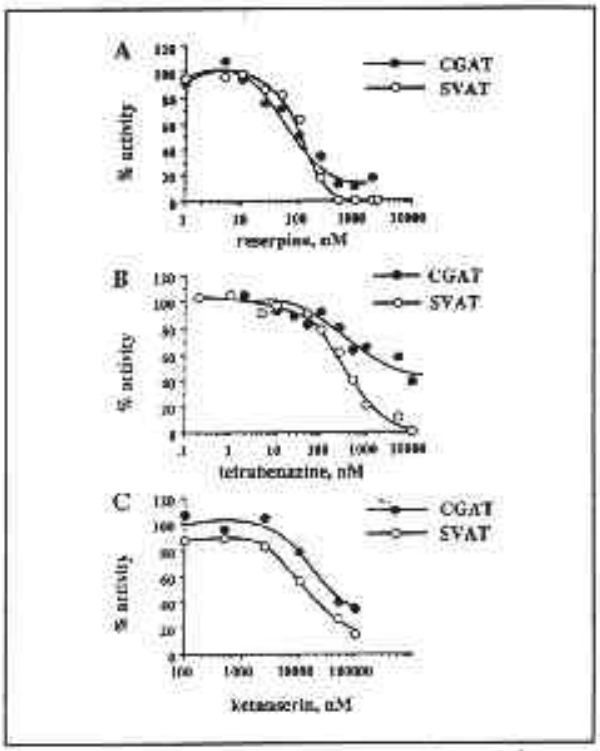

FIGURE 3. *Differential inhibition of VMAT-1 (CGAT) and VMAT-2 (SVAT) by tetrabenazine but not reserpine or ketanserin. Dose-response analysis of ^{3}H-serotonin transport by membranes from COS cells transiently transfected with the two cDNAs. Reserpine inhibits the two vesicular amine transporters with similar potency (A), but tetrabenazine shows substantially higher potency as an inhibitor of VMAT-2 than VMAT-1 (B). Ketanserin is thought to interact with the same site as tetrabenazine but inhibits VMAT-1 and VMAT-2 with similar potency (C).*

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influence binding, indicating that tetrabenazine does not bind at the site of substrate recognition and recognition of the drug does not change during the transport cycle. However, tetrabenazine can prevent reserpine binding, suggesting an allosteric interaction between the two sites (Darchen et al. 1989). In the case of VMAT-1 and VMAT-2, tetrabenazine also inhibits 3H-reserpine binding but with distinct potencies that correspond to the differential sensitivity of transport to tetrabenazine (Peter et al. 1994). Thus, the difference in tetrabenazine sensitivity between VMAT-1 and VMAT-2 does not reflect a difference in the interaction between these two sites. Rather, additional study with 3H-dihydrotetrabenazine has shown binding to VMAT-2 but not VMAT-1, indicating a simple difference in drug recognition (Peter et al. 1994). The vesicular amine transporters also differ in their interaction with psychostimulants.

VESICULAR AMINE TRANSPORT AND PSYCHOSTIMULANTS

The authors have found that the two vesicular amine transporters differ in their interaction with amphetamines. Methamphetamine inhibits the transport of 3H-serotonin by VMAT-2 much more potently than transport by VMAT-1 (figure 4) (Peter et al. 1994). Although amphetamines can inhibit transport by the dissipation of ΔpH (Sulzer and Rayport 1990), the differential inhibition of VMAT-1 and VMAT-2 and the stereospecificity of the inhibition make a direct interaction far more likely. Indeed, meth-amphetamine inhibits reserpine binding to VMAT-2 with greater potency than to VMAT-1, indicating interaction with the site of substrate recog-nition (Peter et al. 1994). Nonetheless, the significance of this interaction for the mechanism by which psychostimulants induce monoamine efflux remains uncertain. In particular, the lipophilic nature of amphetamines may not require specific transport into vesicles to dissipate ΔpH. Alternatively, recognition by the transporter may promote efflux through an exchange mechanism. However, dissection of the role that the transporter plays in efflux induced by dissipation of ΔpH and efflux induced by amphetamines requires the development of an appropriate heterologous expression system.

Since amphetamines act by inducing the release of stored monoamines, the site of storage also appears critical to their action as toxins. Indeed, amphetamines produce free radical injury localized to the sites where vesicles accumulate (Cubells et al. 1994). The site of monoamine storage appears to differ from other classical transmitters. Small (40 nm), clear synaptic vesicles contain such classical transmitters as acetylcholine,

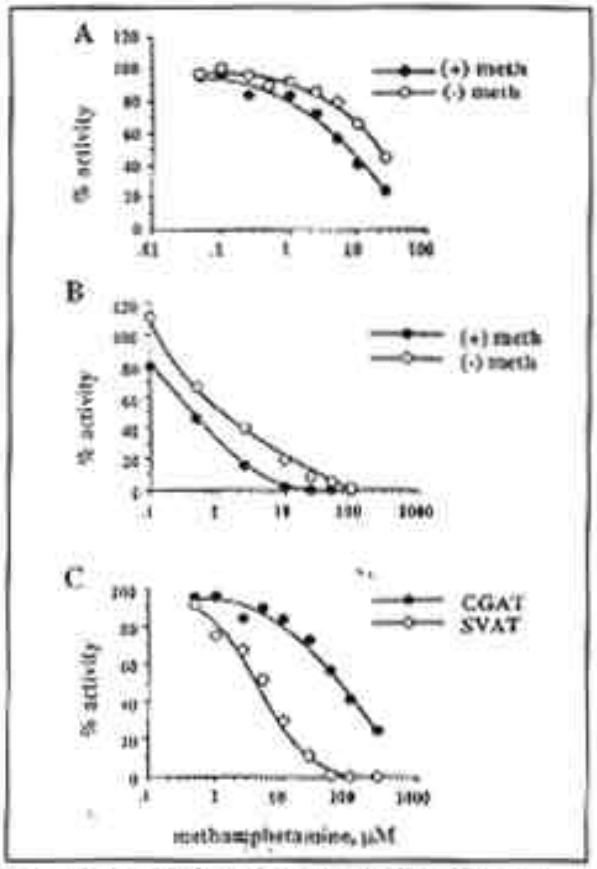

FIGURE 4. *Methamphetamine inhibits VMAT-1 (CGAT) and VMAT-2 (SVAT). Using membranes from stable CHO transformants expressing the two vesicular amine transporters, methamphetamine inhibits serotonin transport by VMAT-1 (A) and VMAT-2 (B) with the anticipated stereospecificity. However, the two transporters differ substantially in their sensitivity to the psychostimulant (C).*

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GABA, glycine, and glutamate; they mediate typical fast synaptic trans-mission and cluster over the nerve terminal (De Camilli and Jahn 1990; Sudhof and Jahn 1991; Trimble et al. 1991). However, monoamines appear to be stored with neural peptides in larger, dense core vesicles in at least some tissues such as the adrenal gland (and PC12 cells). In contrast to synaptic vesicles, dense core vesicles (or secretory granules in endocrine cells) mediate the relatively slow release of neuromodulators and occur in the cell body and dendrites as well as the nerve terminal. Thus, the site of monoamine storage has profound consequences for its role in signaling. Interestingly, smaller, occasionally dense cored vesicles that cluster over the synapse appear to store monoamines in the CNS.

Since localization of the vesicular amine transporters determines the site of monoamine storage, the authors have examined the distribution of endoge-nous VMAT-1 in the neuroendocrine PC12 cell line using a polyclonal antibody generated against a peptide derived from the C-terminus of the protein. By both immunofluorescence and density gradient centrifugation through several different media, VMAT-1 sorts to dense core vesicles (figure 5), accounting for the pattern of monoamine storage (Liu et al. 1994). Only small amounts of immunoreactive material appear in lighter synaptic-like microvesicles. Thus, in contrast to the numerous peptides that sort to the regulated secretory pathway, VMAT-1 is the first integral mem-brane protein identified that is preferentially expressed on dense core vesicles rather than synaptic vesicles. Since neural peptides apparently sort to this pathway by aggregation (Burgess and Kelly 1987; Chanat and Huttner 1991), the availability of a membrane protein may enable identi-fication of a specific sorting signal. The identification of this signal may then help to explain how aggregated lumenal proteins such as neural peptides sort to the pathway. In addition, VMAT-1 contains signals for endocytosis, accounting for the detection of transport activity using membranes from CHO cells (Liu et al. 1992b). However, the storage of central monoamines in smaller vesicles requires explanation. It may derive from the expression of a transporter (VMAT-2) with distinct sorting sequences or from expression within a different cell type. As a result of sorting to different types of vesicle, monoamines may play distinct roles in synaptic transmission and influence the activity of amphetamines.

CONCLUSIONS

The authors have used selection in the neurotoxin MPP+ to isolate a cDNA clone encoding vesicular amine transport. The protein protects

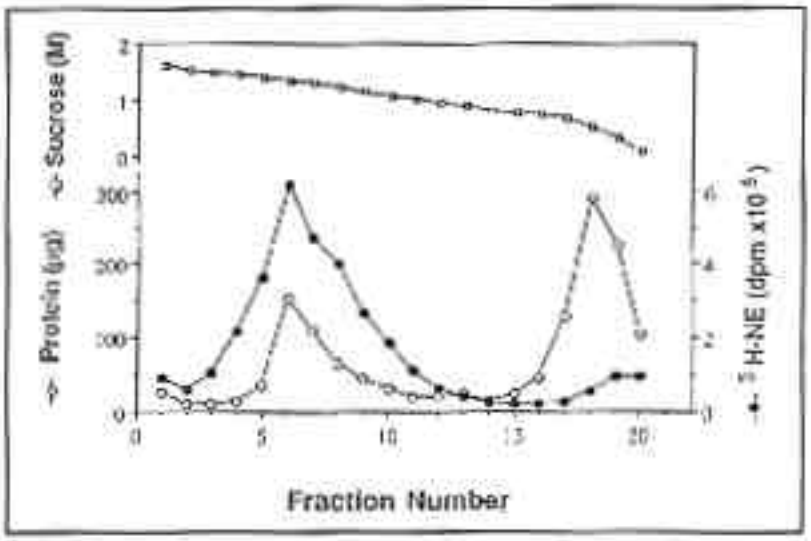

FIGURE 5a. *Distribution of VMAT-1 by equilibrium sedimentation of PC12 membranes through sucrose. The cells were loaded overnight with ^{3}H-norepinephrine, the nuclear debris pelleted, and the supernatant separated by sedimentation through a continuous 0.6 to 1.6 M sucrose gradient. Fractions were collected from the bottom and the accumulation of radioactivity indicates large dense core vesicles centered at fraction 6 (A). Western blot analysis shows that VMAT-1 also centers around fraction 6 together with secretogranin II, away from the peak for synaptic-like microvesicles marked by synaptophysin (B). VMAT-1 occurs in two forms of different molecular weight.*

SOURCE: Reproduced from *The Journal of Cell Biology*, 1994, vol. 127 by copyright permission of The Rockefeller University Press (Liu et al. 1994).

against MPP+ by sequestering it in vesicles, away from its primary site of action in mitochondria. Interestingly, the sequence of the cDNA predicts a protein with 12 transmembrane domains but no strong relationship to previously reported sequences other than several bacterial antibiotic-resistant proteins, suggesting evolution from ancient detoxification systems. Molecular cloning has further demonstrated that two distinct

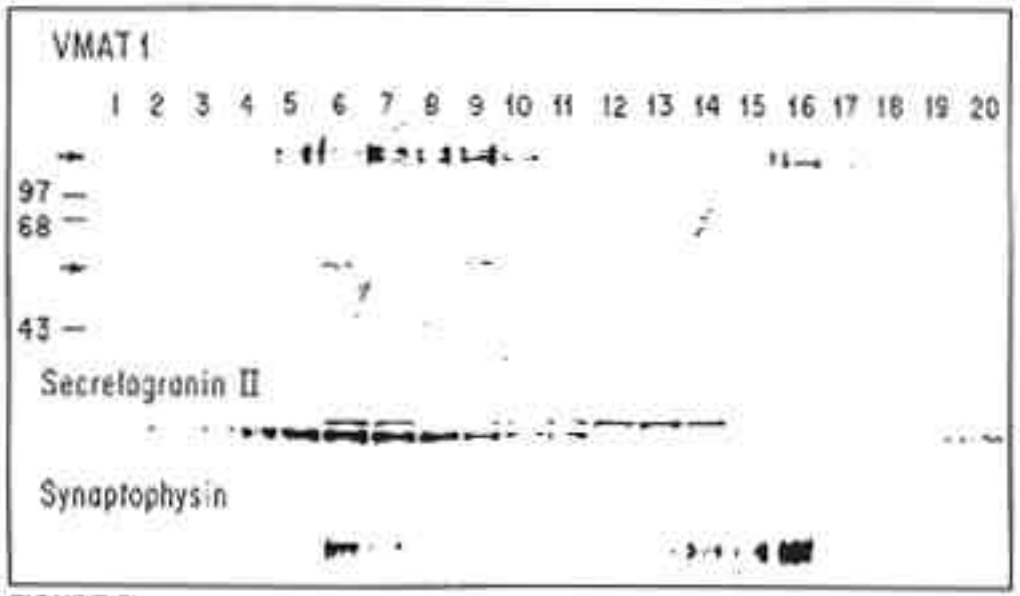
VMAT 1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
97
68
43
Secretogranin II
Synaptophysin

FIGURE 5b.

proteins mediate vesicular amine transport in the adrenal gland and central nervous system (VMAT-1 and VMAT-2, respectively). Using membrane vesicles from cells transfected with the two cDNAs, it has been found that they differ in physiological and pharmacological properties, including the interaction with amphetamines. In conjunction with the development of appropriate expression systems and efflux assays, the availability of the cDNA clones will enable scientists to dissect the mechanism of amphetamine action. In particular, does amphetamine-induced efflux involve reversal of the vesicular transporter? Do amphetamines simply dissipate ΔpH or do they interact directly with the transport protein? The storage of monoamines within dense core vesicles and synaptic vesicles will also influence the site of monoamine release and hence the psychostimulant and neurotoxic action of amphetamines. It has been found that VMAT-1 sorts preferentially to dense core vesicles in PC12 cells. To understand the psychostimulant and neurotoxic actions of amphetamines, researchers must now examine the subcellular localization of VMAT-2 in the CNS. In particular, how does the intracellular trafficking differ from VMAT-1 in the adrenal gland and PC12 cells? What is the mechanism of sorting?

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Strategies for Identifying Genes Underlying Drug Abuse Susceptibility

John C. Crabbe, Kari J. Buck, Pamela Metten, and John K. Belknap

INTRODUCTION

It is becoming widely accepted that most, if not all, drug responses are subject to influence by genetic factors. The magnitude, duration, and even the quality or direction of response can differ among individuals with differing genotypes. Most drug responses are not simple genetic traits; rather, multiple genes can be demonstrated to influence a given response. In addition, any gene is likely to affect multiple traits, a condition called pleiotropism. To the extent that two traits are pleiotropically influenced by some of the same genes, they are said to be genetically correlated. Use of genetic animal models has achieved significant progress in the analysis of drug response traits.

The goal of this chapter is to discuss two related methods for tracing the pathways from genes to drug-related behaviors. An example of one method, Quantitative Trait Locus (QTL) gene mapping, will demonstrate that a gene has been identified that appears to affect the severity of withdrawal from multiple drugs. The utility of this method for extrapolating animal model findings to humans will be discussed.

STUDIES WITH PANELS OF INBRED STRAINS

One of the classic methods of pharmacogenetics depends upon the analysis of inbred strains. In a seminal report, McClearn and Rodgers (1959) reported that inbred mouse strains differed markedly in their consumption of alcohol solutions. Because an inbred strain is created by mating close genetic relatives for many generations, all mice of the same sex within an inbred strain are virtually identical genetically. There are more than 100 inbred strains of mice commercially available. If individuals from several inbred strains are tested for a response in carefully controlled environ-mental conditions, differences among strain mean values are due to genetic differences, while pooled individual differences within strains are by definition

nongenetic or environmental (see figure 1). Therefore, partitioning variance in responses leads to a direct estimate of the proportion of trait variability due to genetic influences, technically known as the heritability (Falconer 1989).

Comparisons of multiple strains on multiple traits can then be used to estimate genetic correlations. Statistically, this is accomplished by partitioning trait covariance into among-strain and within-strain components (Falconer 1989). An experiment testing two strains for two traits is uninformative: multiple strains are required before correlations can be estimated with any degree of confidence. How many strains should be examined depends on the genetic structure of the traits examined, which is usually not known. One estimate of the number suggested in practice as sufficient to detect genetic correlations of modest degree with modest statistical power is 7 to 10 strains (Crabbe et al. 1990).

In one example of this approach, mice from 15 inbred strains were tested for the severity of acute withdrawal following intraperitoneal (IP) injection of 4 grams per kilogram (g/kg) ethanol by assessing the magnitude of their handling-induced convulsions over several hours. The same mice were tested 1 to 2 weeks later for withdrawal severity after 60milligrams per kg (mg/kg) pentobarbital. Strains differed significantly in withdrawal from both drugs. Figure 2 shows the genetic correlation resulting from the analysis of mean strain responses on the two traits. The significant corre-lation seen suggests that about half the genetic variability in each response is shared, and is presumably due to the pleiotropic influence of some genes on both responses (Metten and Crabbe 1994). Significant genetic correlations between ethanol and nitrous oxide withdrawal severity have also been reported in a similar analysis of a set of recombinant inbred strains (Belknap et al. 1993a). Studies with mice selectively bred to express severe (withdrawal seizure prone, WSP) or mild (withdrawal seizure resistant, WSR) ethanol withdrawal handling-induced convulsions have revealed that they differ in severity of withdrawal from diazepam, abecarnil, pentobarbital, phenobarbital, nitrous oxide, several other alcohols, and acetaldehyde. WSP mice have more severe acute or chronic withdrawal than WSR mice from all the above agents (Belknap et al. 1987, 1988, 1989; Crabbe et al. 1991). Together, these results suggest that some genes affect withdrawal from several different central nervous system depressant agents.

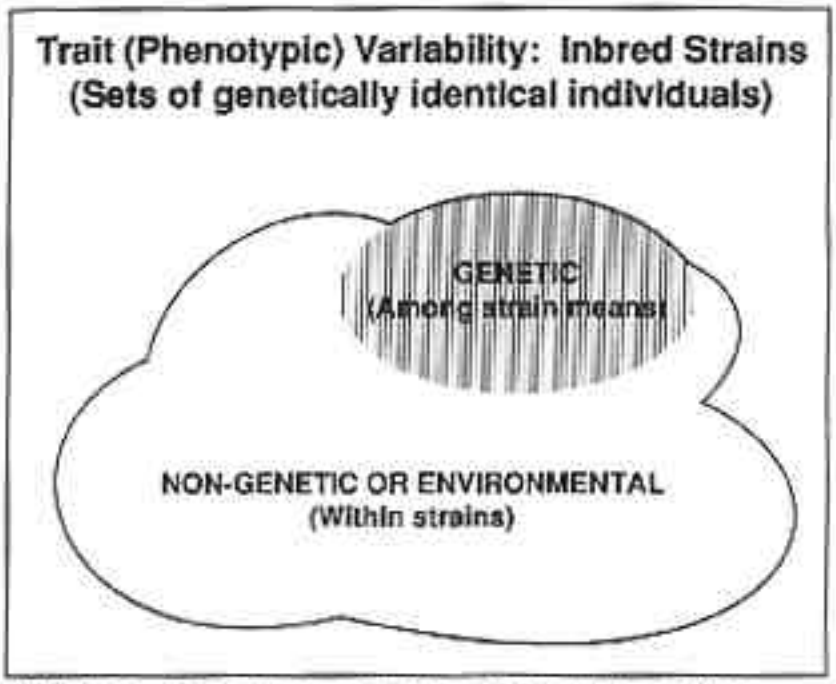

FIGURE 1. *Schematic representation of the partition of drug-response variability in a study employing several inbred strains tested under controlled environmental conditions.*

A large literature documenting strain differences in response to virtually all abused drugs has accumulated over the years. A major advantage of inbred strains is their genetic stability, so studies performed 10 years ago can be directly compared with those employing the same strains performed last week. Strain differences in drug responses have been reviewed (Broadhurst 1978; Crabbe and Harris 1991).

A systematic attempt to determine patterns of genetic commonality has recently been undertaken in Portland. Over a 4-year period, 15 inbred strains of mice were studied after administration of saline or 1 of 4 doses of morphine (Belknap et al., submitted), ethanol (Crabbe et al. 1994a), diazepam (Gallaher et al., submitted), or pentobarbital (Crabbe et al., in process). To the extent possible, the same battery of tests was employed. All drugs were tested for their effects on open-field activity and body temperature; in addition, preference drinking of different concentrations of the drugs in tap water, and subsequently in saccharine solutions, was

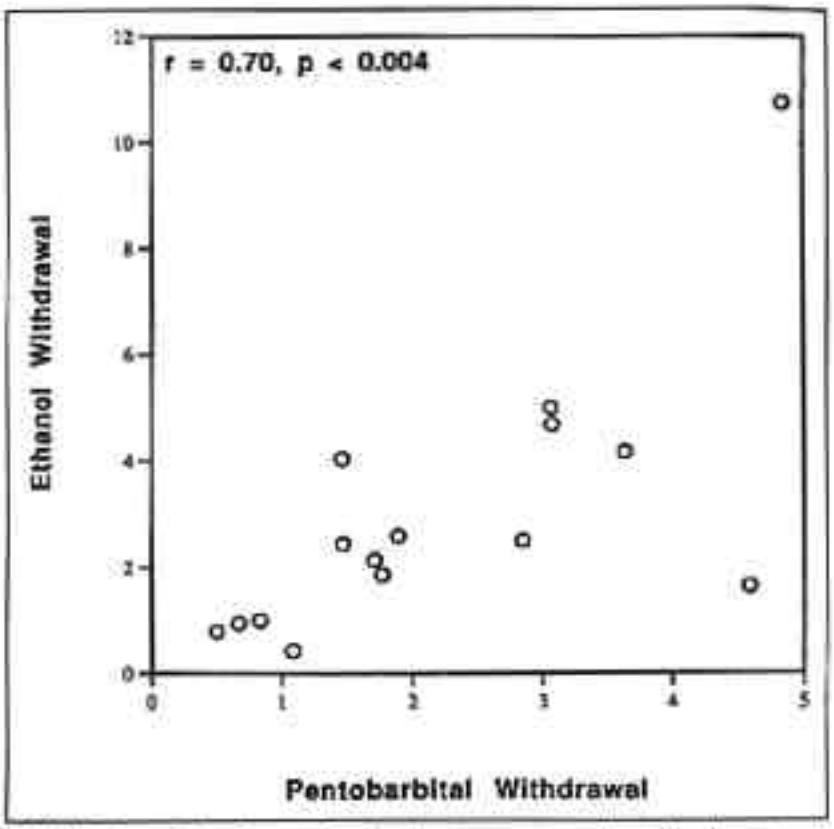

FIGURE 2. *Genetic correlation between acute ethanol and acute pentobarbital withdrawal severity in 15 standard inbred strains of mice. Area under the withdrawal curve for each strain on each measure is plotted as a circle.*

SOURCE: From Metten and Crabbe 1994 with permission.

determined (Belknap et al. 1993b, 1993c; Belknap et al., in process). Other drug-specific responses were also examined (e.g.,loss of righting reflex after ethanol and pentobarbital).

An example of results from these studies is given in figure 3. It can be seen that strains differed markedly in the sensitivity to the locomotor stimulant effects of IP morphine. Not only were there quantitative

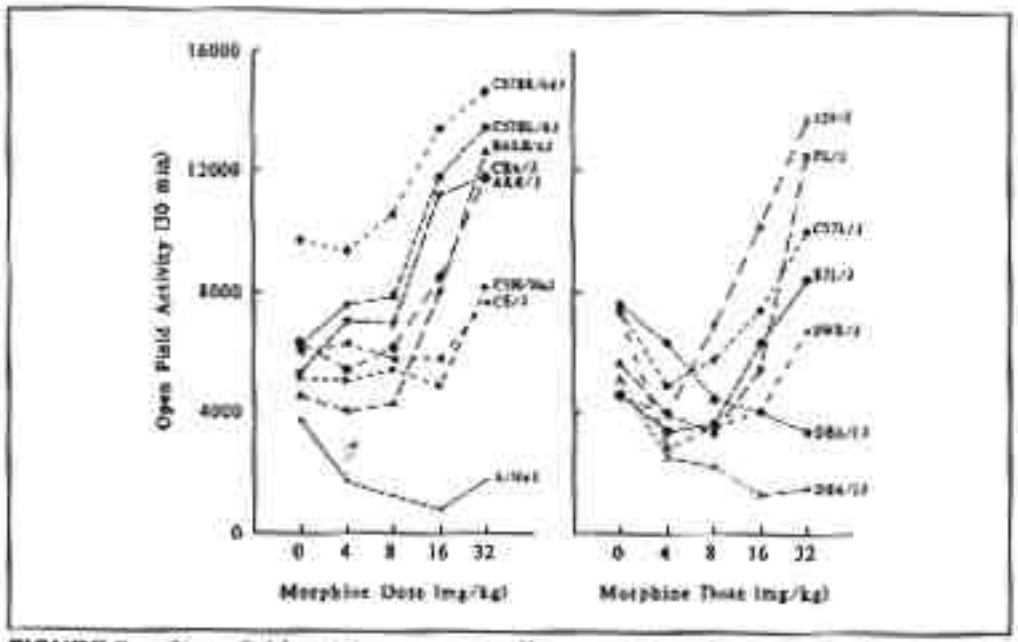

FIGURE 3. *Open-field activity automatically recorded as distance traveled (in cm) 30 min following injection of indicated dose of morphine or saline. Strains are divided into two groups for clarity. Each symbol represents the mean value for the indicated dose and strain. N = 24 to 41 mice per strain, approximately equally divided across doses.*

SOURCE: Data from Belknap et al., submitted.

differences in response magnitude, but qualitative differences could be seen: the A/HeJ, DBA/1J, and DBA/2J strains responded with reduced activity at all morphine doses. Similar qualitative strain differences in morphine-stimulated activity have been known for many years (Shuster et al. 1975). Strain differences of the magnitude shown in figure 3 were detected for virtually all responses to all drugs.

Two traits were tested for all four of the drugs examined. To identify common patterns of genetic influence on these traits, a multidimensional scaling (MDS) analysis was performed (Kruskal 1964; Kruskal and Wish 1978). Mean locomotor activity, monitored for 15 minutes, and the mean change in body temperature 30 minutes after injection were calculated for each strain. For each variable, strain mean values for the saline groups were subtracted from the drug-dosage group means. A (genetic) correlation matrix among strain means was calculated and subjected to least squares MDS. The results of this analysis are shown in figure 4. The resulting plot shows the position of each drug dose response relative to all others, mapped by its weighting on the two principal dimensions identified by the MDS analysis. Linear distance represents similarity among the plotted variables in the pattern of inbred strain (genetic) differences. Points close together share a great deal of common genetic influence (i.e., are genetically correlated), while points far apart do not. The numbers near each point identify the dose in milligrams per kilogram or grams per kilogram of EtOH. The two primary dimensions (linear components) mapped accounted for 85 percent of the total genetic variance in this data set.

Several results of this analysis can be identified. Different doses of the same drug on a given response showed a substantial degree of common genetic influence; this was to be expected. It also appeared that both activity and temperature responses to morphine were genetically related at all four doses. This cluster of responses was reasonably distinct from other clusters of variables/doses/drugs. In addition, most doses of diaze-pam and pentobarbital (for both activity and temperature responses), and the activating effects of moderate doses of ethanol, were grouped in a common cluster. All three of these drugs influence gamma aminobutyric acid A (GABAA) receptor-gated chloride ion conductance (Harris and Allan 1989; Macdonald and Olsen 1994). Therefore, modulation of this receptor complex is a possible common mechanism for the mediation of the genetic similarity of this response cluster. It is of interest that ethanol effects on body temperature were genetically distinct from ethanol-induced activity responses. It seems reasonable to suggest that ethanol

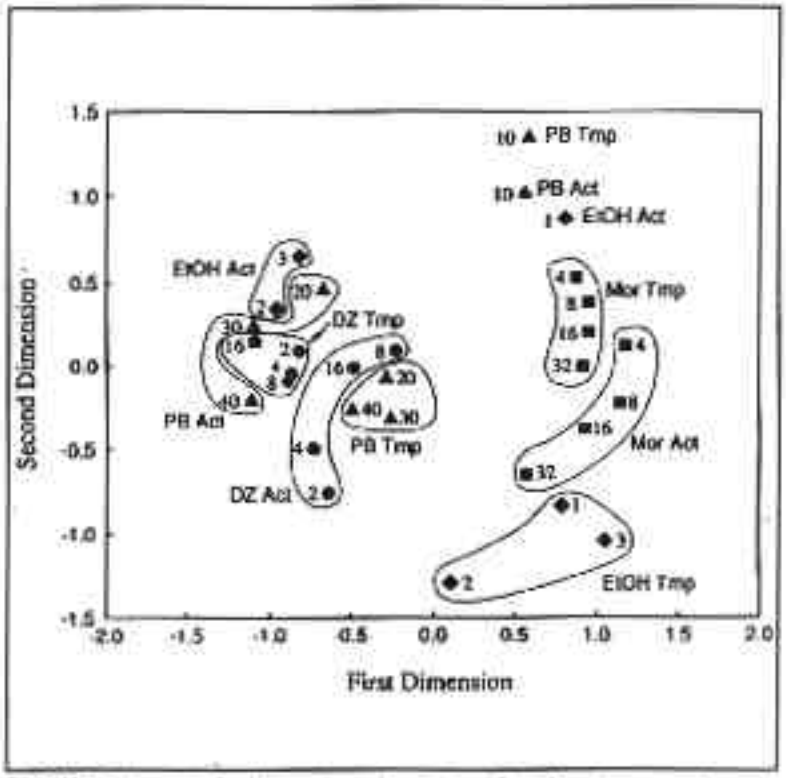

FIGURE 4. *Multidimensional scaling plot showing genetic similarity among responses to given doses of ethanol (EtOH), diazepam (DZ), pentobarbital (PB), or morphine (Mor). Perimeters are drawn to emphasize doses from a single drug. Responses displayed are locomotor activity in an open field (Act) or change in body temperature (Tmp).*

KEY: ♦= EtOH; ●= diazepam; ■= morphine; and ▲= pentobarbital.

SOURCE: Reprinted from Crabbe et al. 1994*b*, with permission. Copyright 1994 American Association for the Advancement of Science.

hypothermia is not likely to be influenced to a major degree by the genetically determined mechanisms underlying pentobarbital and diaze-pam hypothermia. Finally, the effects of the lowest doses of ethanol and pentobarbital on activity appeared to be genetically distinct from the higher doses, and are similar to the effects of the lowest dose of pentobarbital on body temperature.

This is only one example of the application of multivariate methods to the analysis of genetic similarity among drugs and responses. There is an accumulating wealth of knowledge about drug responses in a few specified genotypes. It would be of interest to know, for example, whether the several drug responses hypothesized to index drug reward, intravenous (IV) self-administration, preference drinking, place-conditioned and taste-conditioned responses, and forward locomotion and its sensitization with repeated administration of drug shared a common genetic influence. Extension of multiple-strain studies to include neurochemical and neuro-pharmacological responses would be of utility in identifying common mechanisms of drug action.

QTL GENE MAPPING

Traditional analyses of drug sensitivity using genetic animal models such as those described above are able to estimate degree of genetic influence, and to identify response clusters of genetic codetermination. Many studies not reviewed above have also employed genetic animal models in cases where identified candidate genes are implicated in a given drug effect. (See Crabbe and Harris 1991 and Crabbe et al. 1994b for reviews.) For most drug responses, no clear candidate gene is indicated, and several questions remain to be addressed. It would be useful to determine how many genes influence the trait, what their function is, and where they are located in the genome. Traits affected by multiple genes usually display a continuous degree of response (rather than all-or-none responses), and the genes responsible are referred to as QTLs. Although each QTL may have only a modest effect on the drug response, collectively, the QTLs can determine a major proportion of drug responsiveness.

The recently developed technique of QTL gene mapping (Paterson et al. 1988) can be employed to identify the chromosomal location of QTLs. QTL mapping depends upon linkage, or the tendency of genes physically close to one another, to be inherited as a unit.

Unlinked genes, in con-trast, tend to be inherited independently according to the principles of segregation and independent assortment. Discussions of this method have recently been published (CrabbeandBelknap 1992; Lander and Botstein 1989; Plomin and McClearn 1993; Tanksley 1993).

Most pharmacogenetic research with gene mapping has employed recombinant inbred (RI) strains derived from the cross of C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains. This panel of strains was developed by inbreeding from the F2 population derived from B6XD2 hybrid mice. More than 20 BXD RI strains exist; each represents the more or less random shuffling of the genetic deck for all genes where B6 and D2 mice possess different alleles. Each RI strain is like any other inbred strain in that all its members are homozygous for each gene and are identical to each other. Furthermore, each RI strain can only have inherited either the B6 or the D2 allele (for any gene where the B6 and D2 progenitor strains had the same allele, all BXD RI strains also have only that allele).

The use of RI strains for gene mapping was first recognized by Taylor (1978) and Bailey (1981). The BXD RI panel of strains is powerful for gene mapping because it comprises a substantial number of strains that have been genotyped for more than 1,500 marker loci that are poly-morphic in the B6 and D2 progenitors. Once the utility of restriction fragment length polymorphisms (RFLPs) for gene mapping was clearly recognized (Lander and Botstein 1989), many RFLPs were identified in the BXD RI strains. More recently, markers based on polymerase chain reaction (PCR) amplification of deoxyribonucleic acid (DNA) micro-satellite simple sequence repeats (SSRs) have been typed in great numbers in the BXD and other RI strains (Deitrich et al. 1992). Plomin and colleagues (1991) first applied QTL analysis to behavioral responses in BXD RI mice.

One method for identifying and mapping QTLs is to adopt a two-stage procedure (Belknap et al. 1993a). An example of a QTL analysis for alcohol withdrawal severity will be given to illustrate the procedure. For the first stage, a database was established comprising the allelic status of each RI strain at each mapped marker locus. For a given marker, any RI strain possessing B6 alleles was given a value of 0, and any RI strain possessing D2 alleles a score of 1. The RI strains were then tested for acute ethanol withdrawal and each strain's mean response determined. Ethanol withdrawal severity (the quantitative trait) was then correlated with allelic status for each marker gene in the database.

Six groups of markers on four chromosomes were significantly associated with ethanol withdrawal severity (Belknap et al. 1993a). A similar analysis was performed for nitrous oxide withdrawal severity. The locations of the putative QTLs identified in these analyses are given in figure 5. Each such associated chromosomal region suggests the presence of a QTL affecting withdrawal severity. In this first stage of the analysis, it is undesirable to reject any potential QTL as unassociated; therefore, a relatively weak criterion of significance ($p < 0.05$) was adopted. However, because of the large number of correlations calculated, some QTLs detected during this initial stage will represent false-positive (chance) associations (Belknap 1992; see also other articles in the same issue for methodological considerations and alternative strategies). Therefore, it was necessary to verify the putative QTLs detected with further analyses.

In the second stage of testing, a QTL associated with both ethanol and nitrous oxide withdrawal severity was selected for further study. This apparent QTL appeared to account for as much as 48percent of the genetic variability in ethanol withdrawal severity; the markers most highly associated ($r = 0.69$, $p < 0.001$) between allelic status and withdrawal severity for 19 BXD RI strains were D2Mit9 and Scn2a, which mapped to a position 37 centiMorgans (cM) from the centromere of chromosome 2 (Silver et al. 1993). If a QTL in this region were actually associated with withdrawal severity, then allelic status of individual mice at markers in this region should also predict withdrawal severity. To verify this, approxi-mately 150 F2 mice derived from crosses of C57BL/6J and DBA/2J mice were tested for ethanol withdrawal severity. They were then genotyped for six nearby SSR markers using PCR.

Figure 6 shows how individual F2 mice display segregation and independent assortment at a polymorphic marker, and the codominant inheritance of heterozygotes. For informative markers, about one-half of the F2 mice were homozygous for the B6 (or D2) allele and one-half were heterozygotes. Ethanol withdrawal severity was significantly associated with allelic status only at the marker D2Mit9, but not at other more distant markers (Buck et al., in process). Figure 7 shows that the pattern of the withdrawal response in F2 mice that were B6B6 homozygotes at D2Mit9 was significantly less pronounced and of shorter duration than that in F2 mice that were D2D2 homozygotes (Buck et al., in process).

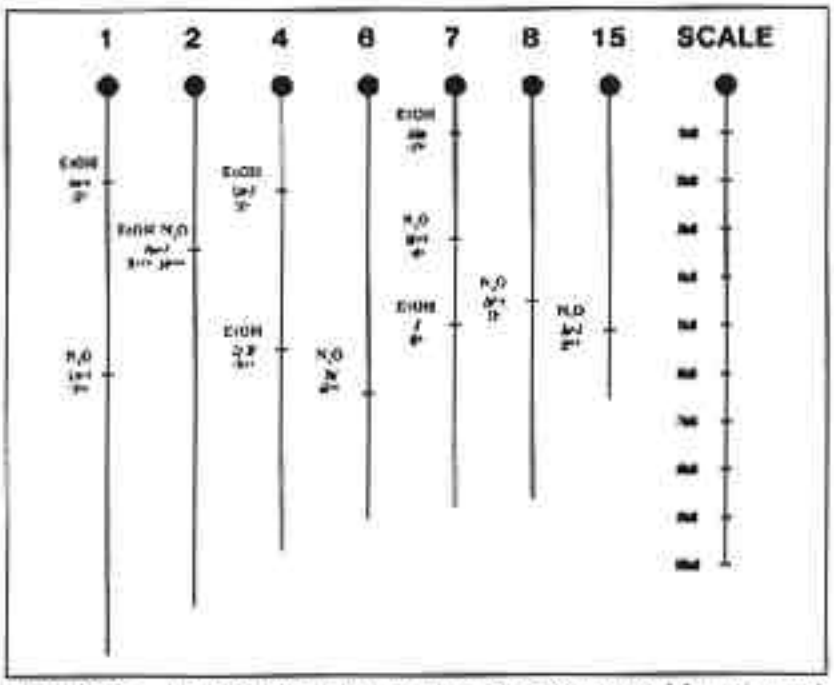

FIGURE 5. *Schematic representation of chromosomal locations of putative QTLs influencing acute ethanol (EtOH) or nitrous oxide (N_2O) withdrawal severity, determined by analysis of BXD RI strains. The most highly significant marker in each cluster of associated markers is given, along with the correlation coefficient and significance level.*

KEY: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

SOURCE: Data are from Belknap et al. 1993a. Markers are shown in their approximate location according to Silver et al. 1993, and chromosomes are approximately to scale.

This region of mouse chromosome 2 is syntenic with human 2q24-q37, which suggests that there is likely to be an analogous gene in humans. The great degree of homology between mouse and human chromosome maps (Copeland et al. 1993) is one of the most attractive features of QTL

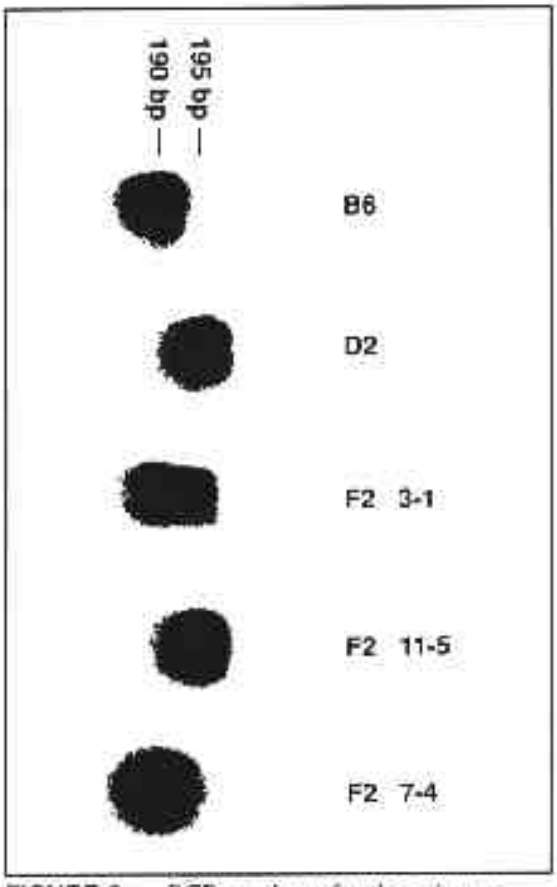

FIGURE 6. *PCR products for the primers amplifying the polymorphism at the marker D2Mit9. Inbred B6 mice have a 190 base pair (bp) PCR product, while inbred D2 mice have a 195 bp product. Three F2 mice from B6XD2 F1 crosses are shown. Of the 150 mice genotyped for D2Mit9, one quarter resembled F2 7-4 and were homozygous B6 at this locus; one quarter resembled F2 11-5 and were D2 homozygotes; about one half resembled F2 3-1 and were heterozygotes that displayed both products.*

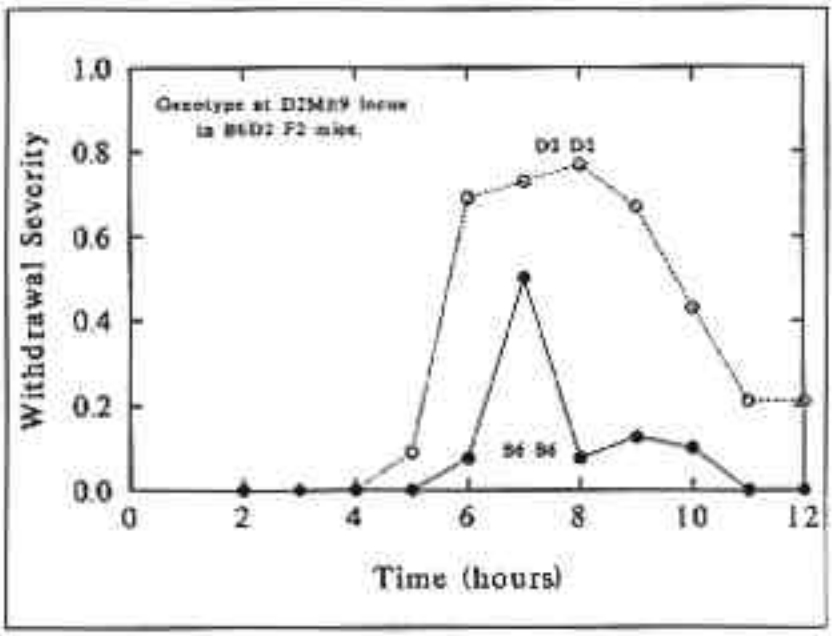

FIGURE 7. *Timecourse for ethanol withdrawal severity in B6D2 F_2 mice. Handling-induced convulsion scores during ethanol withdrawal in mice homozygous for the D2 allele at D2Mit9 (open circles) were markedly increased in magnitude and duration versus mice homozygous for the B6 allele (closed circles). Scores shown as increases over preinjection baseline differed significantly in the two groups ($p < 0.01$, two-tailed). In the first 4 hr after ethanol administration, convulsions were suppressed in both groups. As alcohol was metabolized and eliminated, withdrawal scores increased above baseline, reaching a peak after 6-7 hr.*

SOURCE: Data from Buck et al., in process.

mapping in mice: any map site that can be verified in mice can likely be extrapolated to a human map site. In addition, there are plausible candidate genes near D2Mit9. Gad-1 codes for glutamic acid decarboxy-lase, an enzyme catalyzing synthesis of GABA, and maps to very nearly the same location as D2Mit9. A cluster of genes coding for a-subunits of brain voltage-dependent sodium channels (Scn1a, Scn2a, and Scn3a), which influence the action potential in

excitable cells including neurons, also map nearby. Experiments are underway to determine whether functional differences in these candidate genes influence drug withdrawal severity.

FUTURE DIRECTIONS

Several interesting approaches are made possible by the QTL mapping approach. QTL maps for one drug response trait can obviously be compared with those for another (see figure 5). A recent example was able to identify several QTLs influencing both acute ethanol hypothermia and the development of tolerance to this effect (Crabbe et al. 1994c). In addition, a growing number of potential candidate genes are now mapped in mouse (e.g., neurotransmitter receptors and transporters, synthetic and metabolic enzymes, ion channels). A composite map for multiple drug traits can reveal genomic regions where presumed QTLs affecting multiple responses are located. Combining QTL maps for drug response traits and potential candidate genes can reveal associations across biological levels of analysis (e.g., behavior with candidate gene). A recent preliminary attempt to construct such a composite map compared QTLs from more than 20 responses to ethanol and multiple responses to methamphetamine, morphine, nitrous oxide, and other drugs (Crabbe et al. 1994b). In that analysis, several responses to alcohol, morphine, methamphetamine, and haloperidol all were significantly associated with a region spanning 27 to 31 cM on chromosome 9. This outcome is interesting because the gene encoding the dopamine D2 receptor gene (Drd2) has been mapped to mouse chromosome 9, 27 cM from the telomere. Another region of interest was on chromosome 6, where a putative QTL influencing six methamphetamine responses was identified.

At this Technical Review, the existence of a transgenic mouse absent 5-hydroxytryptamine 1B (5-HT1B) receptors was demonstrated (Hen, this volume). The gene encoding this receptor (Htr1b) has been mapped to chromosome 9 at 40cM, near the dilute coat color locus at 42 cM. The composite QTL map mentioned above detected associations of two methamphetamine, three morphine, and three ethanol responses with markers in this region. One of these traits was methamphetamine-induced hypophagia (Angeli-Gade, unpublished data). Hypophagia is known to be induced by stimulation of 5-HT1B receptors (Kitchener and Dourish 1994). It would be of interest to see whether the 5-HT1B knockout mice differed in the drug responses suggested by the QTL associations. It should be noted that nearly all

the QTL analyses collected on the composite map described are preliminary and represent only first-stage (BXD RI) associations; each will need to be verified in a segregating genetic population. Nonetheless, the rapid advances in gene mapping in the area of pharmacogenetics make the prospect of such studies exciting.

Other avenues are also opened by the QTL approach. Using the infor-mation derived from PCR genotyping of individual mice for markers flanking a QTL of interest, it will be possible to breed mice selectively for the genotype of interest (Plomin and McClearn 1993). This approach in distinct from the usual practice of selective breeding based on phenotypes, where the underlying responsible genes remain anonymous. With selection based on genotype, individuals possessing several risk-promoting QTLs could be generated and studied for their drug responses. A similar approach would be to produce congenic lines by backcrossing individuals possessing selected genotypes to an appropriate background strain (Bailey 1981). For example, F1 mice possessing DBA/2J alleles promoting high pentobarbital withdrawal could be repeatedly backcrossed with low-risk C57BL/6J mice. After many generations of selection and backcrossing, a congenic strain will be produced, which possesses C57BL/6J genotype at all genes except for a very small genomic region surrounding the QTL of interest. This approach is already proving feasible using phenotypic markers for selection of alcohol drinking (Dudek and Underwood 1993).

The potential advantage of congenic selected lines is in their utility for subsequent applications with molecular biological methods. Even once a QTL of interest has been identified, it remains a daunting task to find the (relatively) nearby candidate gene that is actually influencing the trait. It has recently been demonstrated that the technique of representational difference analysis (RDA) can be used to identify polymorphisms that can be amplified by PCR, through comparison of two closely related DNA samples (Lisitsyn et al. 1994). Genetically directed RDA has revealed numerous previously unidentified polymorphisms closely targeted to the region of interest. If such a region were defined by the difference between a normal C57BL/6J and a selected congenic C57BL/6J strain possessing only a small segment of DNA containing DBA/2J alleles leading to high pentobarbital withdrawal, genetically directed RDA might eventually increase the QTL map precision to about 200 kilobases (0.2 cM), or about 0.05 percent of the mouse genome. This might make positional cloning of the nearby gene feasible.

CONCLUSIONS

Pharmacogenetic analyses of drug sensitivity have been fruitful in identifying the important influence of often anonymous genes on virtually all drug response traits. Genetic animal models are now being employed using procedures designed to detect the influence of QTLs on drug responses and to identify their chromosomal location. The rapid identification of such QTLs, and the emerging pattern of influence of drug-related QTLs, show promise as a strategy for linking mouse and human genetic determinants of susceptibility to abuse drugs.

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