Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Pharmacy 310

Preproenkephalin Gene and mRNA

Studies of Structure, Function, Cocaine Responses in an Animal Model, and Genetic Association with Human Opiate Addiction

BY

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ACTA UNIVERSITATIS UPSALIENSIS
UPPSALA 2004

Dissertation for the Degree of Doctor in Philosophy (Faculty of Pharmacy) in Biological Research on Drug Dependence at Uppsala University in 2004

ABSTRACT

LaForge, K.S. 2004. Preproenkephalin gene and mRNA: studies of structure, function, cocaine responses in an animal model, and genetic association with human opiate addiction. Acta Universitatis Upsaliensis. *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy* 310. 85 pages. Uppsala. ISBN 91-554-5889-0

The endogenous opioid enkephalin neuropeptides are mediators of pain perception and have been implicated in human addictions. The preproenkephalin gene and its mRNA have also provided many examples of tissue- and species-specific variations in mRNA structure produced through a variety of transcriptional and post-transcriptional mechanisms. Resultant differences in mRNA structure, in several cases, have impact on translation of enkephalin prepropeptide. The reports and discussion presented herein describe studies of the preproenkephalin gene and mRNA structure in the guinea pig, an animal that may have specific advantages for modeling the human endogenous opioid system. A guinea pig brain cDNA library was constructed and screened for clones of preproenkephalin and preprodynorphin, which were then sequenced. These studies confirmed the predicted mRNA structure that had been previously proposed based on homology with gene sequences and other methods. Multiple transcription initiation sites for each of these prepropeptide genes were also identified. Studies were conducted in the guinea pig to evaluate the effects of the administration of cocaine in a "binge" paradigm for two and seven days on preproenkephalin mRNA levels in several brain regions. "Binge" cocaine administration for seven (but not two) days resulted in differential changes in mRNA levels in different brain regions. Decreases were observed in the nucleus accumbens and hypothalamus, and increases in the frontal cortex, amygdala and hippocampus. These findings differ from those of previous rodent studies and suggest that this species may provide a useful alternative model for the study of the effects of cocaine on preproenkephalin gene expression in the human brain. Human genetic studies were also conducted in opioid-dependent (formerly heroin-addicted) and control subjects to test the hypothesis that the preproenkephalin gene is associated with heroin addiction. In two separate studies, evidence was obtained that this gene may be associated with the development of human heroin addiction.

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ISSN 0282-7484
ISBN 91-554-5889-0
urn:nbn:se:uu:diva-4013 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-4013)

Printed in Sweden by Uppsala University, Tryck & Medier, Uppsala, 2004

To my parents
Gail and Rolfe LaForge

ORIGINAL PAPERS

This thesis is based upon the papers listed below, which are referred to by their Roman numerals I-IV.

I. LaForge, K.S., Nyberg, F., Kreek, M.J. 2004. Primary structure of guinea pig preprodynorphin and preproenkephalin mRNAs: multiple transcription initiation sites for preprodynorphin. Brain Res Bull, in press.

II. LaForge, K.S., Yuferov, V., Zhou, Y., Ho, A., Nyberg, F., Kreek, M.J. 2003. "Binge" cocaine differentially alters preproenkephalin mRNA levels in guinea pig brain. Brain Res Bull 59, 353-357.

III. LaForge, K.S., Khuri, E., Wells, A., Leal, S.M., Ott, J., Nyberg, F., Kreek, M.J. 2003. A $(CA)_n$ repeat polymorphism in enkephalin gene: association with opiate addiction. Manuscript, submitted.

IV. LaForge, K.S., Proudnikov, D., Ball, J., Leal, S.M., Nyberg, F., Kreek, M.J. 2004. Single nucleotide polymorphisms of the human preproenkephalin gene: association with opiate addiction. Manuscript, submitted.

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AFBAC	Affected family based controls
Amy	Amygdala
ANOVA	Analysis of variance
AP	Activator protein
ASI	Addiction Severity Index
ATG	Codon for methionine, translation initiation codon
BETA	Brain-specific [preproenkephalin] transcription activator
cAMP	Cyclic adenosine monophosphate
$(CA)_n$ repeat	Cytosine-adenosine repeat polymorphism
cDNA	Complementary deoxyribonucleic acid
Cer	Cerebellum
CG	Central grey
cM	Centimorgan
COGA	Collaborative Study on the Genetics of Alcoholism
COS	Cohesive end site
CPu	Caudate putamen
CREB	Cyclic adenosine monophosphate response element binding protein
dCTP	Deoxycytosine triphosphate
DNA	Deoxyribonucleic acid
DOP	Delta opioid receptor
DOR	Delta opioid receptor
DSM-III-R	Diagnostic and Statistical Manual of Mental Disorders III-Revised
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders IV
Eco	*Escherichia coli*
ENKTF	Enkephalin transcription factor
ERE	Estrogen response element
FAM	6-Carboxyfluorescein
FCx	Frontal cortex
FRET	Fluorescence resonance energy transfer
G-protein	Guanine-protein
GC	Guanine/cytosine
GIRK	G-protein-activated inwardly rectifying potassium channel
GRE	Glucocorticoid regulatory element
GRK	G-protein receptor kinase
HapMap Project	Haplotype Map Project
Hip	Hippocampus
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HUGO	Human Genome Organization
Hyp	Hypothalamus
IUPHAR	International Union of Pharmacology
IVS	Intervening sequence (intron)
KOP	Kappa opioid receptor
KOR	Kappa opioid receptor
LAAM	L-α-acetyl methadol
LB	Luria broth
LOD	Log of the odds ratio
MOP	Mu opioid receptor
MOR	Mu opioid receptor
mRNA	Messenger RNA
MSH	Melanocyte stimulating hormone
nt	Nucleotide
N-terminal	Amino-terminal
NAc	Nucleus accumbens
NFκB	Nuclear factor κB
Olf	Olfactory tubercle
Oligo(dT)	Oligodeoxythymidine
OPRD1	Delta opioid receptor gene
OPRK1	Kappa opioid receptor gene
OPRM1	Mu opioid receptor gene
OP-1	Delta opioid receptor
OP-2	Mu opioid receptor
OP-3	Kappa opioid receptor
OR	Odds ratio
[^{32}P]-dCTP	^{32}Phosphorous-labeled deoxycytosine triphosphate
PCR	Polymerase chain reaction
PDYN	*Preprodynorphin gene*
PENK	*Preproenkephalin gene*
Pfu	*Pyrococcus furiosus*
Pit	Pituitary
Poly(A)	Polyadenosine
POMC	Preproopiomelancortin
POMC	*Preproopiomelancortin gene*
QTL	Quantitative trait locus
RACE	Rapid amplification of cDNA ends
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
SN	Substantia nigra
SNP	Single nucleotide polymorphism
T3	Bacteriophage T3
T4	Bacteriophage T4
T7	Bacteriophage T7
TATA	Transcription initiation consensus sequence motif
TDT	Transmission disequilibrium test
UTR	Untranslated region
VIC	2'-Chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein
VMH	Hypothalamic ventromedial nucleus
VTA	Ventral tegmental area
Xho	*Xanthomonas campestris*

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1. INTRODUCTION

1.1 Endogenous opioid system

1.1.1 Discovery, anatomy, and function

Compounds derived from the milky sap of the seed case of the opium poppy, *Papaver somniferum*, have been used for their medicinal properties for millennia. Over 20 alkaloids are found in opium, including morphine, first described by Sertürner in 1806 (Gutstein and Akil, 2001). Drugs isolated from opium or congeners synthesized from opium-derived compounds (e.g., morphine, codeine, and thebaine) are termed "opiates" and have been used as analgesics, soporifics, antitussives, and antidiarrheals since the identification of these properties in such compounds. The term "opioid" refers to all substances with opiate-like effects, including those derived from the opium poppy, those synthesized from naturally-occurring opiate compounds (such as heroin, and also classified as "opiates"), and synthetic compounds with opiate properties, such as levorphanol, meperidine and its congeners (e.g., fentanyl and loperamide), as well as methadone and numerous others. The term "opioid" also refers to the endogenous neurotransmitter system on which opiate and opioid drugs act.

In addition to their medicinal properties, opiate and opioid agonists affect mood and cognition. In particular, opioid agonists with rapid pharmacokinetics activate endogenous reward systems, which contribute to the addictive properties of these compounds. Repeated administration of opiate or opioid drugs leads to tolerance and, ultimately, physical dependence. Psychological dependence is also a hallmark of opiate addiction. The changes in neurophysiology wrought by repeated drug exposure are long-lasting or permanent. Intense drug-craving generally occurs following withdrawal from long-term opiate self-administration, particularly in the presence of psychological cues; craving or "drug-hunger" is a major factor in the high relapse rate of untreated former heroin addicts. Addiction to opiates, especially heroin (diacetyl morphine), is a major medical, social, and geo-political problem.

Endogenous receptors for opiate drugs were postulated to exist based on the structural requirements of opiate and opioid drugs (Beckett and Casy, 1954) and first studied using an experimental protocol based on ligand stereoselectivity (Ingoglia and Dole, 1970; Goldstein et al., 1971). Use of high specific-activity radiolabeled stereoselective opioid ligands in binding assays led to the simultaneous discovery of specific opioid receptors by three separate groups (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). In the ensuing years, increasingly selective ligands allowed the pharmacological classification of opioid receptors into three types: mu (μ), delta (δ), and kappa (κ), with subtypes suggested for each receptor type (for reviews of the endogenous opioid system and its functions, see Kreek, 1996b; Akil et al., 1997; Nestler and Aghajanian, 1997; Standifer and Pasternak, 1997; Shippenberg and Elmer, 1998; Connor and Christie, 1999; Law and Loh, 1999).

Following the discovery of specific opioid receptors, investigators began searching to discover their endogenous opioid ligands. Three different classes of endogenous opioid peptides were soon described: first, Met- and Leu-enkephalin (Hughes et al., 1975); then, β-endorphin (Bradbury et al., 1976; Cox et al., 1976; Li and Chung 1976); and, finally, the dynorphins (Goldstein et al., 1979). The word "endorphin" (a contraction of "endogenous" and "morphine") was coined to describe any endogenous opioid peptide agonist, including enkephalins, β-endorphin and dynorphins. Subsequent studies demonstrated that these three classes of endogenous opioid peptides are derived from proteolytic processing of three distinct peptide precursors encoded by three separate genes: preproenkephalin, which yields Met-and Leu-enkephalins and also longer peptides with opioid activity; preprodynorphin, which yields dynorphins -A and -B and α-neoendorphin; and preproopiomelanocortin (POMC), which yields the opioid peptide β-endorphin by cleavage of the non-opioid peptide β-lipotropin, and which also encodes the important peptide hormones ACTH, and α- and γ-MSH (enkephalin: Comb, et al., 1982; Noda et al., 1982a,b; dynorphin: Kakidani et al., 1982; Horikawa et al., 1983; POMC: Nakanishi et al., 1979; Nakanishi et al., 1981; Takahashi et al., 1981, 1983). Two novel opioid peptides, endomorphins -1 and -2, have recently been isolated from brain tissue (Hackler et al., 1997; Zadina et al., 1997); however, no peptide precursors and no genes encoding peptide precursors for the endomorphins have yet been identified and the endogenous physiological importance of these peptides remains uncertain.

Almost twenty years following the discovery of specific opioid receptors, the first molecular cloning of an opioid receptor was reported simultaneously by two groups working independently (Evans et al., 1992; Kieffer et al., 1992). Following this initial report of the cDNA sequence of the mouse delta opioid receptor, cDNA or genomic clones for mu and kappa opioid receptors were soon identified based on their close sequence homology to the delta receptor (mu: Chen et al., 1993; Fukuda et al., 1993; Thompson et al., 1993; Wang et al., 1993; kappa: Li et al., 1993; Meng et al., 1993; Minami et al., 1993; Yasuda et al., 1993). Several groups have now isolated and sequenced cDNA or genomic clones for these receptors in other species, including humans (e.g., mu: Bare et al., 1994; Wang et al., 1994; Mestek et al., 1995; delta: Knapp et al., 1994; Simonin et al., 1994; kappa: Mansson et al., 1994; Simonin et al., 1995, Zhu et al., 1995). A fourth receptor (currently termed the orphanin FQ or nociceptin receptor) was also isolated on the basis of its sequence homology to opioid receptors (Bunzow et al., 1994; Chen et al., 1994; Mollereau et al., 1994). Although this receptor is closely related structurally to opioid receptors, it shows little or no opioid ligand binding and, therefore, is not classified as an opioid receptor. Chromosomal locations for the human opioid receptor and peptide genes have been established and are listed in Table 1.

Cloning of the opioid receptors allowed their classification into the superfamily of seven transmembrane domain guanine-protein (G-protein) coupled receptors. The three opioid receptors show a very high degree of sequence similarity at both nucleotide and peptide levels. The homology is particularly striking in the seven trans-

Table 1. Chromosomal localization of human opioid system genes

Protein	Gene	Location
Mu opioid receptor	*OPRM1*	6q24-25[d]
Kappa opioid receptor	*OPRK1*	8q11.2[e, g]
Delta opioid receptor	*OPRD1*	1p34.3-36.1[f]
Preproopiomelanocortin	*POMC*	2p23.3[a, b, h]
Preproenkephalin	*PENK*	8q23-q24[c]
Preprodynorphin	*PDYN*	20p12-pter[c]

membrane domains and three intracellular loops. The extracellular N-terminal domain, three extracellular loops and the intracellular carboxy-terminal domains are less conserved among the three receptor types.

Opioid receptors and their peptide ligands are widely distributed in the central nervous system and in several peripheral systems, including the gastrointestinal tract, male and female reproductive tissues, adrenal cortex, immune cells and other tissues. The endogenous opioid system mediates or modulates a diverse array of physiologic functions, including perception of pain or other nociceptive stimuli, hypothalamic-pituitary-adrenal (HPA) axis-regulated stress responsivity, hypothalamic-pituitary-gonadal (HPG) axis-regulated reproductive function, gastrointestinal function, immune function, cardiovascular and pulmonary function, thermal regulation, and, possibly, mood, affect, cognition, learning and memory (see Kreek, 1996a; 1996b; Kreek and Koob 1998; Massotte and Kieffer, 1998).

In addition to their importance in regulating normal physiology, and of immense clinical importance, opioid receptors are the primary molecular target of opiate and opioid analgesic drugs, mediating both the analgesic effects of these compounds, as well as the rewarding properties of short-acting opioids. It is the effect on brain reward systems that leads initially to repeated self-exposure to drugs of abuse and contributes to the development and persistence of addiction, and to relapse to addiction following withdrawal. The endogenous opioid system is also important in modulating physiological and behavioral responses to opioids, alcohol, and other drugs, including the psychostimulants cocaine and amphetamine. These substances also alter expression of opioid system genes or other aspects of system functioning (e.g., see Hurd and Herkenham, 1992; Hurd et al., 1992; Spanagel et al., 1992; Unterwald et al., 1992; Daunais et al., 1993; Hurd and Herkenham, 1993; Spangler et al., 1993; Unterwald et al., 1993; Unterwald et al., 1994; Azaryan et al., 1996; Zubieta et al., 1996; Selley et al., 1997; Shippenberg and Rea, 1997; Kreek et al., 1999).

Mu-directed opioid agonist medications with long-acting pharmacokinetics in man (specifically, methadone and L-α-acetyl methadol or LAAM) are clinically important in the treatment of addictive disorders, particularly in long-term agonist maintenance pharmacotherapy for heroin addiction (Dole et al., 1966; Kreek 1996c; d). Although other pharmacological agents are sometimes used in the treat-

[a]Owerbach et al., 1981; [b]Zabel et al., 1983; [c]Litt et al., 1988; [d]Wang et al., 1994; [e]Yasuda et al., 1994; [f]Befort et al., 1994; [g]Simonin et al., 1995; [h]Satoh and Mori, 1997

ment of opiate addiction (such as opiate antagonists), long-term agonist maintenance treatment (particularly with methadone) remains the single most effective treatment modality.

In animal studies with the opioid antagonists naloxone or naltrexone (e.g., Altshuler et al., 1980; Myers et al., 1986; Volpicelli et al., 1986; Ulm et al., 1995) and in human studies with naltrexone (O'Malley et al., 1992; Volpicelli et al., 1992), administration of opioid antagonists has been shown to reduce alcohol consumption. Naltrexone is now approved for the treatment of alcoholism in several countries. In further clinical studies, a third opiate antagonist, nalmefene, has been shown to have greater affinity than naltrexone for kappa opioid receptors (Schluger et al., 1998) and has also been shown to reduce alcohol consumption (Mason et al., 1994).

Intracellular events following agonist binding to opioid receptors are effected through several signal transduction pathways. These include initial inhibition of adenylyl cyclase activity through coupled G-proteins (primarily of the pertussis toxin-sensitive $G\alpha_i$ and $G\alpha_o$ families), inhibition of conductance of voltage-gated calcium channels, and activation of G-protein-activated inwardly rectifying potassium (GIRK) channels.

Like many other G-protein coupled receptors, agonist activation results in a rapid (seconds, minutes or hours) and reversible loss of opioid receptor function, a process known as "desensitization." Many studies provide evidence that desensitization may be mediated by opioid receptor phosphorylation by agonist-dependent G-protein receptor kinases (GRKs), although evidence also exists that there are exceptions to this requirement. Continued agonist exposure (hours or days) can also lead to a sustained loss of cellular receptors, termed receptor "down-regulation." Activation of opioid receptors by peptide ligands and some alkaloid agonists leads to internalization of the phosphorylated receptor into clathrin-coated vesicles, mediated by β-arrestin, followed by dephosphorylation and recycling of the functional receptor to the cell surface. Agonist-induced internalization has been demonstrated both in *in vitro* transfected cellular systems and also *in vivo* (e.g., Keith et al., 1996; Keith et al., 1998). However, specific ligands vary considerably in the extent to which they induce receptor internalization. For example, in one study, mu-directed peptide ligands, as well as the alkaloid ligands methadone, etorphine, dihydroetorphine, etonitazene, and fentanyl, caused internalization of the mu opioid receptor, whereas morphine, heroin, codeine, and buprenorphine did not (Keith et al., 1996). Since mu opioid receptor desensitization is observed for many agonists that do not cause internalization, these processes are apparently dissociable. Also, receptor phosphorylation may not be an absolute requirement for opioid receptor internalization (Murray et al., 1998). Receptor desensitization or downregulation have been postulated to possibly underlie, at least in part, the physiological expression of tolerance and dependence; however, it must be stressed that the cellular processes of desensitization, internalization, or downregulation are dissociable from tolerance and dependence, and that tolerance and dependence are also dissociable phenomena.

Homo- and heterodimers of opioid receptors have recently been shown to form *in vitro* in transfected cellular systems with associated alterations in ligand binding affinity and receptor functioning (e.g., Cvejic and Devi, 1997; Jordan and Devi, 1999). This phenomenon has not yet been conclusively demonstrated to occur *in vivo*.

In vitro studies of chimeric opioid receptor constructs and opioid receptors with engineered deletions or site-directed mutations have provided significant insights regarding the domains and specific amino acids that may be important for specificity of ligand binding and other aspects of receptor function, including signal transduction, desensitization, internalization, and receptor downregulation. Exchanges or deletions of specific receptor domains or alterations of some specific amino acid residues (for example, putative phosphorylation sites), have been shown to affect receptor function (e.g., Meng et al., 1995; Cvejic and Devi, 1997; Koch et al., 1997; Ehrlich et al., 1998; Hirst et al., 1998; Murray et al., 1998; Cavalli et al., 1999; Appleyard et al., 1999; Chaturvedi et al., 2000; Deng et al., 2000; Wang et al., 2000). These findings are of importance for the present discussion, since some single nucleotide polymorphisms (SNPs) in receptor genes result in alterations of specific amino acids, some of which have been demonstrated to alter receptor functioning in cellular assay systems (e.g., Bond et al., 1998; Koch et al., 2000).

The nomenclature of opioid receptors continues to be controversial. The original pharmacological designations mu, delta and kappa (μ-, δ-, κ-) were adopted by most researchers for the cloned receptors and their genes, and have most commonly been abbreviated MOR, DOR, and KOR, respectively. The 1996 decision by the International Union of Pharmacology (IUPHAR) Receptor Nomenclature Committee to rename the cloned receptors as OP-1, OP-2, and OP-3 for the delta, mu and kappa receptors, respectively (see Dhawan et al., 1996) was largely rejected by researchers in the field and has been abandoned. The current proposal from the nomenclature committee to abbreviate the receptors MOP, DOP, and KOP (for mu, delta, and kappa opioid receptors, respectively) has recently been officially adopted (B. Cox, personal communication). However, this nomenclature must still be considered provisional, since the acceptance of these terms has yet to achieve wide usage in the field. The abbreviations of the opioid receptor genes in the Human Genome Organization (HUGO) database is *OPRM1, OPRD1*, and *OPRK1* for the mu, delta, and kappa opioid receptor genes, respectively. Abbreviations for opioid receptors are not used in this thesis.

1.1.2 Opioid peptides

The initial discovery of endogenous peptides with opiate-like activity that bind to and activate the opioid receptors took place in the middle and late 1970s, shortly after the discovery of the receptors. The enkephalins, the dynorphins, and β-endorphin have a four amino acid N-terminal sequence of NH_2-Tyr-Gly-Gly-Phe-[Met/Leu], which confers the opioid properties of these peptides. Removal of the N-terminal tyrosine eliminates opioid activity (see Frederickson and Geary, 1982). Each

of these three classes of peptides is derived from endopeptidic processing of three propeptides, proenkephalin, prodynorphin, and proopiomelanocortin (POMC), the last of which also gives rise to adreno-corticotropic hormone (ACTH), as well as α- and γ-melanocyte stimulating hormones (α- and γ-MSH) and other bioactive peptides.

In addition to the three types of peptides that make up the traditional "endogenous opioid system (enkephalins, dynorphins, and β-endorphin)," a number of other peptides that have opioid-like activity have been discovered. These include the endomorphins, hemorphins, casomorphins, and dermorphins. Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) were first described by Zadina and colleagues (1997). Endomorphin-1 and -2 are potent mu-selective agonists and have been isolated from both bovine (Zadina et al., 1997) and human brain tissue (Hackler et al., 1997). Despite significant efforts toward their identification, a peptide precursor and gene for the endomorphins have eluded discovery. The possibility of non-ribosomal biosynthesis of these peptides should not be ruled out. Hemorphins are derived from hemoglobin and were first reported by Brantl and colleagues (1986). This class of four to twelve residue peptides all contain the Tyr-Pro amino acids at position 35-36 of the β-chain of human hemoglobin (for reviews, see Nyberg et al., 1997; Sanderson-Nydahl, 2000). Casomorphins, which also contain the Tyr-Phe sequence motif, are derived from processing of β-casein, and were first reported by Brantl and Teschmacher (1979). Dermorphins are peptides with opioid activity found in the skin of amphibia, and were discovered in 1981 by Montecucchi and colleagues (1981a, b).

1.1.3 Preproenkephalin gene, processing and peptides

Leucine-enkephalin ([Leu^5]-enkephalin), and methionine-enkephalin ([Met^5]-enkephalin) were the first endogenous opioid peptides identified (Hughes et al., 1975). These peptides are derived from the propeptide proenkephalin, which is transcribed from the preproenkephalin gene. The sequence of this gene was one of the first gene sequences elucidated, and has been subsequently reported for several species, including human, rat, golden hamster, and guinea pig (Noda et al., 1982b; Rosen et al., 1984; Zhu et al., 1994; LaForge et al., 1995). The sequences for the human, rat, mouse, bovine and golden hamster, and *Xenopus laevis* mRNAs (determined by sequencing of cDNA clones) have also been reported (Comb et al., 1982; Gubler et al., 1982; Legon et al., 1982; Noda et al., 1982a; Howells et al., 1984; Martens and Herbert, 1984; Yoshikawa et al., 1984; Beaulieu et al., 1994).

The preproenkephalin gene is highly conserved in those species in which the sequence has been determined. A signal sequence at the N-terminus of the propeptide directs the peptide into the rough endoplasmic reticulum with appropriate conformational folding for processing, transport through the golgi apparatus, and, ultimately, secretion into the synapse. Endopeptidic cleavage of the enkephalin propeptide by enkephalin-converting enzymes produces seven enkephalin peptides, each of which is flanked by the basic amino acid residues arginine or lysine. Four

copies of Met-enkephalin, one copy of Leu-enkephalin, one copy of Met-enkephalin-Arg-Gly-Leu, and one copy of Met-enkephalin-Arg-Phe are produced from a single proenkephalin peptide. Additional longer peptides with opioid activity have been described, which are derived from the sequence between Leu-enkephalin and the following Met-enkephalin (Kilpatrick et al., 1981; Udenfriend and Kilpatrick, 1984); however, whether these longer forms represent processing intermediates or have biological function distinct from the five- to seven-residue enkephalin peptides has not been established. One additional enkephalin peptide has been proposed to exist in the mouse, which was predicted from a cDNA sequence difference in the preproenkephalin gene of that species (Zurawaski et al., 1986). If that reported sequence is correct, a glycine to serine substitution would result in the octopeptide Met-enkephalin-Arg-Ser-Leu instead of the Met-enkephalin-Arg-Gly-Leu peptide observed in all other mammalian species.

Enkephalin peptides are generally selective for delta and mu opioid receptors. They have been demonstrated to have a role in the modulation of pain and other nociceptive perception (see Höllt et al., 1985; Herz, 1987). Recent studies of mice with genetically-engineered deletion of the preproenkephalin gene (or alternatively the delta opioid receptor), have also demonstrated the importance of enkephalin-derived peptides in opioid tolerance, but not physical dependence (Nitsche et al., 2002). In addition, evidence for a role of the enkephalins in the overall response to hedonic experience has been reported. Mice with deletion of the enkephalin gene were reported to have reduced responding to the reinforcing properties of food in a non-deprived state, suggesting a general decrease in the response to feeding, which is one of the strongest and well-established reward experiences (Hayward et al., 2002). Interactions between the mu and kappa opioid receptors also have been reported in studies of opiate analgesia, tolerance and dependence (e.g., Tulunay et al., 1981; Aceto et al., 1982; Khazan et al., 1983; Takemori et al., 1992). For this reason, the guinea pig, with its highly-developed κ-opioid system, may be useful in elucidating these complexities with implications for the processes that occur in the human brain with opiate, cocaine, or other drug exposure.

1.1.4 Preproenkephalin gene and mRNA

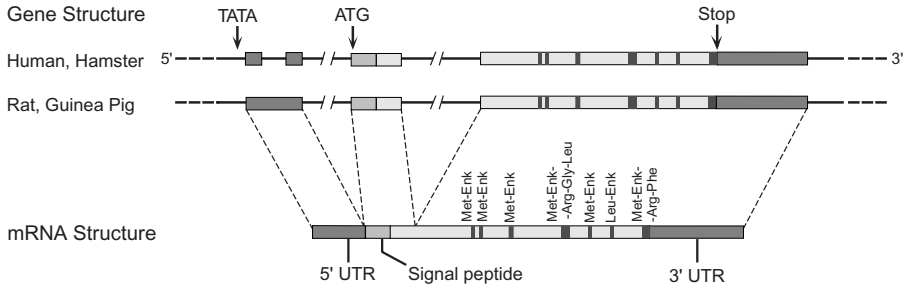
As previously noted, the human, rat, hamster, and guinea pig genes have been sequenced (Noda et al., 1982b; Rosen et al., 1984; Zhu et al., 1994; LaForge et al., 1995) and cDNA sequences have been reported for human, rat, mouse, bovine, golden hamster, and *Xenopus laevis* (Comb et al., 1982; Gubler et al., 1982; Legon et al., 1982; Noda et al., 1982b; Howells et al., 1984; Martens and Herbert, 1984; Yoshikawa et al., 1984; Beaulieu et al., 1994). For species in which both the gene and cDNA (indicating mRNA sequence) have been determined, it is possible to specify the intron/exon structure of the gene. If the cDNA structure is unknown, the gene organization can either be inferred from homology comparisons to other species, or by experimental techniques such as RNase protection mapping.

The intron/exon structure of the preproenkephalin gene as determined by comparison of the cDNA and gene sequences (rat, human, and hamster) and deduced from homology comparisons combined with RNase protection transcript mapping in the 5' and 3' regions of the gene mapping (guinea pig) is shown in Fig. 1. The human and hamster genes are organized into four exons, while the rat and guinea pig have three exons. In the human gene, Exons I (70 nt) and II (56 nt) are separated by a short (87 nt) intron (IVS1). In the rat, and for the predicted structure of the guinea pig gene, the first exon consists of sequences corresponding to Exons I, II and IVS1 of the human and hamster genes. This is caused by the lack of a splice donor site in the rat and guinea pig genes that prevents the mRNA splicing pattern seen in the other two species (LaForge et al., 1995). The first two exons of the human and hamster genes and the first exon of the rat and guinea pig genes encode mRNA in the 5' UTR. In humans, exon III (141 nt) encodes three nucleotides of the 5' UTR, the 24 amino acid signal peptide, and additional 66 residues of proenkephalin (Noda et al., 1982b; Comb et al., 1983). This exon of the gene in other species shows similar structural patterns. Exon IV codes for most of the proenkephalin sequence, including all sequences that yield bioactive enkephalin peptides as well as the 3' UTR.

1.1.5 Regulation of preproenkephalin mRNA expression

Preproenkephalin mRNA is widely expressed in central and peripheral nervous tissues as well as in the pituitary, adrenal medulla lymphocytes, the male and female reproductive tract, and other tissues. In general, enkephalin peptides are also isolated from the tissues in which the mRNA is expressed, however, some exceptions occur, such as in the heart and spermatogenic cells, which will be discussed below.

Figure 1. Intron/exon of the human, hamster, rat and guinea pig genes



The human and hamster genes are organized into four exons and the rat and guinea pig genes contain three exons. This difference is due to the lack of a splice donor consensus sequence in the human and hamster genes that is present in the others. Coding sequences for the preproenkephalin genes in all four species exhibit high homology. The enkephalin peptides (Met-Enkephalin, Met-Enkephalin-Arg-Gly-Leu, Met-Enkephalin-Arg-Phe) are encoded in the final exon.

A sequence of approximately 100 nucleotides located directly upstream (5') from the transcription initiation site of the preproenkephalin gene is highly conserved in all species for which sequence data has been determined. The most highly conserved region is a 30 nucleotide sequence that is virtually identical in the human, guinea pig, rat, and hamster genes. *In vitro* studies of transcriptional regulation, conducted primarily using reporter constructs containing the 5' flanking region of the human gene have documented several *cis*-acting regulatory elements in these sequences. Two response elements (ENKCRE 1 and ENKCRE 2), as well as an AP-2 consensus site, that are important for regulation of expression by the cAMP/phorbol ester, as well as the calcium-dependent signaling pathways, reside in this region (e.g., Comb et al., 1986; 1988; Hyman et al., 1988; 1989; Van Nguyen et al., 1990; Giraud et al., 1991). Additional transcription factor protein complexes interact at this position in the regulation of enkephalin transcription, including AP-1, AP-2, AP-4, CREB proteins and a novel enkephalin transcription factor that was named ENKTF-1 (Comb et al., 1988; Hyman et al., 1988; Comb and Goodman, 1990; Chu et al., 1991; Vallejo et al., 1993; Spiro et al., 1993, Spiro and McMurry, 1997).

In vitro cellular studies using the rat gene 5' flanking region have identified a functional element (B2) with partial homology to the canonical NFκB binding site. This transcription factor was shown to induce preproenkephalin mRNA transcription in activated T-lymphocytes (Rattner et al., 1991). A brain-specific proenkephalin transcription activator (BETA), which was isolated from the hippocampus, also binds to this site (Korner et al., 1989). A 13 nucleotide sequence in the 5' flanking region of the guinea pig gene was previously identified that has partial sequence homology to both the NFκB and B2 sites (LaForge et al., 1995). This site has the sequence (GGGGGAGTCACCC) at nucleotide positions -72 to -84 of the guinea pig gene sequence and exhibits partial dyad symmetry that is characteristic to NFκB binding elements. Functional studies have not yet been performed to determine whether this site contributes to regulation of preproenkephalin transcription following activation in T-lymphocytes.

Enkephalin gene expression has been shown to be regulated by glucocorticoids in several tissues and cell types (e.g., Glaser et al., 1981; Yanase et al., 1984; Naranjo et al., 1986; Inturrisi et al., 1988). Glucocorticoids also function to maintain basal preproenkephalin levels in several brain regions (Chao and McEwen, 1990; Ahima et al., 1992), as well as in the adrenal medulla (LaGamma and Adler, 1987). Sequences similar to glucocorticoid regulatory element GRE sequences have been located in the first intron of the rat and hamster preproenkephalin genes (see LaGamma and Adler, 1987; Zhu et al., 1994), but it is not yet clear if these sites are responsible for regulated transcription by glucocorticoids. GRE regulatory element sequences have not been found in 1165 nucleotides of the 5' UTR region of the guinea pig gene (LaForge et al., 1995).

In the ventromedial nucleus of the hypothalamus (VMH), gonadal steriod hormones regulate expression of preproenkephalin mRNA (Romano et al., 1989). The patterns of this regulation are gender-specific. An estrogen response element

(ERE)-like sequence is located in the 5' promoter region of the rat preproenkephalin gene (Romano et al., 1989). No sequences homologous to ERE regulatory elements have been identified in the guinea pig gene.

1.1.6 Preproenkephalin mRNA structure and translational regulation

Transcription of the preproenkephalin gene results in several interesting examples of species-, tissue-, and treatment-specific variations in the mRNA primary structure. Some of these variant forms have significant impact on translational efficiency. Other tissue-specific and yet undefined mechanisms also lead to apparent lack of correlation between levels of preproenkephalin mRNA and enkephalin peptides. In addition, in previous reports, we have identified an unusual post-transcriptional cleavage at a specific site in the 3' UTR of preproenkephalin mRNA in the guinea pig brain that results in a truncated non-polyadenylated mRNA (LaForge et al., 1995; 1997).

In the rat and mouse, most tissues that express the preproenkephalin mRNA, including the brain and adrenal gland, the mRNA has a transcript length that corresponds to the approximately 1400 nucleotide size predicted from the gene intron/exon organization described above. However, in haploid spermatogenic cells in these species, preproenkephalin mRNA is approximately 300 nucleotides longer than the form found in somatic tissues (Kilpatrick et al., 1985; 1987; 1990). Additionally, and unlike most somatic tissues, there is a lack of correlation between mRNA and peptide levels, with abundant mRNA and virtually no detectable peptides (Pittius et al., 1985; Kilpatrick et al., 1985; Kilpatrick and Rosenthal, 1986; Cox et al., 1987). Polysome gradient profiles also provide evidence that the longer testicular mRNA is not efficiently translated (Garrett et al., 1989; Kew et al., 1989). The difference in transcript size in these cells was shown to be caused by a difference in transcription initiation, with transcription starting at several sites within the first intron (intron A) (Yoshikawa, et al., 1989; Kilpatrick et. al., 1990; Zinn et al., 1991). This longer mRNA includes all of exons II and III, which contain all the coding region of the prepropeptide and might be expected to be transcribed. However, Rao and Howells (1993) demonstrated that sequences with predicted high secondary structure within intron A, and present in the longer testicular mRNA, inhibit translation of this mRNA (Rao and Howells, 1993). This variation of the preproenkephalin mRNA is species-specific: in the hamster and guinea pig, the mRNA found in the testis is the same size as the somatic form (Kilpatrick and Rosenthal, 1986; LaForge et al., 1995).

In the rat heart, a lack of correlation is also observed between enkephalin peptide and mRNA levels, with abundant mRNA and low peptide levels detected (Lang et al., 1983; Pittius et al., 1985; Howells, et al., 1986). However, the mechanism that leads to this disconnect between mRNA and peptide levels is unclear. Preproenkephalin mRNA isolated from the heart is the same size as that from other somatic tissues in which it is efficiently transcribed (Howells et al., 1986; Rao and Howells, 1993) and polysome gradient profiles suggest that this mRNA is actively translated

(Low et al., 1990). Presumably, some post-translational mechanism such as rapid secretion or degradation of peptides is the cause of low levels in this tissue (Low et al., 1990).

Transcription of preproenkephalin mRNA in the rat brain and adrenal gland has been reported to be initiated at one primary site located approximately 30 nucleotides downstream from the TATA sequence and also at several additional sites up to 30 nucleotides upstream from the TATA site (Weisinger et al., 1990; 1992). The utilization of these sites varied in a tissue- and treatment-specific manner, but a functional importance for the differences in length has not been established. In our previous study, we also found that a small proportion of mRNA isolated from the guinea pig caudate putamen begins at sites up to 30 nucleotides 5' with respect to the primary transcription initiation site (LaForge et al., 1995).

1.1.7 Guinea pig in experimental models of opioid system

Animals of many different species have long been used for experiments that model human physiology, neurochemistry, behavior, and diseases. In one example of use of an animal model that made use of a relatively primitive species, Eric Kandel and colleagues studied the marine sea slug *Aplysia californica* in their work elucidating the cellular and molecular mechanisms of neuronal conditioning (e.g., Castellucci et al., 1978; Hawkins, et al., 1983).

Species more closely evolutionarily related to humans are frequently used to study neurochemistry and behavior with the belief that processes and mechanisms in the model species reflect the workings of the human brain. Non-human primates are, of course, the most closely related species to humans, and some researchers take advantage of this to perform studies of behavior and pharmacology that are likely to have the most direct relevance to humans. However, it is impractical to use non-human primates for most studies that require animals, and several other animal species, particularly rodents, are commonly used. Each species has specific advantages and disadvantages in modeling human neurochemistry or behavior. For example, the most widely-studied rodent in terms of neurobiology and behavior is the rat species (*Rattus norvegicus*). The common house mouse (*Mus musculus*) is increasingly used in neurochemical studies because of the availability of targeted-gene deletion or "knock-out" mice, which offer investigators powerful tools to explore functioning of specific gene products, such as specific neuroreceptors, in living animals.

For the animal studies described in this thesis, we have selected another animal species: the guinea pig (*Cavia porcellus*). The guinea pig has had an important historical role in discovery and elucidation of the endogenous opioid system. An early established assay of opiate and opiate-like activity makes use of the guinea pig ileum, since one important property of these compounds is to reduce smooth muscle contractile activity (which results in reduced gastrointestinal motility, an important side-effect of opiate and opioid drugs). This effect is readily measurable and was utilized in the discovery of endogenous opioid peptides, the first of which to be found being Met- and Leu-enkephalins (Hughes et al., 1975). The guinea pig was also

used in the discovery and early elucidation of the kappa opioid receptor (Chavkin and Goldstein, 1981; Young et al., 1983).

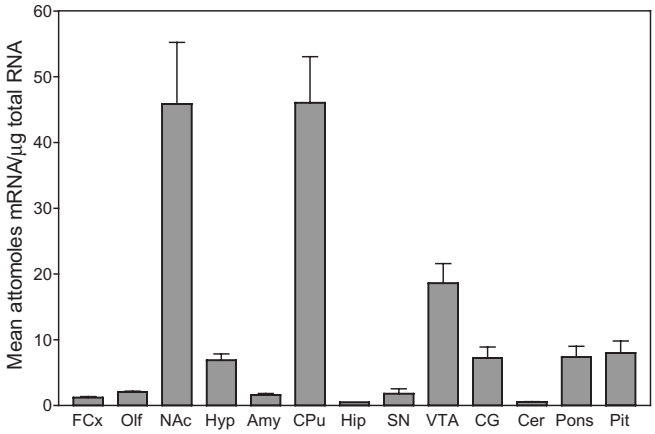
The guinea pig offers some specific advantages for modeling the brains of higher primates, including the human. In particular, as in humans and other primates, and unlike the rat, the kappa opioid receptor is the most common type of opioid receptor in the brain; in primates and humans, the kappa opioid receptors constitute 35% to 50% of total opioid binding (Young et al.,1983; 1986; Mansour et al., 1988; Sharif and Hughes, 1989). This is also true for the guinea pig gastrointestinal tract (Tavani et al., 1984; Culpepper-Morgan et al., 1988; 1995). In addition, studies of autoradiographic binding with selective kappa ligands have suggested that there may be two populations of kappa opioid receptors: a high-affinity type (κ_1) and a lower-affinity subtype (κ_2). Guinea pigs have a higher proportion of the high affinity subtype (Zukin et al., 1988; Unterwald et al., 1992). Since subsequent studies with knock-out mice have now established that only one gene exists for the kappa opioid receptor, the interpretation of these binding data are open to question. It may be that the two subtypes represent different coupling states to G-proteins, or alternatively, that the single kappa opioid receptor gene gives rise to mRNA splice variants that encode receptors that exhibit different affinities for selective ligands. However, the fact remains that with respect to selective kappa opioid receptor binding data from autoradiographic studies, the guinea pig brain more closely resembles the human brain than does the rat brain.

1.1.8 Site-specific cleavage of guinea pig preproenkephalin mRNA

Following the cloning and sequencing of the guinea pig preproenkephalin gene, we were able to analyze features of mRNA expression and structure in that species. In general, the distribution of mRNA in different brain regions corresponds to that in other species, with the highest levels in the caudate putamen, nucleus accumbens, and ventral tegmental area intermediate levels in the hypothalamus and pituitary, and lower levels in other regions studied (LaForge et al., 1997). This quantification was performed using a highly specific, sensitive, and accurate solution hybridization RNase protection method (Branch et al., 1992; LaForge et al., 1997; Pham et al., 1998). Figure 2 shows levels of mRNA in 12 brain regions and the pituitary.

Some differences exist in mRNA distribution between the guinea pig brain and the rat and hamster brains, two species in which the mRNA has been quantified using identical methodology used for measurement of the guinea pig mRNA (Franklin et al., 1991; Branch et al., 1992). For example, in the caudate putamen from all three species, approximately equivalent amounts are observed; however in the nucleus accumbens, whereas the guinea pig and hamster have approximately comparable levels, the rat nucleus accumbens contains approximately half the level found in the other two species. Other differences in brain region distributions have also been noted (LaForge et al., 1997).

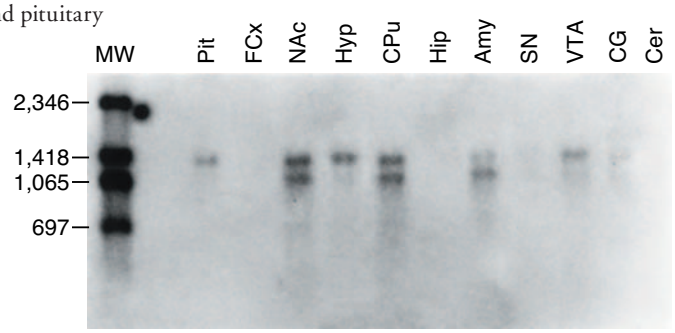
Figure 2. Preproenkephalin mRNA levels in 12 brain regions and pituitary of the guinea pig



Measurements were made by RNase protection assays. Data is expressed as mean attomoles of preproenkephalin mRNA per microgram of total RNA, with 18S ribosomal RNA used as a standard to measure total RNA. Abbreviations are: FCx, frontal cortex; Olf, olfactory tubercle; NAc, nucleus accumbens; Hyp, hypothalamus; Amy, amygdala; CPu, caudate putamen; Hip, hippocampus; SN, substantia nigra; VTA, ventral tegmental area; CG, central grey; Cer, cerebellum; Pit, pituitary (data from LaForge et al., 1997).

When the size of guinea pigpreproenkephalin mRNA transcripts was analyzed using northern blots, a striking finding was observed. In several brain regions, particularly the nucleus accumbens and caudate putamen, the mRNA was present in two forms of different lengths. Part of the mRNA was of the predicted length of approximately 1400 nucleotides; in addition, a shorter form was observed with a transcript length of about 1130 nucleotides (LaForge et al., 1995). The extent of the amount in the longer and shorter forms varies from region to region. Figure 3 shows a northern blot of guinea pig preproenkephalin mRNA from 10 brain regions and the pituitary.

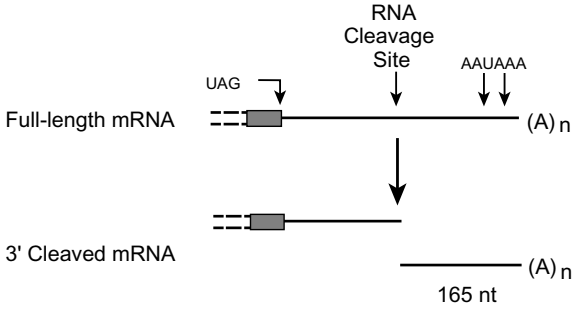
Figure 3. Northern blot analysis of preproenkephalin mRNA from selected regions of the guinea pig brain and pituitary



In several brain regions, notably the nucleus accumbens and caudate putamen, the guinea pig preproenkephalin mRNA is found in two sizes, one of approximately 1400 nucleotides, and a shorter form of transcript length approximately 1130 nucleotides. Abbreviations are: Pit, pituitary; FCx, frontal cortex; NAc, nucleus accumbens; Hyp, hypothalamus; CPu, caudate putamen; Hip, hippocampus; Amy, amygdala; SN, substantia nigra; VTA, ventral tegmental area; CG, central grey; Cer, cerebellum. (Data is from LaForge et al., 1995.)

We used several types of experiments to identify the nature of this transcript size difference. In studies of 5' transcript mapping with RNase protection, differences in transcriptional initiation were ruled out. Northern blot hybridization using probes from exons I, II, III, and the 3' UTR suggested that the difference resided in the 3' UTR, as did the fact that the shorter transcript form was not polyadenylated, but the difference in size was due not only to the lack of poly(A) tails. Finally, we used additional studies of RNase protection transcript mapping to demonstrate that mature full-length polyadenylated transcripts are cleaved at a specific site in the 3' UTR (located approximately 150 nucleotides downstream from the termination codon) to generate the truncated transcript and a small polyadenylated fragment of the 3' UTR (LaForge et al., 1995). Figure 4 shows a diagram of the position of the cleavage site and resultant RNA fragments.

Figure 4. Unusual post-transcriptional processing of the guinea pig preproenkephalin mRNA

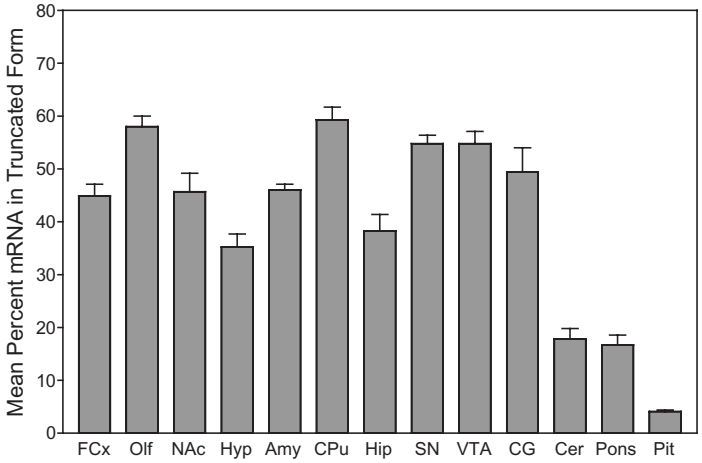


Cleavage of mature, polyadenylated transcripts in the 3' untranslated region (UTR) of the guinea pig preproenkephalin gene results in truncated mRNA containing the coding sequence and a polyadenylated 165-nucleotide 3' fragment (LaForge et al., 1995).

In a subsequent study, we used RNase protection assays to quantify the amount of preproenkephalin mRNA in the full-length and truncated forms in several brain regions and pituitary (Figure 5) (LaForge et al., 1997). The distribution of the cleaved RNA accounted for 40 to 60% of the RNA transcripts in most brain regions except for the cerebellum and brain stem. Only a small amount (4.1%) was detected in the pituitary.

The 3' UTR in several mRNAs have been shown to contain elements that regulate mRNA stability (see Belasco and Higgins, 1988; Cleveland and Yen, 1989; Jackson, 1993; Guhaniyogi and Brewer, 2001). Examples of site-specific cleavage in the 3' UTR are limited, but often have important functional implications, particularly for mRNA stability and degradation rates. The sequence of the cleavage site in the guinea pig preproenkephalin mRNA is distinct from 3' UTR mRNA cleavage sites identified in other species, but the RNA folding program of Zuker and Stiegler (1981) suggests that the position of the cleavage site may be located in the loop of a stable stem-loop structure. Also, we hypothesized that the 3' truncated form may be less efficiently translated than the full-length form, since it lacks a poly (A) tail, which has been shown to function in transcription initiation (e.g., Drummond et

Figure 5. Distribution of 3'-cleaved preproenkephalin mRNA in guinea pig brain and pituitary



Measurements were made by RNase protection assays and expressed as mean percent of total preproenkephalin mRNA content in each region that is observed in the 3' truncated form. Abbreviations are: FCx, frontal cortex; Olf, olfactory tubercle; NAc, nucleus accumbens; Hyp, hypothalamus; Amy, amygdala; CPu, caudate putamen; Hip, hippocampus; SN, substantia nigra; VTA, ventral tegmental area; CG, central grey; Cer, cerebellum; Pit, pituitary (data from LaForge et al., 1997).

al., 1985; Galili, et al., 1988; Munroe and Jacobson, 1990). Sucrose gradient profiles of preproenkephalin mRNA isolated from the guinea pig caudate putamen offer some support for a decrease in translational efficiency of the truncated form, although both short and long forms are associated with polysomes and are apparently both translated (LaForge et al., 1997).

1.1.9 Cocaine and the endogenous opioid system

In the brain, cocaine blocks reuptake of dopamine into presynaptic terminals by binding to and inhibiting the dopamine transporter, thereby increasing synaptic dopamine and dopaminergic neurotransmission. Acute or chronic cocaine administration also alters the endogenous opioid system, and, conversely, opioids influence the brain's response to cocaine. For example, studies of receptor autoradiography have demonstrated that chronic (14 d) "binge" cocaine administration increases in mu and kappa opioid receptor binding density in brain regions containing dopaminergic terminals (Unterwald et al., 1992; 1994). Cocaine has also been shown to cause release of dynorphin from striatal tissue (Sivam, 1989). Levels of mRNA for opioid receptors (mu and kappa) and mRNA for opioid peptide precursors, particularly preprodynorphin, but also, in some studies, preproenkephalin, have been shown to be altered by cocaine, as well as other aspects of opioid system functioning such as basal and opioid-regulated adenylyl cyclase levels (e.g., Hurd et al., 1992; Hurd and Herkenham, 1992; Daunais et al, 1993; Spangler et al., 1993; Steiner and Gerfen, 1993; Unterwald et al., 1993; Azaryan et al., 1996; Spangler et al., 1997;

Yuferov et al., 1999). Conversely, the opioid peptide dynorphin, as well as other κ-opioid receptor ligands, reduces basal dopamine levels and attenuates cocaine-induced dopamine increases in brain regions (nucleus accumbens and caudate putamen) known to be important for reward and locomotor effects of the drug (e.g., Di Chiara and Imperato, 1988; Werling et al., 1988; Spanagel et al., 1992; Claye et al., 1997; Zhang et al., 2004, in press).

In some animal studies (primarily rat), but not others, acute or semi-acute cocaine administration (in a variety of administration paradigms) or withdrawal from cocaine following acute or chronic administration has been reported to cause increases in striatal preproenkephalin mRNA. In general, in studies where a change was found, transient increases have been reported (Steiner and Gerfen, 1993; Przewlocka and Lason, 1995; Spangler et al., 1996; Daunais et al., 1997; Svensson and Hurd, 1998). Other studies reported no alterations in preproenkephalin mRNA levels following acute or chronic cocaine administration (e.g., Branch et al., 1992; Hurd and Herkenham, 1992). Additional studies have reported decreases in preproenkephalin mRNA following cocaine withdrawal. In one such rat study, reductions in preproenkephalin mRNA were reported to occur in the nucleus accumbens core and rostral striatum at a 30 min time point (but not at 3 hours) following withdrawal from an acute cocaine injection (Adams et al., 2000). Another study reported decreases in dorsal striatum and nucleus accumbens 1 or 2 days following withdrawal from a chronic cocaine administration paradigm (Przewlocka and Lason, 1995). Short-term effects of cocaine withdrawal on expression of this gene are probably transient (Svennson and Hurd, 1998).

Evidence from a human postmortem study and a primate study suggests that the effects of cocaine on preproenkephalin mRNA expression in primates may differ from those in the rat. In postmortem brains from individuals with evidence of cocaine abuse, preproenkephalin mRNA levels were found to be lower in the nucleus accumbens compared to control brains (Hurd and Herkenham, 1993). Striatal preproenkephalin mRNA levels were also lower in brains of rhesus monkeys following long-term cocaine self-administration (Daunais et al., 1997). Experimental design, mode of drug administration, age of animals, but also neurobiological differences in species may underlie these differences in the effects of cocaine on preproenkephalin mRNA expression. Additional animal models (such as the guinea pig) may be useful in modeling cocaine effects on gene expression of this neuropeptide mRNA. This hypothesis forms the basis of the experiments that were conducted using the guinea pig model and which are reported in Paper II of this thesis.

1.2 Human Genetics

1.2.1 Human genetic studies to estimate heritability

Almost a century and a half have now passed since the publication of *On the Origin of Species,* Darwin's revolutionary work that defined the fundamental concepts of contemporary evolutionary theory. Following the synthesis of the ideas of

Darwinian mechanics with the emerging fields of genetics (including theoretical population genetics), development, cytology, and systematics that occurred during the second, third and fourth decades of the previous century, it has been generally accepted by biological scientists that individual traits or differences among organisms result from an interaction between environmental (or developmental) factors and heritable (or genetic) factors that may have selective advantage within a particular environmental context.

Studies to estimate heritability commonly use family-based studies, including risks to relatives of a proband, or alternatively, twin and adoption study designs, to define the relative contributions of heritable factors, contrasted with environmental influences, to a particular phenotype (see Farrer and Cupples, 1998). These approaches can be used in determination of heritability of mono-genetic (Mendelian) traits or disorders, but are also applicable to heritability estimation of complex traits that involve multiple genes, as well as gene-gene and gene-environment interactions (Pericak-Vance, 1998a; b).

These types of studies can provide evidence that a particular trait (including increased or decreased risk for a disease, for example) has a heritable or genetic basis, as well as the extent of genetic influence on the trait. However, these methods do not identify the specific variant of specific genes that cause the phenotype, disease, or difference in risk for a disease. Pinpointing the specific alleles (variants) of genes that underlie the heritable contribution to specific phenotypes requires molecular genetic methods (see below).

1.2.2 Heritability of addictions

Early studies, dating back to the 1960s, established a heritable basis for alcoholism using twin, family, and adoption designs (e.g., Kaij, 1960; Partanen et al., 1966; Goodwin et al., 1973; Cloninger et al., 1981; Hrubec and Omenn, 1981; Cadoret et al., 1985). Many of these early studies were carried out in Sweden and Finland. Differences in types of alcoholism were defined with heritability patterns that differed; also, the highest estimates of heritability were found in males (e.g., Cloninger et al., 1981; Pickens et al., 1991).

Family-, twin-, and adoption-based studies have also shown that abuse of, or dependence on, other drugs is also influenced by genetic as well as environmental factors (e.g., Cadoret et al., 1986; Grove et al., 1990; Tsuang et al., 1996; Bierut et al., 1998; Kendler and Prescott, 1998; Merikangas et al., 1998; Tsuang et al., 1998; Kendler et al., 1999; Tsuang et al., 2001; Kendler et al., 2003). In one large study of 3372 twin-pairs from the United States Viet Nam Era Twin Registry, Tsuang and colleagues (1996; 1998) investigated use, abuse, or dependence for several drugs of abuse (defined using DSM-III-R criteria developed by the American Psychiatric Association, 1987). In that study, they found that heroin abuse or dependence had the greatest degree of heritability of all drugs studied, with over 50% of the variance attributable to genetic factors. In addition, they reported that abuse or dependence had genetic factors general for all of the drugs studied, but also that each drug had

genetic factors that were specific for that drug. Heroin abuse or dependence was also found to have the greatest proportion of genetic variance (38%) specific for that drug (Tsuang et al., 1996; 1998).

Although all studies of genetic epidemiology that have examined drug and alcohol abuse or dependence have reported evidence for a genetic contribution to disease risk, with heritability estimates of 0.3 to 0.5, the question of whether the genes that influence addiction susceptibility do so in a non-drug-specific manner, or are specific to a particular substance is of great interest, and also one for which there is conflicting data. In contrast with the findings of Tsuang and colleagues (1996; 1998), Kendler and colleagues (2003), also studying male twin pairs, reported a heritable contribution to substance abuse (including to heroin) but reported no evidence of genetic causes that were substance-specific. Two other (non-twin) family studies also examined this question with conflicting findings. Bierut and colleagues (1998) studied subjects from the Collaborative Study on the Genetics of Alcoholism (COGA) sample and found genetic influences in familial transmission that were specific for cannabis and cocaine dependence. In contrast, in a family study of individuals recruited from drug-treatment clinics, Merikangas and colleagues (1998) reported that although addiction was transmitted through families, the transmission was not drug-specific.

Given the conflicting evidence on the question of specific versus non-specific genetic influences, clearly more studies are needed. Appropriate phenotyping and endophenotyping will be crucial for this effort. Clinical observation suggests that most addicts exhibit a clear "drug-choice." This lends credence to the idea that the individual influences on specific addictions, whether they are genetic or environmentally based, are drug-specific. Since addictive drugs, such as nicotine, alcohol, cocaine, amphetamines, or heroin have distinct neural substrates, but also share some common pathways (such as increasing levels of dopamine in reward areas of the brain) the neurophysiological and neurochemical evidence supports the hypothesis that some genetic variants may be common to several or all addictions but that other genes may contribute to specific addictions. Also, the progression from first use, to abuse, to addiction is likely to involve different contributions of environmental and genetic influences at each stage of these diseases, and these differences are likely to be drug-specific (see Tsuang et al., 2001).

1.2.3 Identification of disease alleles by molecular genetic studies

Once a heritable contribution to a specific trait or disease has been established, the search for the specific gene variants involved can begin. Two general approaches are applied in such molecular genetic studies. In the first approach, specific genes are selected based on prior evidence from studies of physiology, cell biology, molecular biology, or genetic studies in other species that indicate that a particular gene product contributes to the trait. This type of hypothesis-based research is commonly termed a "candidate gene" study. The second type of investigation makes use of genetic markers at defined locations on chromosomes to identify positions or regions

that may be associated or linked with a particular trait or disease. These methods are termed "positional" approaches.

1.2.4 Candidate genes

The selection of candidate genes for human molecular genetic studies is guided by a variety of considerations. Clearly, genes that encode proteins that are directly involved in a trait should be studied. For example, to investigate individual differences in the responses to a particular drug, a gene that encodes the endogenous molecule (such as a receptor or enzyme) with which the drug interacts would be a logical target for study. Other potential genes for investigation are those whose expression is altered by the condition under study (be it disease, drug administration, or other perturbation). Microarray expression analyses may be particularly valuable in identifying such genes. Also, evidence from strain differences in animal models or genetic analyses in animal models may point to genes or chromosomal positions that are involved in particular phenotypes. Antisense, "knockout" and quantitative trait locus (QTL) analyses are examples of these types of studies.

1.2.5 Positional methods to identify genetic traits

The second general approach for identification of gene variants that influence specific traits or diseases is through positional methods. For positional methods, no prior assumptions are necessarily made regarding genes that may be involved. Markers at defined positions on a chromosome are used to assess whether a gene that resides in that region may be linked or associated with the trait under study. Further studies, including replication of findings of a study pointing to a particular genetic region being involved in a specific trait, followed by fine-mapping with additional genetic markers, and ultimately biochemical or molecular genetic studies, are necessary to identify the specific genes and polymorphisms that are causative or contributory to the phenotype under question.

1.2.6 Tests of genetic linkage and association

In the search to identify a gene variant that is causally related to a particular genetic trait, two general statistical methods can be utilized. Genetic "linkage" of a marker with a disease or phenotypic trait is evaluated by testing whether the marker and trait co-segregate in families. Genetic "association" of a trait with a marker is a separate type of genetic test which utilizes a population-based sample and which measures differences in genotype or allele frequencies between case and control individuals or in a family-controlled allele transmission test. These concepts are related, but distinct in their experimental and statistical methodology, and the application of each method to specific traits or diseases has particular advantages and disadvantages.

Genetic linkage is one of the methods that has been used to map the specific location of genes that contribute to a particular phenotype. Statistical linkage analyses have been useful in mapping disease genes in many disorders including those involv-

ing Mendelian traits as well as disease susceptibility loci in complex disorders (for a review of these principles, see Pericak-Vance, 1998a). The primary measure of linkage is the recombination fraction, which represents the probability that homologous recombination will occur between two particular loci in an offspring. In human studies, this value is referred to as (θ), which can range in value from 0 (loci that are completely linked) and 0.5 (completely unlinked loci, either distantly located on the same chromosome or on different chromosomes).

For tests of genetic linkage, the most powerful statistical method is the likelihood ratio approach developed initially by Morton (1955) that builds on early statistical genetic methods developed by Haldane (1919), Haldane and Smith (1947) and models of sequential sampling by Wald (1947). The lod score, first defined by Morton (1955), is the *l*og$_{(10)}$ of the o*d*ds for linkage of a trait with a specific locus. In practical application, the likelihood ratio of observing a particular combination of loci (disease and marker) is calculated over a range of recombination fractions (θ = 0.0 to 0.5), and the most probable genetic distance determined by the maximal lod score obtained for a particular value of θ. These analyses can be performed for two- or multi-point analyses (Elston and Stewart, 1971; Ott, 1974, 1991; see also Bridge, 1997; Pericak-Vance, 1998a). It is important to note that lod score linkage analyses are parametric, and require the specification of an inheritance model and other specific parameters (such as mutation rate, allele frequencies, and penetrance). If incorrect assumptions about these parameters are made, the power to detect linkage is markedly reduced (Pericak-Vance, 1998a). Other non-parametric linkage analyses are also available for linkage studies, such as the commonly used sib-pair analyses (Penrose, 1935; Haseman and Elston, 1972; Risch, 1990). Although less powerful than the traditional large-pedigree lod score analyses, these methods are non-parametric, or model-independent; that is, they do not require knowledge or assumption of the mode of inheritance (Pericak-Vance, 1988a).

A second type of genetic test of the potential contribution of a specific allele to a phenotype is that of "association." Rather than assessing the co-segregation of a genetic marker and a disease (or other phenotype) in families, association is a population-based statistical analysis that tests whether an allele is more or less likely to be represented with a disease or other phenotypic trait. Association analyses have been suggested to be particularly powerful in defining genes that contribute to complex traits (e.g., Risch and Merikangas, 1996). Association of a particular allele with a trait may be because the assessed allele is the actual biological cause of (or contributor to) the trait or because it is in "linkage disequilibrium" with a nearby causative (or contributory) allelic variant.

Linkage disequilibrium refers to allelic association in populations that is maintained by strong linkage. Normally, evidence of linkage disequilibrium indicates that two (or more) allelic markers or polymorphisms are proximally co-localized on a chromosome and that homologous recombination has not occurred between them over many generations, leading to their co-inheritance in many apparently unrelated individuals in a population (see Pericak-Vance, 1998b). Over many generations,

linkage disequilibrium between two sets of markers (a, b) decays at a rate which is proportional to the recombination fraction between them ($\theta_{(a,b)}$) and the number of generations since the variants appeared on the same chromosome. The concept of linkage disequilibrium is similar to linkage, but the time scale relevant for studies of linkage disequilibrium is in tens or hundreds of generations, whereas for linkage, it is a few generations. In practical application, the chromosomal distances over which linkage disequilibrium can be detected is much smaller than for linkage. This has important implications: for association analyses that rely on linkage disequilibrium, markers must be located quite close to the causative or contributory polymorphism, generally less that 1 cM (Pericak-Vance, 1998b), whereas markers used to identify linkage can be considerably more distantly located (5 to 10 cM).

Two types of studies are used to detect allelic association with a disease or other phenotype. "Case-control" studies compare allele frequencies in a population sample of unrelated individuals who have the phenotype ("cases") with those that do not express the trait ("controls"). Differences in genotype or allele distributions between cases and controls are taken as evidence that a particular allele is causative/contributory to the trait or is in linkage disequilibrium with the causative/contributory allele. Careful matching of case and control groups is essential for this type of study because population stratification caused by recent admixture or non-assortative mating can produce allelic associations that result from population phenomena that bear no relation to the connection between an allele and a trait. Although allelic association caused by population admixture or genetic drift is expected to decay significantly more rapidly than that due to actual linkage disequilibrium from linked loci, it is still critical in this type of study to closely match case and control groups carefully with respect to ethnicity/geographic ancestry, and, in some cases, age or other factors (Pericak-Vance, 1998b).

A second approach to study genetic association makes use of family-based controls, which avoids the problem of matching population case and control groups. One example of this type of test was first introduced by the transmission disequilibrium test (TDT) described by Spielman and colleagues (Spielman et al., 1993, Spielman and Ewens, 1996). This method tests whether a suspected disease allele is transmitted from heterozygous parents to affected probands more often than would be expected by chance, i.e., for linkage in the presence of association, and is robust in the presence of population admixture. This approach is attractive since only the probands need to be assessed for phenotypic classification, and other factors, such as admixture and environmental factors, are controlled for; however, this approach is limited by the fact that only heterozygous parents are informative. The TDT test has traditionally been applied to candidate gene analyses; however, it has also been proposed for genome-wide scans (Risch and Merikangas, 1996). Other family-based tests for association include the haplotype relative risk test (Falk and Rubenstein, 1987; Terwilliger and Ott, 1992) and affected-family-based controls (AFBAC) methods (Thomson, 1995).

Although there are significant differences in the extent of linkage disequilibrium in different regions of the genome (e.g., Dunning et al., 2000; Taillon-Miller et al., 2000; Abecasis et al., 2001; Patil et al., 2001; Pritchard and Przeworski, 2001; Reich et al., 2001; Bonnen et al., 2002; Reich et al., 2002), recent studies that have investigated linkage disequilibrium over large distances on chromosomes provide evidence that the genome, or at least portions of it, is organized into relatively large regions of high linkage disequilibrium separated by other regions (recombination "hot-spots") with higher recombination rates (Daly et al., 2001; Jeffreys et al., 2001; Patil et al., 2001; Gabriel et al., 2002). Regions of relatively low historical recombination separated by regions of higher recombination have been termed "haplotype blocks" (for a recent review, see Wall and Pritchard, 2003). For example, in the study by Daly and colleagues (Daly et al., 2001), the haplotype structure of a 500-kb region of chromosome 5 could be defined by haplotype blocks with a relatively low number (2-4) of common haplotypes found with sizes that ranged from 3-92 kb. The existence of such haplotype blocks has immediate implications for association mapping of disease genes by greatly reducing the number of markers that would need to be genotyped in order to define the common haplotypes in a chromosome-wide or genome-wide association scan (Wall and Pritchard, 2003). A large-scale effort to map the haplotype structure of the human genome (the International HapMap Project) has recently been launched by the United States National Human Genome Research Institute. Haplotype analyses can have greater power in detecting association than individual markers, and many approaches to this method have been developed (e.g., Xie and Ott, 1993; Hoehe et al., 2000). A recent study provides an example in which specific haplotypes, rather than individual polymorphisms acting alone, may functionally influence gene expression through regulation of mRNA stability or other means (Duan et al., 2003).

1.2.7 Types of genetic polymorphisms and genetic markers

The word "polymorphism," from the Greek word "polymorphos," is derived from the roots "poly" or "many" and "morphe" or "shape." In genetics, polymorphism refers to any change or rearrangement of nucleotides that make up the sequence of a particular chromosome. Many types of polymorphisms can occur, either through mutation or chromosomal rearrangements. Some polymorphic changes are lethal, some produce devastating diseases, and others are completely benign, with no selective consequences. The use of polymorphic genetic markers to identify adjacent disease mutations heralded the modern era of molecular genetics and was introduced by Kan and Dozy (1978), in that case, by the use of restriction fragment length polymorphism (RFLP) markers. For polymorphisms to be used as genetic markers, they must be located closely enough to the causative/contributory gene to be appropriate for linkage or association studies; as noted above, association-based methods require much tighter linkage of the marker to the disease polymorphism than do linkage-based methods.

The markers most commonly used in linkage studies, particularly those that involve genomic scans, are short tandem repeats or "microsatellite" polymorphisms first proposed and used as markers by Weber and May (1989) and Litt and Luty (1989). These polymorphisms are typically di-, tri-, or tetra-nucleotide repeats with alleles containing variable numbers of repeats. If present within genes, these repeats can produce serious genetic disease (for example, Fragile X syndrome is caused by a high-number trinucleotide repeat located at chromosome X q27.3). However, most microsatellite polymorphisms lie in intergenic regions and are selectively neutral, and therefore have utility as genetic markers for nearby genes. Microsatellite markers are located throughout the genome and are highly polymorphic and thus informative in genetic analyses. For example, the most common repeat element, the $(CA)_n$ repeat, has a highly polymorphic variant located approximately each 0.4 cM (Weber, 1990). The number of repeats at a particular locus is readily analyzed by amplifying the section of genomic DNA containing the marker using polymerase chain reaction (PCR) with primers radioactively- or fluorescently-labeled, followed by conventional or automated gel electrophoresis. Microsatellite markers have been used in studies of both linkage and association; however, because they have high mutation rates, they are more suited for linkage analyses.

The most common type of genetic variation in the human genome is the single nucleotide polymorphism, commonly abbreviated "SNP". In this type of polymorphism, a single nucleotide substitution or deletion is represented in a particular allele. Early large-scale resequencing studies suggested that a SNP occurred at approximately each 1 kb of genomic sequence (Cargill et al., 1999; Halushka et al., 1999). Subsequent studies have shown that SNPs are more common than originally reported, with SNPs separated at an average of each 200 nucleotides, and with approximately 3.4 SNPs per 1 kb of coding sequence of genes (e.g., Stephens et al., 2001). The allelic frequency of most SNPs is low (< 1%). Rare SNPs do not have utility as genetic markers; also, rare polymorphisms of any type, including SNPs, are, by virtue of their rarity, unlikely to be important as causative factors in common genetic disorders.

Unlike microsatellite polymorphisms, which can have many alleles, SNPs are in most cases biallelic, and therefore cannot have a heterozygosity greater than 0.5, which makes them less informative than microsatellite polymorphisms as genetic markers. On the other hand, because SNPs are very stable (i.e., they have a low recurrent mutation rate), they are excellent indicators of genomic history. This property, along with their frequent placement in the genome, makes SNP markers particularly suitable for association analyses, especially those that evaluate association with specific haplotypes.

1.2.8 Physiogenetics and pharmacogenetics

Over the past decade, researchers have increasingly focused attention on understanding the basis of individual differences in responses to medications, and, in particular, determining whether a specific genotype or combination of genotypes is

predictive of responses to specific drugs. Differences in physiology and the contribution of specific genotypes to these differences are now seen as potentially important in the development of pharmacologic regimens "tailored to the individual." This new area of study is widely known as "pharmacogenetics."

The potential payoff of pharmacogenetic research is twofold: identification of genotypes that allow practitioners to direct specific medications or dosages to individuals who carry specific alleles offers the promise of improving pharmacologic responses and treatment outcomes. Secondly, several potentially useful medications have failed to obtain approval, or have been withdrawn from use, because of severe adverse reactions in a small number of individuals. If a genetic test could identify individuals who would suffer an adverse reaction, these medications could be available to the majority of the population who could benefit from their use.

We have introduced the companion term "physiogenetics" to describe the study of genetic influences on individual differences in endogenous physiology, such as responses to specific hormones (Kreek, 2000; LaForge et al., 2000a, b, c). These innate genetically-based differences may have resultant effects in many aspects of physiology, such as stress responsivity, as well as neurobiology and behavior, and may form the bases, in part, of complex neurological diseases or behavioral disorders, such as the addictions. A better understanding of how genetically-based differences in physiology contribute to the development of these diseases may suggest new strategies for prevention and treatment.

The practical clinical application of pharmacogenetic principles will require the development of rapid, accurate, and inexpensive tests for analysis of diverse types of polymorphisms from patient specimens. Although researchers in genetics will require assessment of increasingly large numbers of genotype determinations, particularly for genome-wide SNP association analyses, the clinical practitioner will more likely find useful genotype information for only those polymorphisms relevant to the diagnosed (or suspected) disorder. We have developed one such approach for SNP identification using hybridization or single nucleotide extension on custom oligonucleotide gelpad microarrays (LaForge et al., 2000b).

1.2.9 Mu opioid receptor gene: an example

A gene that serves as a particularly instructive example of pharmacogenetic and physiogenetic importance in the addictions is the human mu opioid receptor gene, which, as previously noted, has the designated locus name *OPRM1*, and has been experimentally assigned to chromosomal location 6q24-25 (Wang et al., 1994). As previously described, the mu opioid receptor is the primary molecular site of action of many opiate and opioid narcotic drugs, including heroin and its biotransformation products morphine, oxy- and hydro-codeine and -morphone, and other potent analgesic narcotics, such as fentanyl and its congeners.

The gene for this receptor was targeted as a natural candidate in genetic studies of opiate addiction because of its known role in mediating the effects of opiate drugs, as well as from evidence from quantitative locus traits in mice that mapped the

chromosomal region containing the mu opioid receptor to several traits including morphine analgesia and self-administration (Belknap and Crabbe, 1992; Berrettini, et al., 1994; Kozak et al., 1994; Belknap et al., 1995; Bond et al., 1998; Crabbe et al., 1999). Also, the most effective pharmacotherapeutic medication for treatment of opiate addiction is methadone, an agonist which selectively targets the mu opioid receptor. Later studies of mice with targeted deletion of the mu opioid receptor gene further confirmed that this receptor is obligatory for morphine analgesia and reward, using several experimental paradigms (Matthes et al., 1996; Sora et al., 1997; Kitanaka et al., 1998; Loh et al., 1998; Becker et al., 2000). Additional studies of knockout mice using self-administration have also shown that this receptor is involved in mediating reward for cocaine and alcohol (Roberts et al., 2000; Hall et al., 2001; Becker et al., 2002).

Many polymorphisms of the mu opioid receptor gene have been defined. The most common coding region polymorphisms (the C17T and A118G SNPs in the first exon of the gene) were first described independently by three groups in 1997 and 1998 (Bergen et al., 1997; Berrettini et al., 1997; Bond et al., 1998). Both of these SNPs are common in many human populations, and both alter predicted amino acid structure of the receptor. The most common A118G polymorphism results in the predicted amino acid substitution of an asparagine to an aspartic acid residue at amino acid position 40 of the receptor. This substitution eliminates one of five putative glycosylation sites in the N-terminal domain. This receptor domain has been shown to be glycosylated, although the precise sites and patterns of glycosylation have yet to be determined in the N-terminal region (Chaturvedi et al., 2000). The 118G allelic variant has been reported to occur at frequencies between 2% and nearly 50% in different populations (e.g., Bond et al., 1998; Gelernter et al., 1999).

In the first study to examine the possible function of this polymorphism, we used AV-12 cells expressing the variant 118A and 118G receptors and found that one important endogenous ligand of the mu opioid receptor, β-endorphin, bound the 118G variant receptor three times more tightly than the 118A prototype (Bond et al., 1998). Also, when the 118G variant receptor was co-expressed in *Xenopus* oocytes with G-protein activated inwardly rectifying potassium channels, we observed an approximately three-fold greater β-endorphin-induced potassium current, compared to oocytes transfected with the prototype receptor; this effect was observed for β-endorphin only, and not with other agonists tested (Bond et al., 1998).

Recent human studies have confirmed a "physiogenetic" role and suggested a "pharmacogenetic" significance of the A118G polymorphism. In two separate studies of normal volunteers administered the opioid antagonist naltrexone, individuals with one or two copies of the 118G allele exhibited a higher response of the hypothalamic-pituitary-adrenal (HPA) stress-responsive axis, which is under tonic inhibitory control of the opioid system; these studies demonstrate a difference in innate physiology resulting from this polymorphism (Wand et al., 2002; Hernandez-Avila et al., 2003). In another recent study, it was reported that individuals

carrying the 118G allele responded more successfully to the treatment of alcoholism with naltrexone than individuals expressing the 118A prototypic variant (Oslin et al., 2003). This study establishes a potential prognostic pharmacogenetic role for this polymorphism.

1.2.10 Preproenkephalin gene in human addictions

The hypothesis that the preproenkephalin gene is associated with specific addictions has been evaluated in two previous studies. The genetic marker used was a $(CA)_n$ repeat that was identified in the initial sequencing of the gene (Comb et al., 1983) and later found to be polymorphic with six identified alleles (Weber and May, 1990; Chan et al., 1994). Allelic frequencies for this marker have been shown to vary in different populations, necessitating stratification of data with matched control subjects compared to cases (Chan et al., 1994). In their study of alcoholism, Chan and colleagues found no differences in allele frequencies between alcoholics and controls in Chinese, Atayal, Caucasian or African-American populations (1994).

A second study, using the same genetic marker, tested the hypothesis that the preproenkephalin gene is associated with opiate dependence (Comings et al., 1999). In this study, 31 non-Hispanic Caucasian opioid-dependent inpatients from an addiction treatment program in California were compared to two control groups (patients dependent on other substances from the same treatment unit, and students from a nearby university) as well as to ethnically-matched individuals previously reported in the scientific literature. The authors reported significant differences in grouped allele and genotype frequencies between each of the control groups and the opioid-dependent cases, suggesting an association of the preproenkephalin gene and opiate addiction (Comings et al., 1999).

2. OBJECTIVES OF THE STUDIES

1) To isolate and sequence cDNA clones of the preproenkephalin and preprodynorphin genes from the guinea pig to confirm their gene structures. [Paper I]

2) To evaluate the effects of "binge" cocaine administration on preproenkephalin mRNA expression in the guinea pig brain. [Paper II]

3) To test the hypothesis that the preproenkephalin gene is associated with heroin addiction in humans. [Papers III and IV]

3. METHODS

3.1 Guinea pig preproenkephalin mRNA structure [Paper I]

3.1.1 Construction and screening of guinea pig brain cDNA library

For this study, as well as the animal studies described in objective 2, the specific research protocol was approved by the Institutional Animal Care and Use Committee of The Rockefeller University.

To accomplish the first objective of the thesis, it was necessary to construct a guinea pig brain cDNA library, since no commercial library was available. Two male Hartley guinea pigs (approximately 280 g each) were sacrificed and brains and pituitaries removed. Total RNA was isolated from the combined brains and pituitaries by the acid-phenol method of Chomczynski and Sacchi (1987), which has proved to be one of the most effective methods for the rapid isolation of intact, full-length RNA from a variety of tissues. The first step of this method involved immediate homogenization of the tissue in a 4 M solution of guadinium thiocyanate, a chaotropic salt that inactivates RNase activity. The subsequent step is extraction with phenol at an acidic pH, which causes partitioning of RNA into the aqueous phase, with protein and DNA in the organic phase or at the interface between the aqueous and organic phases. Total RNA is then collected by isopropanol or ethanol precipitation. Polyadenylated RNA (mature mRNA) was isolated from the total RNA by chromatography on oligo(dT) cellulose, in which poly(A) containing RNA sequences were bound to the column in a solution containing a high salt concentration. The column was then washed with a salt solution of intermediate molarity to remove non-adenylated RNA (primarily ribosomal and tRNAs) and the poly(A) RNA eluted with water.

To construct the cDNA library, double-stranded cDNA was prepared from the poly(A) mRNA. This procedure requires several steps. First, an oligo(dT) linker primer was annealed to the mRNA. This oligonucleotide contained a linker sequence that encodes the *Xho* I restriction endonuclease site as well as oligo(dT), which anneals to the poly(A) tails at the 3' end of the mRNA. Moloney murine leukemia virus reverse transcriptase was then used to polymerase the first strand of cDNA using the mRNA as template. This reaction was performed in the presence of 5-methyl dCTP as well as the other three nucleotide triphosphates. This procedure prevents the synthesized cDNA from being digested by restriction endonucleases in subsequent steps of the procedure. To synthesize the second strand of cDNA complementary to the first, we used a combination of RNase H and DNA polymerase. The RNase H produces single-strand "nicks" in the RNA strand of the cDNA: RNA duplexes at multiple positions along the molecule. The RNA fragments serve as primers for the DNA polymerase to synthesize the second cDNA strand, and the mRNA strand is displaced/digested in the process. Uneven termini of the double-stranded cDNA were then "polished" using *Pfu* DNA polymerase, and a double-

stranded oligonucleotide adaptor containing an *Eco* RI restriction site was then covalently attached to both the 5' and 3' ends of the cDNA using bacteriophage T4 DNA ligase. Termini were then phosphorylated using bacteriophage T4 polynucleotide kinase. Finally, the cDNA was digested with the restriction endonuclease *Eco* RI, producing a double-stranded cDNA molecule that has an *Xho* I site at the 5' terminus, and an *Eco* RI site at the 3' terminus, and, following size-fractionation on Separaose CL-2B to remove cDNA inserts smaller than ~600 nucleotides, was ready for directional cloning into the lambda phage vector. Each of the above steps was assayed by the incorporation of a trace amount of [^{32}P]-dCTP in the DNA synthesis and detected using a Geiger monitor or by electrophoresis on polyacrylamide gels followed by autoradiography.

The vector used in the cloning (λ ZAP Express™, Stratagene, La Jolla, California) has several engineered features that make it particularly suited for cDNA library cloning. This vector contains promoters for both procaryotic and eucaryotic expression, allowing for both expression and hybridization screening. As well as the COS (*C*ohesive *E*nd *S*ites) necessary for packaging of the right and left arms into lambda phage, the vector contains multiple unique restriction sites 5' and 3' to the cloning sites, primer sites for bacteriophage T7 and T3 RNA transcription, a β-galactosidase fusion reporter sequence for blue/white screening of recombinant clones, and ampicillin and kanamycin resistance genes. Using a helper phage, cloned inserts (up to 12 kb) can be excised in the form of the pBK-CMV® phagemid vector (Stratagene), from which either double-stranded replicative form (plasmid) DNA or single-stranded (filamentous phage) DNA can be prepared.

The double-stranded cDNA was ligated to the right and left arms of the lambda phage vector using T4 DNA ligase. The arms have termini that are cohesive to the restriction fragment sites on the end of the cDNA (*Eco* RI and *Xho* I) so that the insert will be directionally cloned into the phage in the 5' to 3' orientation appropriate for expression of the mRNA in the sense direction for expression screening, if that method is to be used. Following ligation of the cDNA into the vector, the concatenated DNA was packaged into lambda phage using two packaging extracts that are each deficient in packaging ability, but when mixed, complement each other and allow for efficient packaging (GigaPack® III Gold Cloning Kit, Stratagene). Following packaging, phage were grown and plated using the XL1-Blue MRF'®strain of *E. coli* (Stratagene), which prevents digestion of the hemimethylated cDNA, since it is deficient in the restriction systems necessary for digesting this modified DNA. The library titer was determined by plating serial dilutions on agar plates with a lawn of *E. coli*, and was found to contain a total of 1.42 x 12^6 individual clones. Twelve clones were picked and the ExAssist™ helper phage (Stratagene) was used to excise phagemid clones, which were grown in LB (Luria Broth). Plasmid DNA was prepared from the bacteria and analyzed on agarose gels following digestion with *Eco* RI and *Xho* I restriction endonucleases. A range of insert sizes were observed (from 0.8 kb to 4.5 kb), with an average size of 2.7 kb.

We used the method first described by Maniatis for hybridization screening of the library (see Maniatis et al., 1982). A total of 6 x 10^5 clones were plated with *E. coli* on agar plates at a density of 2 x 10^4 per plate, nitrocellulose filters placed on the plates, removed, and phage DNA bound to the filters by incubation first with NaOH, which lyses the phage and denatures their DNA, followed by a neutralizing buffer. Filters were then baked at 80°C, which covalently binds the DNA, maintaining the position of the phage plaques on the filters.

Probe DNA for the preproenkephalin and preprodynorphin screening were gene fragments from the previously-cloned guinea pig genes (LaForge et al., 1995; Yuferov et al., 1996). Probes were DNA, labeled with [^{32}P]dCTP to a high specific activity (~1 x 10^9 dpm/µg) using the random-primer method (Melton et al., 1984) and hybridized under conditions of high stringency to filters. Following washing, filters were exposed to X-ray film and positions of plaques of phage hybridizing to the probe identified by the signals on autoradiographs. Positive plaques were picked and purified by three or more rounds of plaque purification at lower phage density, until pure clones were obtained. Phagemids were then excised and double-stranded plasmid DNA isolated from the clones using Qiaquick™ DNA isolation kits (Qiagen, Valencia, California).

3.1.2 Sequence analysis of cDNA clones

For DNA sequence analysis of cDNA clones, we used a "primer-walking" approach, since the gene sequence had previously been reported, and the primary purpose of this part of the study was to confirm the predicted mRNA structure of the prepropeptides. For the 3' and 5' termini of the clones, we used primers complementary to the bacteriophage T7 or T3 sites in the pBK-CMV® vector. Sequencing was performed at The Rockefeller University DNA Sequencing Center Resource using an Applied Biosystems, Inc. 3700™ automated DNA sequencer. The longest individual clones for preproenkephalin and prodynorphin that were identified were then sequenced with primers designed using the previously-reported gene sequences. Oligo® (version 5) software was used for primer design and sequences were assembled using SeqMan II® (version 5.5) software (DNASTAR, Madison, Wisconsin). Oligonucleotide primers were synthesized at The Rockefeller University Protein/DNA Resource Center.

3.1.3 Mapping transcription initiation sites

To map the transcription initiation sites of the preproenkephalin and preprodynorphin mRNAs we used RNase protection assays. We have previously reported a detailed description of the methods (Pham et al., 1998). The general principle of the method is as follows: a radioactive probe that contains (usually) a gene fragment is hybridized to mRNA from the tissue of interest. Hybridized RNA is then subjected to digestion with RNases (in this case, RNase A and T_1), which digests single-stranded RNA. RNase is then inactivated by digestion with proteinase K in the presence of sodium dodecyl sulfate (SDS), and removed by phenol extrac-

tion. Protected duplex RNA is then denatured and electrophoresed on denaturing polyacrylamide gels, which are exposed to X-ray film and protected size fragments determined from resultant autoradiographs.

To confirm the 5' terminus of the preproenkephalin mRNA from the guinea pig brain, and to determine if intron A sequences were present to an appreciable extent, we used a gene fragment probe that contained all of intron A and exon 1, and a second probe that contained all of intron A, but terminated immediately following exon 1 (see Paper I, Figure 4). To evaluate the transcription initiation sites for the preprodynorphin gene, we used a probe generated from the longest of the isolated cDNA clones, which contained 192 nucleotides of sequence 5' with respect to the previously-reported transcription initiation site (See Paper I, Figure 2). This upstream sequence exhibits high homology to the human and rat gene sequences and was hypothesized to correspond to the 5' upstream guinea pig gene sequence.

3.2 Cocaine effects on preproenkephalin mRNA in guinea pig brain [Paper II]

3.2.1 The "binge" model of cocaine administration

Many modes of drug administration have been used in animal modeling of human addiction. Although animal self-administration models are expected to closely model human drug self-administration, they are experimentally complex, laborious, and require expensive equipment. To observe many of the important neurochemical effects of abused drugs, self-administration paradigms may not be necessary. For the studies described in Paper II, we elected to use an investigator-administered paradigm known as "binge" pattern cocaine administration. This paradigm is designed to model the mode of administration of human cocaine addicts, who usually self-administer cocaine in several "binges" over the course of a few hours during the evening or night hours (i.e., corresponding to the usual sleep period). In the "binge" cocaine paradigm, rats or other rodents are administered cocaine (in this study, 15 mg/kg/injection) in three intraperitoneal injections, spaced at hourly intervals, with the first injection 30 min after the start of the light (sleep) cycle. This pattern of cocaine administration has been shown to cause greater changes in opioid receptor density compared to the same daily dose of cocaine administered in one or two doses (Unterwald et al., 2001).

3.2.2 Animal treatment

As with the studies described previously, all protocols involving use of animals were approved by The Rockefeller University Institutional Animal Care and Use Committee prior to initiation of the study.

To study the effects of "binge" pattern cocaine administration on preproenkephalin mRNA in the guinea pig brain, we used male Hartley guinea pigs (320-350g in weight). Animals were handled and weighed daily for five days following arrival at

The Rockefeller University animal facility, with a 12 h light/dark cycle used. Animals were randomly assigned to treatment and control groups. Access to food and water was *ad libitum*. Three daily injections of saline (1 ml/kg) or cocaine hydrochloride, 15 mg/kg per injection (1mg/ml saline), were administered by intraperitoneal injection as described, for a total daily dose of 45 mg/kg. This dose was selected based on previous studies in which changes in gene expression, receptor density, and behavior were demonstrated in the rat (e.g., Unterwald et al., 1992; Spangler et al., 1993; Unterwald et al., 1994; Spangler et al., 1997; Yuferov et al., 1999; 2001).

In the first study, guinea pigs were administered saline or cocaine for either two or seven days. To control for injection stress, all animals received the same number of injections: saline animals received seven injections of saline; two-day cocaine animals received five days of saline followed by two days of cocaine; seven-day cocaine animals received seven days of cocaine injections. All animals were handled and weighed daily prior to injections.

3.2.3 Preproenkephalin mRNA quantification in total RNA

Thirty minutes following the last injection, animals were sacrificed and brains removed and dissected on ice. RNA isolation was performed as previously described by the acid-phenol method (Chomczynski and Sacchi, 1987). RNase protection assays for the quantification of preproenkephalin mRNA were performed as described above, except that quantification of protected hybrids was by direct measurement of radioactive decay of hybridized RNA by liquid scintillation counting. This modification of the RNase protection assay was first introduced by Devi and colleagues (1987) and further developed by the introduction of 18S ribosomal RNA as an internal standard to control for variations in RNA extraction (Branch et al., 1992). The 18S RNA levels were also determined using RNase protection assays. Standard curves for preproenkephalin measurements were produced using sense RNA synthesized from transcription plasmids with the same insert used for the radioactive probe in the opposite orientation. RNA standards for total RNA were prepared from total RNA isolated from the guinea pig brain. Concentrations of standards were determined by optical absorbance at 260 nm.

3.2.4 Statistical analyses

Two studies of cocaine administration to guinea pigs were performed. In each study, the method of Dixon (1953) was used to eliminate outlier values from the analysis. In Study 1, animals were administered cocaine for two or seven days. Differences in preproenkephalin mRNA levels between treatment and saline groups were analyzed by one-way analysis of variance (ANOVA) followed by Neuman-Keuls *post hoc* tests. Unexpected results were obtained from the analysis of Study 1 data, and therefore a second study was undertaken. In this second study, preproenkephalin mRNA levels were measured in seven-day saline compared to seven-day cocaine-treated animals. If in the first study a hypothesis was generated regarding the alteration of preproenkephalin mRNA levels by cocaine, a one-tailed unpaired

t-test was used to assess an increase or decrease. A two-tailed *t*-test was used if no significant alteration was observed in the first study. Finally, to provide greater power in the statistical analysis, Studies 1 and 2 were combined in the analysis of the effects of seven-day cocaine on preproenkephalin mRNA levels in two-way ANOVA analyses (drug treatment x study).

3.3 Human preproenkephalin gene and opiate addiction [Papers III & IV]

3.3.1 Human molecular genetic methods

In the studies reported in Papers III and IV, we tested the hypothesis that specific polymorphisms in or near the human preproenkephalin gene are associated with a history of heroin addiction. A positive finding would suggest that this gene is involved in the development or persistence of this disorder. In Paper IV we also resequenced the promoter region, as well as exons I and II and the first intron of the gene from over 200 study subjects in an effort to identify additional polymorphisms that might reside in functional elements that could affect transcription or splicing.

3.3.2 Study subjects, recruitment, and assessment

Subjects included in the studies reported in Papers III and IV were drawn from a group of consecutive volunteers participating in ongoing human genetic studies at the Laboratory of the Biology of Addictive Diseases located at The Rockefeller University in New York City. The specific research protocol for genetic studies using DNA was approved by the Institutional Review Board (for ethical review of studies involving human subjects) of The Rockefeller University Hospital. Prior to participation, the study was explained to the subject, including the purpose, study procedures, potential risks and benefits, and subject rights. All subjects read and signed IRB-approved written informed consent.

For Paper III, all subjects were recruited and assessed between July 17, 1997, and September 2, 1999. For Paper IV, additional subjects who had been recruited earlier (from February 7, 1995, to July 17, 1997) were also studied in addition to those described in Paper III.

Following screening, all subjects were assessed by a research nurse, clinical psychologist, or physician (psychiatrist or internist). Clinical researchers participating in the project are thoroughly trained in all instruments and experienced in study of subjects with substance abuse and dependence. All study subjects, including controls, were administered tests for urine toxicology, which was screened for several drugs of abuse to confirm reported histories.

Opioid-dependent subjects were all former heroin addicts, primarily recruited from two methadone maintenance treatment clinics associated with the Weill Medical College of Cornell University. These clinics are located a short distance from The Rockefeller University in New York City. All subjects met the United

States Federally-regulated criteria for admission into methadone maintenance treatment: one or more years of daily self-administration of heroin or other illicit (or illicitly-obtained) opiates, and the development of tolerance, physical dependence, and drug-seeking behavior despite harm to self or others (Rettig and Yarmolinsky, 1995; National Consensus Development Panel, 1998). These criteria are significantly more stringent than either DSM-III-R or DSM-IV (American Psychiatric Association, 1987; 1994) diagnoses for abuse or dependence. Opioid-dependent subjects were also administered the Addiction Severity Index (ASI) (McLellan et al., 1980) to establish years of use of heroin and other illicit drugs and alcohol, and years in methadone treatment. For many subjects, this information was confirmed by hospital chart review. Abuse of other drugs or alcohol was not used as an exclusion criterion for opioid-dependent subjects as long as addiction to opiates was the primary diagnosis. Our extensive assessment procedures allowed us to include in the opioid-dependent group only individuals with severe opiate addiction.

Inclusion and exclusion criteria for control subjects were also rigorous to assure that these individuals had no ongoing or prior history of alcohol or drug abuse or dependence. The primary instrument used to establish drug-free status was the ASI. For inclusion as controls, subjects from the potential study pool must not have used illicit drugs or alcohol to intoxication at any point during the previous 30 days (with the exception of use of cannabis, up to 12 of the prior 30 days). Also, individuals were excluded as controls if at any point in their life they had had a period of use of an illicit drug or alcohol to intoxication three times per week for a period of six months or more. Modest prior use of cannabis was not used as an exclusion criterion for control subjects, nor was use of nicotine or caffeine.

3.3.3 Genomic DNA isolation

Venipuncture was used to draw blood specimens from study subjects. Lymphocytes (buffy coat) were isolated from blood by centrifugation on a step-gradient of Ficoll-Pac™ (Pharmacia, Uppsala, Sweden). Genomic DNA was isolated from lymphocytes using a simple salting-out procedure (Miller et al., 1988). Briefly, cells are suspended in buffered saline solution, and then lysed by the addition of SDS and proteinase K, with incubation at 55°C for two hours to digest proteins and inactivate nucleases. A high-salt solution is then added to precipitate proteins, which are removed by centrifugation. Isopropanol is then added to precipitate DNA, which is collected by spooling on a glass rod, and resuspended in a buffered aqueous solution. For TaqMan® assays [Paper IV], DNA concentrations were determined using the PicoGreen® DNA quantification kit (Molecular Probes, Eugene, Oregon) using a Fusion™ microplate reader (Perkin-Elmer, Wellesley, Massachusetts) located at The Rockefeller University High-Throughput Screening Resource Center. Genomic DNA was stored at -80°C until needed for assays.

3.3.4 Genotyping $(CA)_n$ repeat polymorphism

For genotyping the $(CA)_n$ repeat polymorphism in the 3' flanking region of the preproenkephalin gene, we used a standard microsatellite genotyping approach. This repeat was identified in the original sequencing of the gene (Comb et al., 1983) and was later shown to be polymorphic, with six alleles identified in the populations in which it has been studied (primarily Caucasian, African-American, and Chinese) (Weber and May, 1990; Chan et al., 1994). The repeat is located 297 nucleotides downstream from the termination codon of the gene.

Using Oligo® (version 5) software, we designed custom primers which were tested in a PCR reaction (see below). A primer-pair that provided good amplification was synthesized by GeneLink® (Hawthorne, New York) and used to prepare amplified DNA from several study subjects. These samples were then purified on Qiaquick™ DNA isolation kits (Qiagen, Valencia, California) and sequenced at The Rockefeller University DNA Sequencing Center Resource using the same primers used in amplification. DNA sequencing was performed to confirm that the correct sequences had been amplified, and to accurately quantify the number of repeats in each of the identified alleles.

The forward primer was synthesized with a fluorescent marker (FAM) on the 5' end by Applied Biosystems. Genotyping was then performed at The Rockefeller University Genotyping Resource Center. DNA from subjects was amplified by PCR and then electrophoresed on an Applied Biosystems model 3700™ automated DNA sequencing system. Allele-calling was performed using Genotyper™ (version 5) software (Applied Biosystems) by two separate investigators who had no knowledge of phenotypic classification of subjects.

3.3.5 DNA sequence analysis of human preproenkephalin gene

Because several functional regulatory elements have been demonstrated to reside in the 200 bases upstream from the preproenkephalin transcription initiation site, we used DNA sequence analysis of study subject DNA in an effort to identify novel polymorphic variants that might reside in that region. Methods for PCR amplification and sequencing were as described above. Briefly, we used Oligo® (version 5) software to design custom primers to amplify a sequence of DNA that included exons I and II, the intron (IVS1) between these two exons, and approximately 300 nucleotides of sequence 5' upstream from the transcription initiation site. PCR of study subjects' genomic DNA was performed with a step-down protocol using a GeneAmp® PCR system. Because of the high GC content of this region of the gene, it was necessary to use Platinum® Taq enzyme and PCRx Enhancer® solution (both from Invitrogen, Carlsbad, California) to obtain specific PCR products. The amplified DNA was purified and sequenced at The Rockefeller University DNA Sequencing Resource Center on automated DNA sequencing systems. Primers were the same as used for amplification. Sequences were assembled using SeqMan II® (version 5.05) (DNASTAR) and electropherograms were visually read by two trained investigators separately to identify polymorphisms.

3.3.6 TaqMan® assays for genotyping single nucleotide polymorphisms

For genotyping three previously-reported common SNPs of the preproenkephalin gene, we used an allele-specific TaqMan® assay. This method is based on the principle of fluorescence energy resonance transfer (FRET), coupled with an enzymatic reaction (PCR). If two fluorescent molecules are closely adjacent (within approximately 100 Å) and one of these molecules (termed the "donor") is excited by light that is at a wavelength within its absorbance spectrum, rather than emitting the energy as a photon, the energy is transferred to the adjacent fluorophore (termed the "quencher"), as long as the absorption spectrum of the quencher overlaps the emission spectrum of the donor fluorophore (keeping in mind that the emission wavelength of a fluorophore is always lower than its absorption wavelength). The quencher fluorophore will then emit the photon at a (lower) wavelength characteristic of its emission spectrum.

For the TaqMan® genotyping assays used in this study, two oligonucleotide probes were designed to hybridize to a specific region of the human preproenkephalin gene known to contain a SNP with a high allelic frequency. Each probe was designed so that it had a perfect sequence match to one of the two possible substituted bases of the SNP. The probes were synthesized so that they contained a donor fluorophore on the 5' end and a quencher fluorophore on the 3' end. Each of the two probes for a given SNP are labeled with a different donor fluorophore, in this case, FAM or VIC. The quencher fluorophore on each included a "minor groove binding" domain to increase the annealing temperature of the probe. This method enables the use of shorter probes with greater allele discriminative properties. Primers that flank each SNP position and that amplify a sequence of 100 to 200 bases were also designed. A PCR reaction was then performed that includes the primers, both probes, and genomic DNA. As the reaction proceeds, DNA is denatured and the probes (and primers) hybridize to the strand complementary to them. The assay performance is based on the difference in discrimination of hybridization efficiency of the perfectly-matched compared to the single-base-mismatched probe. As each new strand of DNA is synthesized in each round of DNA, the probe oligonucleotide that is hybridized to the amplicon template is digested by the 5' to 3' exonuclease activity of Taq DNA polymerase. This releases the fluorophore attached to the probe into the solution, and away from the quencher fluorophore at the other end of the probe. The fluorescent properties of the reaction solution are thereby changed, with a greater proportion of the fluorophore from the perfectly-matched probe released into the solution. The fluorescence of the solution is then measured at the emission spectra of VIC and FAM to determine which of the alleles (or both, indicating a heterozygous individual) are present in the starting genomic DNA.

For the preproenkephalin gene, we selected three SNPs that have previously been reported to have high allelic frequencies (Mikesell et al., 1996; 1997). These included two single nucleotide substitutions (IVS1 C28A, C81T) and a single nucleotide deletion (C808Δ). IVS1 C28A is located in the first intron of the gene, C81T is a synonymous substitution at amino acid position Ser27 in exon III of the recep-

tor, and C808Δ is a single nucleotide deletion located four nucleotides downstream from the termination codon in the 3' UTR of the mRNA. Mikesell and colleagues (1996; 1997) who studied primarily Caucasian and African-American schizophrenic and control subjects, reported overall allele frequencies of 40.5%, 8.9%, and 39.2% for the IVS1 C28A, C81T, and C808Δ SNPs, respectively. An assay for a fourth previously-reported SNP (A1106G) with a high allelic frequency was also designed; however, the SNP is in an unfavorable context for a TaqMan® probe, and assay performance for this SNP was not satisfactory. Primers and probes for each SNP were designed using Primer Express® software (Applied Biosystems) TaqMan® probes, labeled with FAM or VIC, were synthesized by Applied Biosystems. Primers were synthesized by Midland Certified Reagent Company (Midland, Texas). PCR cycling was performed using a GeneAmp® system 9600 and, following PCR reactions, fluorescence measurements were performed using Applied Biosystems Prism® 7900 Sequence Detection System. Analysis of allele discrimination was performed using software provided with that instrument.

3.3.7 Data treatment and statistical methods

For the study reported in Paper III, allele frequencies were calculated. Five alleles were detected with the majority of alleles containing either 13 or 14 copies of the $(CA)_n$ repeat. For analysis of genotypes, alleles were grouped into genotypes of short (≤13 repeat) alleles and long (≥14 repeat) alleles, since the numbers of the 11, 12, and 15 repeat alleles were low. Chi-square tests were performed to assess deviations from Hardy-Weinberg equilibrium in study groups. To test for differences in allele frequencies among the three ethnic/cultural groups with sufficient sample size (control subjects only), the Fisher-Freeman-Halton exact test was used (Freeman and Halton, 1951). Chi-square tests were used to evaluate differences in grouped genotype frequencies among ethnic/cultural groups for controls. We established 95% confidence intervals for allele frequencies of control subjects for the three ethnic groups using the binomial distribution. With data stratified by ethnic/cultural groups, Odds Ratios were calculated for grouped genotypes and Chi-square tests performed to test the hypothesis that the preproenkephalin gene is associated with opiate addiction. Also, we compared differences in genotype distributions using a *t*-test. For this approach, the numbers of CA repeats contributed by both chromosomes in an individual were summed and a *t*-test performed on summed genotypes between cases and controls, with data stratified by ethnicity.

For the study reported in Paper IV, genotyping data from DNA sequencing and TaqMan® methods were compiled and compared. Only subjects for whom the genotyping was unambiguous and complete for all three SNPs were included in the analysis (see Paper IV for details). As in the previous study, departures from Hardy-Weinberg equilibrium for each SNP in all study groups with sufficient sample size were assessed using Chi-square tests. Confidence intervals (95%) for allele frequencies were established using the binomial distribution. With data stratified by ethnic/cultural group, we calculated Odds Ratios and used Chi-square analyses to

test for an association of each SNP with opioid-dependence. A genetic model-free analysis for each SNP was also performed using Chi-square or Fisher-Freeman-Halton tests.

4. FINDINGS

4.1 Primary structure of guinea pig neuropeptide mRNAs [Paper I]

4.1.1 Summary of findings

The primary finding of these studies was the confirmation of the mRNA primary structure (as determined by sequencing of cDNA clones) of the structure predicted from the previously-reported gene sequences of preproenkephalin and preprodynorphin (as well as 5' and 3' RACE studies for the preprodynorphin gene). In addition to confirming the predicted sequence, transcription initiation sites were mapped. For preproenkephalin, one primary site was mapped, with a limited amount of transcription initiating near the TATA signal, located 27 nucleotides upstream from the primary transcription initiation site. Transcripts of preprodynorphin also are initiated at a primary site located approximately 30 nucleotides downstream from the TATA signal; however several upstream sites were also identified between 160 and 190 nucleotides upstream from the primary site.

4.1.2 Results of library screening

From the screening of the guinea pig brain/pituitary library, six individual preproenkephalin cDNA clones were isolated, as well as three clones for preprodynorphin. Sequence analysis of the 5' and 3' termini was performed for all clones and the longest clone for each of the two genes was completely sequenced using the "primer walking" approach.

4.1.3 Preproenkephalin gene and mRNA structure

The organization of the guinea pig preproenkephalin gene into three introns and its predicted mRNA sequence was confirmed in this study. Also confirmed was the existence of a small amount of transcription initiation at or near the TATA sequence, which is located 27 nucleotides 5' upstream from the primary transcription initiation site. The poly(A) addition site for each of the six preproenkephalin clones was 17 nucleotides downstream of the second of two polyadenylation signals, confirming that the second of the two was utilized in polyadenylation. We were able to correct the position of the poly(A) addition site previously predicted using RNase protection mapping from the gene sequence (LaForge et al., 1995). That previous report suggested that the site was located six nucleotides downstream from the actual site determined by sequencing the clones isolated in the present study.

4.1.4 Intron A sequences of preproenkephalin mRNA

One of the cDNA clones contained the sequences of exons I and II, but lacked exon I and initiated at a site within the first intron (intron A) of the gene. This finding was interesting, since initiation in intron A has been demonstrated in spermatogenic cells of the rat and mouse, with longer transcripts that are largely translationally

inactive (e.g., Kilpatrick et al., 1985; Kilpatrick and Rosenthal, 1986; Kilpatrick et al., 1987; Garrett et al., 1989; Kew et al., 1989; Yoshikawa, et al., 1989; Kilpatrick et al., 1990; Zinn et al., 1991; Rao and Howells, 1993). In addition, high heteronuclear concentrations of intron A sequences have been reported to accumulate in cells located in the rat reticular thalamic nuclease and basal forebrain (Brooks et al., 1993), although the functional significance of this finding has not been determined. In this study, however, using RNase protection assays, we were unable to detect intron A sequences in the guinea pig brain or pituitary. The RNase protection assay is quite sensitive, and capable of detecting (and measuring) less than a picogram of a specific mRNA. Therefore, we can conclude that no appreciable intron A sequences accumulate in the guinea pig brain or pituitary. The clone identified in the library screen most likely was produced from an incompletely-spliced nuclear mRNA transcript.

4.1.5 Multiple transcription initiation sites of preprodynorphin gene

The previously-deduced sequence of the guinea pig preprodynorphin gene and mRNA primary structure was confirmed by sequencing of the cDNA clones isolated in this study. We confirm here that the guinea pig mRNA lacks sequences that correspond to the sequences of the human and rat exon II, due to alternative splicing. In this respect, the mRNA from the guinea pig brain resembles the form found in the rat testis, which also does not contain sequences of exon II in that species (Garrett et al., 1989).

Unexpectedly, we identified one clone that had a 5' sequence that was extended by 192 nucleotides at the 5' end of the sequence compared to the previously-defined transcription start site in the guinea pig brain and adrenal gland, as determined by the 5' RACE method (Yuferov et al., 1996), and which is located 23 nucleotides downstream of the TATA sequence. Previous evidence for upstream transcription initiation in the rat brain and testis was reported by Garrett and colleagues, who used RNase protection assays in the identification of transcription initiation sites 30 nucleotides and 70 nucleotides upstream from the primary site (Garrett et al., 1989). The functional consequences, if any, of the observed differences in length of the preprodynorphin mRNAs has not yet been established.

4.1.6 Commentary

The studies reported in Paper I confirm and extend previous studies of the mRNA and gene structure of preproenkephalin and preprodynorphin in the guinea pig brain. The guinea pig enkephalin mRNA had previously been deduced using the gene sequence, and RNase protection assays (LaForge et al., 1995), and the preprodynorphin mRNA predicted using a partial gene sequence and the RACE method. However, the method to establish the primary structure of an mRNA with the most confidence is the method used in this study: direct sequencing of cDNA clones.

The findings of differential transcription initiation sites in the guinea pig brain are also of interest. Although we did not experimentally explore the potential functional

consequences or regional distribution of the variant forms, sequences in the 5' UTR can have dramatic effects on stability and translational efficiency. One example of this is the variant form of preproenkephalin found in rat spermatogenic cells, as noted above. In particular, Rao and Howells (1993) demonstrated that specific sequences in the 5' UTR of the variant testicular form are responsible for the lack of translation of the mRNA. These sequences have high predicted secondary structure, and apparently impede the ribosomal movement along the mRNA, or, alternatively, prevent translation initiation through some unknown mechanism.

Finally, while these studies are unlikely to have a major immediate impact on our understanding of these neuropeptides, the findings made, and the cDNA clones themselves, provide the basis for potentially important structure-function studies of the mRNAs for these important peptide precursors.

4.2 "Binge" cocaine affects preproenkephalin mRNA in guinea pig brain [Paper II]

4.2.1 Summary of findings

In this Paper, we report on the findings of our studies on the effects of two and seven days of "binge" cocaine administration on preproenkephalin mRNA levels in several regions of the guinea pig brain. Animals were administered cocaine in a "binge" paradigm designed to model the common human pattern of cocaine self-administration. Drug or saline was administered for either two or seven days with all animals receiving the same number of injections to control for injection stress. Following final drug or saline injection, animals were sacrificed, brains removed and dissected, and total RNA isolated from several brain regions. Quantification of preproenkephalin mRNA was by solution hybridization RNase protection assays. Two separate studies were performed. In Study 1, animals received either two or seven days of cocaine administration. Two-day cocaine treatment resulted in no observed differences in preproenkephalin mRNA between treated and control animals in six brain regions studied. However, in brains of animals administered cocaine for seven days, we detected increases in preproenkephalin mRNA in the frontal cortex, amygdala and hippocampus and a decrease in the nucleus accumbens. Because findings of this study for the seven-day time point were unexpected (based on previous rat studies), particularly with respect to a decrease in preproenkephalin mRNA in the nucleus accumbens, we performed a second study at the seven-day time point using identical methodology. In the second study, we again observed an increase in preproenkephalin mRNA in the frontal cortex and also a decrease in the hypothalamus.

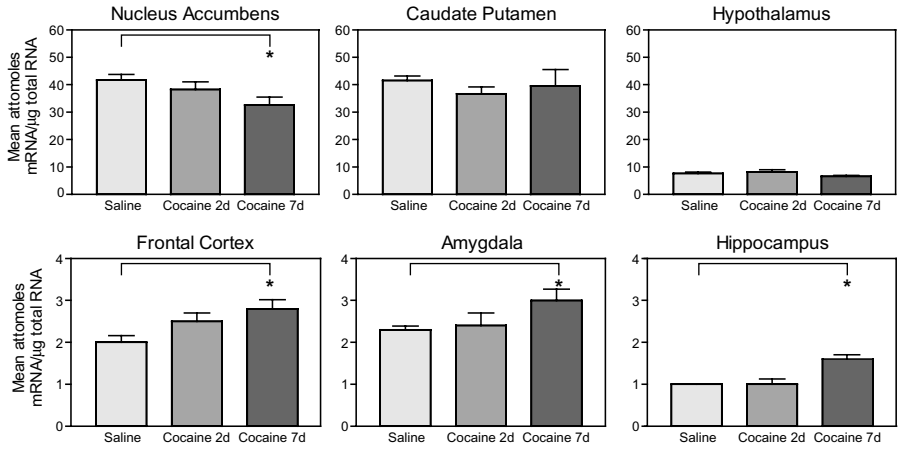
To provide greater statistical power, results from the seven-day time point of the two studies were combined and analyzed by two-way analysis of variance (ANOVA). In this combined analysis, increases of preproenkephalin mRNA in the frontal cortex, amygdala and hippocampus, as well as decreases in the nucleus accumbens and hypothalamus, were found to be significant. The differences

between the preproenkephalin mRNA responses to cocaine in the guinea pig and rat provide evidence that this animal model may be an additional useful species in neurobiological studies of cocaine-induced changes in neuropeptide expression.

4.2.2 Results of Study 1

As noted above, in this study we evaluated the effects of two and seven days of "binge" cocaine administration on preproenkephalin mRNA in six brain regions of the guinea pig. Cocaine-induced changes observed in this study are shown in Figure 6.

Figure 6. Study 1: effects of two and seven day "binge" cocaine administration on preproenkephalin mRNA in the guinea pig brain



Measurements of preproenkephalin mRNA and total RNA were made by ribonuclease protection assays. Data are expressed as mean attomoles of preproenkephalin mRNA per microgram of total RNA. No alterations in mRNA levels were observed at two days, but at the seven-day time point a significant decrease in preproenkephalin mRNA levels was found in the nucleus accumbens and significant increases were found in the frontal cortex and hippocampus.

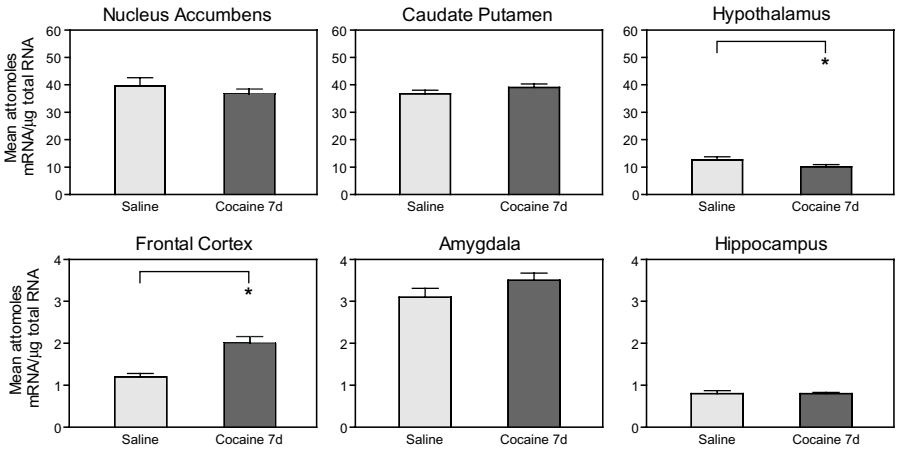
Significance of alterations was assessed by ANOVA followed by Neuman-Keuls *post hoc* tests. No alterations at the two-day time point were significant. However, at the seven-day time point, a significant decrease in the nucleus accumbens ($p<0.05$) was found, as well as significant increases in the amygdala ($p<0.05$), frontal cortex ($p<0.05$), and hippocampus ($p<0.01$) (significance values are for Neuman-Keuls *post hoc* tests; for details of ANOVA results, see Paper II). No effect of cocaine on preproenkephalin mRNA levels in the caudate putamen or hypothalamus was observed.

4.2.3 Results of Study 2

Since the findings of the first study were unexpected based on previously-reported studies in the rat, in which transient cocaine-induced increases in preproenkephalin mRNA had been observed in striatal tissues, we performed a second study at the seven-day time point using identical methodology. The results of Study 2 are shown in Figure 7.

In this study, a significant effect of cocaine was observed in two brain regions, with an increase in the frontal cortex ($p<0.0001$, Mann-Whitney one-tailed *U* test) and a decrease in the hypothalamus ($p<0.05$, unpaired two-tailed *t*-test). No significant changes were observed in other brain regions studied. Two additional brain regions were evaluated in Study 2, the thalamus and cerebellum (not shown in Figure 7). No significant effect of cocaine on preproenkephalin mRNA levels was observed in these brain regions.

Figure 7. Study 2: effects of seven days of "binge" cocaine on preproenkephalin mRNA in six guinea pig brain regions



Measurements of preproenkephalin mRNA and total RNA were made by ribonuclease protection assays. Data are expressed as mean attomoles of preproenkephalin mRNA per microgram of total RNA. A significant decrease in preproenkephalin mRNA in the hypothalamus and a significant increase in the frontal cortex were found in this study. Differences in mRNA levels in other brain regions did not reach significance at a level of $p<0.05$.

4.2.4 Analysis of combined data from Studies 1 and 2

At first glance, the findings from Studies 1 and 2 appear to differ, except for the increase in preproenkephalin mRNA in the frontal cortex, which was significant in both studies. However, although the statistical significance of the differences in the two studies was not the same for the other brain regions in which a change was observed in one or the other of the studies, the *direction of change* was the same in all regions except the hippocampus, in which a change was observed in the first study and not the second. Since the two studies were performed using precisely the same methodology, in animals of the same strain and age, and at the same time of year, spaced one year apart, we combined the data for the seven-day time point from the two studies for six brain regions and analyzed the data using a two-way ANOVA (treatment group x study).

In the combined analysis, we observed a significant increase in preproenkephalin mRNA in the frontal cortex ($p<0.0001$), amygdala ($p<0.01$) and hippocampus ($p<0.05$). Decreases in mRNA levels were observed in the nucleus accumbens ($p<0.05$) and hypothalamus ($p<0.01$), with no change in the caudate putamen

(for details of results of ANOVA analyses, see Paper II). We calculated a weighted percentage change for the five brain regions with significant results in the combined analysis. These results are summarized in Table 2.

Table 2. Combined results from Studies 1 and 2 of the effects of seven-day "binge" cocaine administration on preproenkephalin mRNA levels in six guinea pig brain regions

Region	Percent change	Significance level
Frontal cortex	52% increase	$p < 0.0001$
Nucleus accumbens	16% decrease	$p < 0.05$
Caudate putamen	No change	-----
Amygdala	24% increase	$p < 0.01$
Hypothalamus	17% decrease	$p < 0.01$
Hippocampus	27% increase	$p < 0.05$

Weighted percent changes were calculated using numbers of animals in each group from the two studies. Significance was determined by two-way ANOVAs (Treatment x Study). Details of analysis are reported in Paper II.

4.2.5 Commentary

The finding of differential "binge" cocaine-induced alterations of preproenkephalin mRNA expression in different brain regions of the guinea pig is striking for several reasons. First, in most previous studies in the rat in which cocaine has been shown to induce alterations to preproenkephalin mRNA expression in specific brain regions, increases have been reported (e.g., Steiner and Gerfen, 1993; Przewlocka and Lason, 1995; Spangler et al., 1996; Daunais et al., 1997; Mathieu-Kia and Besson, 1998; Svensson and Hurd, 1998). It should be noted that several other studies have not observed cocaine-induced alterations in the rat preproenkephalin mRNA levels (Branch et al., 1992; Daunais and McGinty, 1994; Daunais et al., 1995; Sorg et al., 1995; Spangler et al., 1996). Also, in two previous rat studies, decreases in mRNA levels for this prepropeptide have been reported. Transient decreases in preproenkephalin mRNA levels in the nucleus accumbens core and rostral striatum were reported to occur following acute cocaine administration (Adams et al., 2000), and, in another study, it was reported that 24 or 48 hours following the last injection of chronic (5-day) cocaine administration, decreases in preproenkephalin mRNA were found in the nucleus accumbens and dorsal striatum (Przewlocka and Lason, 1995). The current findings of decreases of this mRNA in the nucleus accumbens of the guinea pig brain are more in accord with the latter two studies. Clearly, additional studies will be necessary to establish the reasons for the differences between the responses in the previous studies. Strain differences or differences in specific experimental conditions and treatment paradigms, may well underlie differences in these reported findings.

A second reason for interest in the findings of the study in guinea pigs is that no previous study in the rat has reported alterations in the hypothalamus (particularly not a decrease) or increases in the frontal cortex or amygdala. Admittedly, most studies have not evaluated these regions; however one study in the rat that used methods

identical to those in the present study (Branch et al., 1992) found no changes in preproenkephalin mRNA in these regions following seven days of cocaine administration. This finding points to the possibility that a species difference is responsible for the changes seen in the guinea pig.

The finding of a cocaine-induced reduction of preproenkephalin mRNA in the guinea pig nucleus accumbens is also interesting in light of two studies, one a post-mortem study of brains from human cocaine abusers and control subjects (Hurd and Herkenham, 1993) and also, a study of rhesus monkeys that had long-term histories (two years) of cocaine self-administration (Daunais et al., 1997). In both of these studies in higher primates, significant reductions in striatal preproenkephalin mRNA levels were detected. In the rhesus study, the nucleus accumbens had the greatest reduction in preproenkephalin mRNA expression (83%) of any region examined. In the human study, the decreases in the nucleus accumbens (20%) did not reach significance ($p=0.09$), but in the caudate and putamen the finding were significant. The decrease of preproenkephalin observed in the guinea pig nucleus accumbens suggests that, for this brain region, this species may be a better model of cocaine effects on the human brain than the rat. However, it is also important to note the lack of a decrease in the guinea pig caudate putamen, whereas in the corresponding regions of higher primates a significant decrease was reported.

Finally, it is interesting that treatment with a single drug, cocaine, results in differential alterations in preproenkephalin mRNA expression in different brain regions. The molecular mechanisms that produce the observed effects of cocaine administration on gene expression are not yet well understood. Presumably, the increased dopamine levels caused by chronic administration lead to compensatory neurochemical changes, including gene expression of a variety of neuroreceptors, neurotransmitters, second messenger systems or other proteins. The guinea pig, with its differential cocaine response for the preproenkephalin gene, may prove to be a useful model for elucidation of these mechanisms of the action of this psychostimulant.

4.3 Preproenkephalin gene and human opiate addiction [Papers III & IV]

4.3.1 Summary of findings

Papers III and IV of this thesis address the same hypothesis and will be considered together. That hypothesis is that the preproenkephalin gene is associated with human heroin addiction. In Paper III, we tested for association of this gene with opiate addiction using a $(CA)_n$ repeat polymorphism (microsatellite marker) at the preproenkephalin gene locus; this marker had previously identified this gene as possibly associated with opiate dependence (Comings et al., 1999). In Paper IV, we used SNPs within the preproenkephalin gene to test for an association of the gene with heroin addiction. In that study, we also screened 5' regulatory sequences and the first two exons (as well as the first intron) in a search for novel polymorphisms.

In both studies, we found evidence for an association of the human preproenkephalin gene and a history of heroin addiction. This finding was the most robust within the Caucasian study group in each of the studies. Highly significant differences were also observed for allele and genotype frequencies among the three ethnic/cultural groups with sufficient sample size for analysis (Caucasians, African-Americans and Hispanics). The findings of these studies support a role of the preproenkephalin gene in the development or persistence of human opiate addiction.

4.3.2 Association of $(CA)_n$ repeat polymorphism and opiate addiction

In this study we genotyped opioid-dependent and control subjects for a polymorphic $(CA)_n$ repeat polymorphism in the 3' franking region of the human preproenkephalin gene. Consistent with previous reports, we identified five alleles at this locus. Our study population consisted primarily of three ethnic/cultural groups: Caucasians, African-Americans and Hispanics. This distribution reflects the ethnic/cultural demography of the primary catchment area for the study, which is the Upper East Side of Manhattan in New York City. The balance between male and female study subjects also reflects the numbers of individuals entering treatment for opiate addiction in New York City (approximately 60% male, 40% female). After application of all inclusion and exclusion criteria, a total of 266 individuals were included in the study, including 137 opioid-dependent cases and 129 control individuals. For details of study subject demographics, see Paper III.

Allele frequencies for the five identified alleles were similar to those previously reported in the ethnic/cultural groups we studied. Using DNA sequencing, we established that five alleles identified in our study contained 11, 12, 13, 14, or 15 repeats, with overall allele frequencies of 1.2%, 5.3%, 43.9%, 42.7% and 6.9%, respectively. In the total study population, alleles that contained 13 and 14 repeats constituted the majority of all alleles. In the Caucasian subjects, the 13 and 14 alleles comprised 96% of all alleles; in African-American subjects, these alleles accounted for 73% of all alleles; for the Hispanic subject group, these alleles accounted for 83%. These overall allele frequency distributions for the Caucasian and African-American groups are in accord with previous studies (Chan et al., 1994; Comings et al., 1999).

Table 3 gives the allele distributions and frequencies for each of the five alleles of this polymorphism for cases and control subjects stratified by each of the three main ethnic/cultural groups. In Table 4, alleles were grouped into short (≤13 repeat) and long (≥14 repeat) alleles; these were used to construct genotype groups (homozygous ≤13/≤13, heterozygous ≤13/≥14, and homozygous ≥14 /≥14). Distribution and frequencies of these grouped genotypes are shown in Table 4. Individuals in other ethnic groups or those who reported mixed ancestry were not included in analyses with data stratified by ethnicity due to small sample size and concerns of population admixture.

Table 3. Allele distributions and frequencies for opioid-dependent cases and control subjects

Study	Subject Group	Allele					Total
		11	12	13	14	15	
Caucasian							
	Control	0	1	40	70	1	112
		(0.00)	(0.01)	(0.36)	(0.62)	(0.01)	
	Opioid-dependent	1	4	59	56	2	122
		(0.01)	(0.03)	(0.48)	(0.46)	(0.02)	
African-American							
	Control	1	7	27	13	6	54
		(0.02)	(0.13)	(0.55)	(0.24)	(0.11)	
	Opioid-dependent	4	2	27	22	13	68
		(0.06)	(0.03)	(0.40)	(0.32)	(0.19)	
Hispanic							
	Control	0	1	24	16	5	46
		(0.00)	(0.02)	(0.52)	(0.35)	(0.11)	
	Opioid-dependent	0	6	34	19	7	66
		(0.00)	(0.09)	(0.51)	(0.29)	(0.11)	

Data are stratified by the three largest ethnic/cultural groups studied. Alleles are characterized by the number of CA repeats they contain (11-15). Allele frequencies are consistent with previously-reported studies. Deviation from Hardy-Weinberg equilibrium was found in the Caucasian opioid-dependent group when all five alleles were considered ($\chi^2_{(10)}$=77.44, p<0.0001). Significant differences in allele frequencies existed among the three ethnic groups, controls only (p>0.0001, Fisher-Freeman-Halton test). Details of analyses are in Paper III.

Table 4. Grouped genotype frequencies of the $(CA)_n$ repeat polymorphism at the preproenkephalin gene locus

Study	Subject Group	≤13 Repeats	Heterozygotes	≥ Repeats	Total
Caucasian					
	Control	8	25	23	56
		(0.14)	(0.45)	(0.41)	
	Opioid-dependent	17	30	14	61
		(0.28)	(0.49)	(0.23)	
African-American					
	Control	10	15	2	27
		(0.37)	(0.56)	(0.07)	
	Opioid-dependent	7	19	8	34
		(0.21)	(0.56)	(0.23)	
Hispanic					
	Control	6	13	4	23
		(0.26)	(0.57)	(0.17)	
	Opioid-dependent	8	24	1	33
		(0.24)	(0.73)	(0.03)	

Alleles are defined as short (≤13 repeats) and long (≥14 repeats). Study subjects were classified as heterozygous or homozygous for each allele type. With analysis performed assuming a genetic model of autosomal dominant inheritance (i.e., short/short individuals combined with heterozygous individuals and compared to homozygous long/long individuals) a significant association of grouped genotype with opioid dependence was detected in the Caucasian subjects (Odds Ratio=2.34 [95% CI 1.1-5.0], $\chi^2_{(1)}$=4.40, p=0.036), No difference was observed in the other two ethnic groups in this analysis. Evidence for a significant difference in summed genotypes between cases and controls was also found using a *t*-test in the Caucasian group (two-tailed *t* = 2.35, degrees of freedom=115, p=0.021), but not in the other groups. For details of these analyses, see Paper III.

We observed significant deviations from Hardy-Weinberg equilibrium in the Caucasian opioid-dependent group ($\chi^2_{(10)}$=77.44, p<0.0001), as well as for grouped allele frequencies ($\chi^2_{(1)}$=9.03, p<0.005) in the Hispanic opioid-dependent subjects. Since deviations from Hardy-Weinberg equilibrium can cause bias in association analyses if allele frequencies are used (Sasieni, 1997; Schaid and Jacobson, 1999; Knapp, 2001), we used grouped genotype frequencies in comparisons of cases to controls.

Significant differences in allele (p<0.0001, Fisher-Freeman-Halton test) as well as grouped allele frequencies ($\chi^2_{(2)}$=12.68, p<0.005) were observed among control subjects in the three ethnic/cultural groups with sufficient sample size for analysis. In addition, the 95% confidence intervals for the calculated allele frequencies of the Caucasian and African-American control subjects did not overlap. For comparisons of case and control data, the data was stratified into three ethnic/cultural groups and analyzed independently.

Assuming an autosomal dominant inheritance model with the short alleles dominant (short/short + short/long vs. long/long), we observed a significant difference in grouped genotype frequencies in the Caucasian group (Odds Ratio=2.34 [95% CI 1.1-5.0] , $\chi^2_{(1)}$=4.40, p=0.036). In the Hispanic group, the difference in allele frequencies did not reach significance at the p<0.05 level, but the significance was at a borderline level (Odds Ratio= 6.74 [95% CI 0.9-28.4], $\chi^2_{(1)}$=3.38, p=0.066). Additionally, if no genetic model was assumed, the differences in grouped genotypes in the Caucasian group nearly reached significance at a p<0.05 level ($\chi^2_{(2)}$=5.68, p=0.058). These findings provide evidence of an association of the preproenkephalin gene and heroin addiction in Caucasians.

4.3.3 A novel single nucleotide polymorphism

We sequenced PCR-amplified DNA from 230 subjects as part of this study (described in Paper IV) to identify novel SNPs that may reside in exons I and II, the first intron (IVS1) and approximately 300 nucleotides 5' upstream from the transcription initiation site. The subjects of study were primarily from three ethnic/cultural groups (Caucasians, Hispanics, and African-Americans), so population groups with diverse ancestry were included in the sample. In this effort, we identified one previously-reported common SNP (IVS1 C23A, with frequency of the minor allele that in this study ranged from 0.25 to 0.51, depending on ethnic/cultural group). In addition to the previously-identified SNP (first reported by Mikesell et al., 1996), we identified a rare novel SNP C-334T in the 5' flanking region of the gene (205 nucleotides upstream of the transcription initiation site). This polymorphism was found in two heterozygous individuals and has an allelic frequency of less than 1%. It is not located within any previously-identified *cis*-acting transcription regulatory sites.

4.3.4 Association of single nucleotide polymorphisms and opiate addiction

Following application of inclusion and exclusion criteria including exclusion of subjects for whom reliable genotype data was not obtained, a total of 380 individuals were included in the study, which is described in Paper IV. Three SNPs (IVS1 C28A, C81T, and C808Δ) were genotyped using TaqMan® assays with some additional genotype data for the IVS1 C23A SNP (15 subjects) derived from DNA sequencing of IVS1. The three primary ethnic groups represented were Caucasian (40%), Hispanic (28%), and African-American (21%). Approximately 40% were male, and there were 228 opioid-dependent cases and 152 control subjects. Details of demographics of these study subjects can be found in Paper IV.

Genotype and allele distributions for the three SNPs studied in the three primary ethnic/cultural groups are in Table 5. All SNPs in all groups were in Hardy-Weinberg equilibrium. There were significant differences in genotype and allele frequencies among the three ethnic/cultural groups. For the IVS1 C28A SNP, genotypes: $\chi^2_{(4)}=23.99$, $p<0.0001$; alleles: $\chi^2_{(2)}=30.05$, $p<0.000001$. For the C81T SNP, genotypes: $p<0.001$, Fisher-Freeman-Halton test; alleles: $\chi^2_{(2)}=27.72$, $p<0.000001$. For the C808Δ SNP genotypes: $\chi^2_{(4)}=40.49$, $p<0.000001$; alleles: $\chi^2_{(2)}=47.24$, <0.000001. These differences are further confirmed by the fact that the 95% confidence intervals for the calculated allele frequencies in Caucasian and African-American individuals did not overlap for any of the SNPs (see Paper IV). These analyses establish the need for stratification of data by ethnic/cultural group when comparing genotype or allele frequencies of cases and controls.

In Caucasian subjects, a significant association of two SNPs, the IVS1 C28A and C808Δ polymorphisms with opiate addiction was found for genotypes with analysis performed using an autosomal recessive model (IVS1 C28A: Odds Ratio=2.85, $\chi^2_{(1)}=3.87$, $p=0.049$; C808Δ: Odds Ratio=2.20, $\chi^2_{(1)}=4.55$, $p=0.033$). No differences between cases and controls in the other ethnic groups at a significance level of $p<0.05$ were found. Also, in the African-American subjects, evidence for an association of genotypes (but not alleles) of the C81T SNP and opiate addiction was detected assuming an autosomal recessive mode of inheritance at a borderline significance level (CC vs. CT+TT, OR=2.49; $\chi^2_{(1)}=3.78$, $p=0.052$).

4.3.5 Commentary

Taken together, the findings reported in Papers III and IV support the conclusion that the preproenkephalin gene is associated with opiate addiction in Caucasians, and possibly in other ethnic/cultural groups. A significant association of opioid-dependence with the preproenkephalin gene was reported in the study by Comings and colleagues (1999), although in contrast with that earlier study, our findings suggest that individuals carrying short alleles (rather than longer alleles) may be at increased risk for development of opiate addiction. The conclusions of our study using the $(CA)_n$ repeat as a marker are further supported by the findings described in Paper IV, in which SNPs of the preproenkephalin gene were found to be associated with opiate addiction in Caucasians. The failure to identify a positive

Table 5. Three common SNPs of the enkephalin gene: IVS1 C28A, C81T, C808Δ

IVS1 C28A

Ethnicity	Category	CC	CA	AA	Total		C	A	Total
Caucasian	Control	15 (0.22)	27 (0.41)	25 (0.37)	67		57 (0.43)	77 (0.57)	134
	Opioid	25 (0.30)	40 (0.48)	19 (0.23)	84		90 (0.54)	78 (0.46)	168
Hispanic	Control	15 (0.56)	9 (0.33)	3 (0.11)	27		39 (0.72)	15 (0.28)	54
	Opioid	37 (0.46)	37 (0.46)	6 (0.08)	80		111 (0.69)	49 (0.31)	160
African-American	Control	21 (0.66)	9 (0.28)	2 (0.06)	32		51 (0.80)	13 (0.20)	64
	Opioid	26 (0.54)	17 (0.35)	5 (0.10)	48		69 (0.72)	27 (0.28)	96

C81T

Ethnicity	Category	CC	CT	TT	Total		C	T	Total
Caucasian	Control	63 (0.94)	4 (0.06)	0 (0.00)	67		130 (0.97)	4 (0.03)	134
	Opioid	82 (0.98)	2 (0.02)	0 (0.00)	84		166 (0.99)	2 (0.01)	168
Hispanic	Control	21 (0.78)	6 (0.22)	0 (0.00)	27		48 (0.89)	6 (0.11)	54
	Opioid	63 (0.79)	16 (0.20)	1 (0.01)	80		142 (0.89)	18 (0.11)	160
African-American	Control	15 (0.47)	16 (0.50)	1 (0.03)	32		46 (0.72)	18 (0.28)	64
	Opioid	33 (0.69)	13 (0.27)	2 (0.04)	48		79 (0.82)	17 (0.18)	96

C808Δ

Ethnicity	Category	CC	C Δ	Δ Δ	Total		C	Δ	Total
Caucasian	Control	15 (0.22)	28 (0.42)	24 (0.36)	67		58 (0.43)	76 (0.57)	134
	Opioid	26 (0.31)	41 (0.49)	17 (0.20)	84		93 (0.55)	75 (0.45)	168
Hispanic	Control	16 (0.59)	11 (0.41)	0 (0.00)	27		43 (0.80)	11 (0.20)	54
	Opioid	39 (0.49)	37 (0.46)	4 (0.05)	80		115 (0.72)	45 (0.28)	160
African-American	Control	25 (0.78)	7 (0.22)	0 (0.00)	32		57 (0.89)	7 (0.11)	64
	Opioid	39 (0.81)	8 (0.17)	1 (0.02)	48		86 (0.90)	10 (0.10)	96

Genotype and allele distributions are shown for opioid-dependent cases and controls with no history of drug or alcohol abuse or dependence. Data are stratified by ethnicity. Genotype and allele frequencies are shown in parentheses. All SNPs in cases and controls in each ethnic category were in Hardy-Weinberg equilibrium. Significant differences in genotype and allele frequencies among ethnicities for each of the SNPs were found (for details of statistical analysis see Section 4.1.6, and Paper IV). In Caucasian subjects, a significant association of two SNPs, the IVS1 C28A and C808Δ polymorphisms with opiate addiction was found for genotypes with analysis performed using an autosomal recessive model (IVS1 C28A: Odds Ratio=2.85, $\chi^2_{(1)}$=3.87, p=0.049; C808Δ: Odds Ratio=2.20, $\chi^2_{(1)}$=4.55, p=0.033). No differences between cases and controls in the other ethnic groups at a significance level of $p<0.05$ were found.

association of the C81T SNP in the Caucasian group may simply reflect the lower frequency of this SNP in that population and, therefore, the lack of power to detect an association.

In addition to an association with opiate addiction, we found significant differences in genotype and/or allele frequencies among ethnic/cultural groups we studied. This finding is also in accord with previous reports of both the $(CA)_n$ polymorphism and the SNPs (Weber and May, 1990; Chan et al., 1994; Mikesell et al., 1996; 1997; Comings et al., 1999). For example, in our study, 13 and 14 repeat alleles accounted for 96% of all alleles. Previous reports of frequencies of these two alleles in Caucasian subjects from unrelated *Centre d'Etude de Polymorphisme Humain* individuals (Weber and May, 1990), from Byelorussia (Chan et al., 1994), from Indianapolis (Chan et al., 1994), and from Central California (Comings et al., 1999), were 96%, 97%, 100%, and 99% respectively. In the African-American subjects in our study, these alleles constituted 73% of all alleles, similar to the previous report of 77% in the African-American population (Chan et al., 1994).

5. SUMMARY AND CONCLUSIONS

The endogenous opioid system is central to numerous aspects of physiology, neurobiology, and behavior. In particular, it is the primary mediator of the effects of opiate and opioid drugs and has a modulatory role in responses to cocaine, alcohol and other addictive substances, which makes this system an important focus for studies of specific addictions. The papers presented herein describe our investigation of several molecular features of the gene and mRNA of one of the endogenous opioid system components, preproenkephalin, which is the precursor of the enkephalin neuropeptides. Enkephalins have long been known to play a role in pain perception. More recently, increasing evidence suggests that enkephalins are also important for aspects of specific addictions.

We studied this important neurotransmitter through a range of approaches from "the test tube to the clinic," including basic molecular biology, animal modeling of neurochemical responses to cocaine administration, and human molecular genetic analyses of opioid addiction. In each case the preproenkephalin gene or its mRNA was the central focus.

The guinea pig was selected as an animal model because this species has a well-developed kappa opioid system, as do higher primates (including humans), and unlike the rat, which has a lower overall abundance of kappa opioid receptors that are predominantly of a lower affinity subtype. The endogenous kappa peptide ligand dynorphin A(1-17) attenuates cocaine-induced dopamine release into the nucleus accumbens, a brain region critical for reward; rapid increases in dopamine are believed to be important, if not central, to cocaine reward, which contributes to human cocaine addiction. The guinea pig offers, therefore, an attractive model for studies of cocaine effects on molecular neurochemistry.

Prior to the study of cocaine-induced alterations in preproenkephalin mRNA in the guinea pig brain, appropriate molecular tools were required. For the ribonuclease protection assay used in these studies, long species-specific probes give the most specific and sensitive results. We had previously cloned the guinea pig preproenkephalin gene and, in that work, several unusual features of the mRNA structure were discovered. In Paper I of this thesis, we report on our studies to confirm and further elucidate the mRNA structure predicted from that earlier work. Paper I describes the construction of a cDNA library from mRNA isolated from the guinea pig brain and pituitary. This library was screened by hybridization to fragments of the cloned preproenkephalin and preprodynorphin genes. Several clones for each neuropeptide precursor were isolated and sequenced, confirming the mRNA structure of these neuropeptides. We also identified clones with unexpected features, in particular, a clone for the preproenkephalin mRNA that initiated within the first intron (intron A) of the gene, and a clone for preprodynorphin that contained approximately 200 additional nucleotides at its 5' end, compared to the previously-predicted sequence. We further studied mRNA for each of these neuropeptide precursors using ribonuclease protection transcript mapping. We demonstrated that no appreciable intron A sequences are found in the guinea pig brain and pituitary,

which suggests that the clone containing these sequences was most likely derived from nuclear pre-spliced mRNA. We also observed a small amount of transcription initiation of the preproenkephalin gene at the TATA sequence in addition to the primary transcription initiation site located 30 nucleotides downstream from the transcription initiation signal. The ribonuclease protection studies also confirmed the evidence from cDNA cloning that multiple transcription initiation sites exist for the preprodynorphin gene: in addition to the primary site located approximately 30 nucleotides downstream to the TATA signal, transcription is also initiated at several upstream sites located between 160 and 190 nucleotides 5' with respect to the primary transcription initiation site.

In these studies we did not experimentally investigate the functional significance of these longer mRNAs. However, differences in the 5' untranslated region can have a large impact on translational efficiency and can also affect rates of mRNA degradation. With the cDNA clones now in hand, these present studies provide the basis for future investigations of preproenkephalin and preprodynorphin mRNA structure and function relationships, including those of translational regulation and mechanisms of transcription initiation for these important neuropeptides.

In some studies, cocaine administration in the rat has been shown to alter (generally increase) preproenkephalin mRNA levels in striatal brain regions. However, in other studies in this species, no changes have been found. Prior to the investigations reported in Paper II, this question had not been addressed in the guinea pig. We used the "binge" paradigm of cocaine administration, which is designed to mimic the pattern and timing of self-administration common to human cocaine abusers. Two studies were performed and the data from the two studies combined to provide greater statistical power. Following seven, but not two, days of "binge" cocaine administration, we found differential alterations in preproenkephalin mRNA levels in selected brain regions, with increases in the frontal cortex, amygdala, and hippocampus and decreases in the nucleus accumbens and hypothalamus. These findings were striking for several reasons. In the rat, most studies in which cocaine-induced alterations have been observed have noted an increase. The findings of a decrease in mRNA levels in the guinea pig nucleus accumbens are particularly interesting in light of a human postmortem study and a non-human primate study in which decreases in the nucleus accumbens were reported. These findings suggest that the guinea pig may be a good model for human cocaine-induced regulation in this brain region. The model may not be perfect, however, since the primate and human study also found decreases in the caudate putamen, which were not observed in the guinea pig. Additionally, cocaine-induced increases in the frontal cortex, amygdala and hippocampus have not been reported before (except for increases in hippocampal preproenkephalin mRNA by cocaine-induced seizures); the finding of a decrease in the hypothalamus is also novel. The question of a mechanism for the observed differential changes in specific brain regions is one of interest, and this species could serve as a useful model for neurobiological and molecular mechanisms of differential gene regulation of this peptide precursor.

In the final set of investigations of the preproenkephalin gene and its potential importance for addictions, we turned to human molecular genetic studies of opiate addiction. In the studies described in Papers III and IV, we investigated polymorphisms at the preproenkephalin gene in case-controlled studies of opiate addiction. As with any other complex disorder, precise and thorough phenotyping and endophenotyping are expected to be critical. Subjects for study were drawn (using rigorous inclusion and exclusion criteria) from a large ongoing study of the genetics of addictions conducted at The Rockefeller University in New York City. All subjects were thoroughly characterized by highly-trained clinical researchers. Former heroin addicts were patients in methadone maintenance treatment, which has stringent requirements for entry, thus insuring that they were severely affected by this disease. Control subjects had no history of drug or alcohol abuse or dependence. In Paper III, we analyzed a microsatellite marker in the 3' flanking sequence of the preproenkephalin gene, and in Paper IV, in a larger number of subjects, three common single nucleotide polymorphisms in the gene.

In these studies two findings were made. First, significant differences in genotype and allele frequencies existed among the ethnic groups studied. This result is not surprising, since population phenomena, including migration, genetic drift in isolated populations, and admixture are all expected to produce differences in allele frequencies among individuals of different geographic origin and ethnic heritage. These findings underscore the need to accurately assess ancestry and ethnic/cultural affiliation of study subjects in case-control studies and to match cases and controls as carefully as possible.

The second and most important finding made in these studies was that both the microsatellite marker and two of the three single nucleotide polymorphisms were significantly associated with heroin addiction in Caucasians. This was not observed in the other ethnic/cultural groups studied (African-Americans and Hispanics), although a trend toward a significant association was observed for one single nucleotide polymorphism in African-Americans. The positive findings in the Caucasian group and not the others may be due to several reasons. First, the sample size of the non-Caucasian subject groups was smaller, thus resulting in lower power to detect an association. Also, African-Americans and American Hispanics, due to patterns of historical migration, are expected to have had a greater degree of population admixture, which could obscure a positive finding. Finally, the genetic context in which a particular allelic variant occurs may be important for expression of the final phenotype. We know that allelic forms of genes vary widely in different populations. An allelic variant of a gene that influences a particular trait in one genetic setting may be offset by other genetic variants in other populations. The enkephalin alleles that influence risk for the development of opiate addiction in Caucasians may be specific for that population.

It is hoped that the studies described herein contribute to a further understanding of the basic neurobiology of the endogenous opioid system and its relationship to cocaine and opiate addictions that will ultimately lead to better treatment of

individuals with these devastating diseases. In particular, the findings of evidence, in two separate studies, of an association of heroin addiction with the preproenkephalin gene, suggest that further studies of this question are warranted. A genetic association is not proof that the gene is etiologically involved in a disease. Indeed, the preproenkephalin gene may simply be linked to the disease-influencing polymorphism. Replication of these findings in different populations will be important. Further genetic and molecular biological studies will be necessary to identify the specific functional alleles responsible for increased risk or protection from heroin addiction. Ultimately, clinical research studies must be performed to bring these findings into useful practice for the betterment of humankind.

6. ACKNOWLEDGEMENTS

First and foremost, I wish to acknowledge Professor Fred Nyberg. I thank him for giving me the opportunity to study at Uppsala Universitet and for many stimulating scientific discussions. I especially thank him for making Sweden my second home.

No lesser thanks and acknowledgment is due Professor Mary Jeanne Kreek. She has been a mentor for me in the theoretical and practical aspects of biomedical science and research ethics. Her energy and dedication are an inspiration.

I wish to acknowledge my mother and father Gail and Rolfe LaForge. They have waited, very patiently, a very, very long time for this. Perhaps they knew it would eventually come about, but without their continuous support and love, and their faith in me, from the beginning to the last, I could not have made it through.

I wish also to acknowledge the memory of my grandfather, Herbert McLean Evans, a man of brilliance, passion, and love of life; a man of letters and science. His memory and example have inspired me in all my endeavors.

I wish to thank my siblings and their families for their continued love and support: Eric, Carolyn, Matthew and Lindsey; Mark, Marie, Nicholas, Justin and Kristen, Liat and Peggy, and Eric. I also acknowledge the memory of my departed brother Frank.

I acknowledge and thank my fellow students at Uppsala who introduced me to Swedish and university customs and have helped me throughout my graduate years. In particular, I thank Erika Roman, who induced me to join with her in the Göteborg Nation, and took care of many things for me that I could not have managed myself. Anna Kindlundh also helped me in many ways particularly by her work to arrange matters pertaining to my thesis. Mathias Hallberg has been a great friend and colleague since we met years ago. I would also like to thank Ingrid Nylander for her advice, help, and friendship.

I have also learned to always rely on Agneta Bergström, who knows everything and can accomplish anything in no time at all.

I would particularly like to thank three people who helped me produce this thesis: Susan Russo, who proofread many versions, Magnus Jansson, who turned my words and graphs into this book, and Sue Ann Fung-Ho, for help with the figures.

I would like to offer special thanks to Gudrun Nyberg. Her kindness and hospitality have always made me feel welcome in Sweden.

I thank and acknowledge my co-authors and many colleagues and friends at The Rockefeller University and elsewhere in New York, including Ann Ho, Harry Harper, Vadim Yuferov, Stefan Schlussman, Yan Zhou, Jurg Ott, Suzanne Leal, Gavin Bart, Elizabeth Khuri, Aaron Wells, Kathy Bell, Dorothy Melia, Brian Reed, Alexis Bailey, Yong Zhang, Adena Svingos, Scott Kellogg, Charles Lilly, Elizabeth Ducat, Dmitri Proudnikov, Eduardo Butelman, David Nielsen, Tessa Cigler, Sagar Kapadia, Jonathan Ball, Dave Fussell and Laura Nuñez.

I also thank and acknowledge a few special friends in science outside the University of Uppsala and The Rockefeller University, including Thomas Kroslak, Johan Franck, Yasmin Hurd, Eija Kalso, Brigitte Kieffer, Chris Evans, Niall Murphy, Eric and Irene Simon, Mikko and Piia Airavaara, and Jukka Kallijärvi.

I especially acknowledge Leif Grönbladh, Ola Thulin, and Markus Heilig.

Outside the world of science there are many to whom I owe acknowledgment and gratitude, friends who have indulged me in my absence during the time I was preparing this thesis: Steve Messner, Jennifer, Kosmo, Jack and Charley Houlton-Vinyl, Tod Aubrey, Patricio Vallejo, Dianne Pohlsander, David and Tracy Finn, Pat Kennedy and Jean-Paul Caulin, Roman Shvets and Nadia, Maria and Razvan State, Ann Hellandsjö, and Sami Ratia.

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Editor: The Dean of the Faculty of Pharmacy

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ISSN 0282-7484
ISBN 91-554-5889-0