Mechanisms of Prodynorphin Gene Dysregulation in the Brain of Human Alcoholics

MALIK MUMTAZ TAQI
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Abstract


The endogenous opioid system (EOS) including dynorphin opioid peptides and κ-opioid receptor (KOR) plays a critical role in alcohol dependence. Aims of the thesis were to evaluate whether the EOS undergoes molecular adaptations in brain areas involved in cognitive control of addiction in human alcohol dependent subjects, and to analyze the impact of genetic and epigenetic factors on these adaptive changes. The main findings were that (1) the dynorphin/KOR system including PDYN mRNA and dynorphins in the dorsolateral prefrontal cortex (dl-PFC), dynorphins in the hippocampus, and KOR mRNA in the orbitofrontal cortex (OFC), is upregulated in human alcoholics. No other significant changes in the EOS were found. (2) Three PDYN single nucleotide polymorphisms (SNPs), which show the most significant association with alcohol dependence, form CpG sites that are methylated in human brain at different levels. Methylation of the C, non-risk variant of the 3'-untranslated region (3'-UTR) SNP (rs2235749; C>T) was increased in dl-PFC and positively correlated with dynorphins. The DNA-binding factor that differentially targeted the T, risk allele and methylated and unmethylated C allele of this SNP was identified in human brain. We hypothesize that influences of the genetic, epigenetic and environmental factors may be integrated through alterations in methylation of the PDYN 3'-UTR CpG/SNP and, as a consequence, affect PDYN transcription and vulnerability to develop alcohol dependence. (3) The principal component analysis suggested that PDYN expression in the dl-PFC may be related to alcoholism, while in the hippocampus may depend on the genotype of the PDYN promoter SNP (rs1997794; T>C). The T, low risk allele of this SNP resides within non-canonical AP-1-binding element and may be targeted by JUND and FOSB proteins, the dominant AP-1 constituents in the human brain. The T to C transition abrogated AP-1 binding. The impact of genetic variations on PDYN transcription may be relevant for diverse adaptive responses of this gene to alcohol. (4) It was proposed that PDYN transcription may be regulated by intragenic DNA regulatory elements controlling the DNA-protein interactions through formation of non-canonical DNA secondary structures. The dynorphin-encoding sequence in PDYN was found to have potential to form such DNA structure in vitro, and this formation was affected by CpG methylation in this region. This methylation sensitive non-canonical DNA structure formation may be involved in regulation of initiation of PDYN transcription from alternative start sites located within this region, or in splicing of non-canonical mRNA.

In conclusion, the dynorphin/KOR system has been identified as the site of robust adaptive changes associated with alcohol dependence in the areas of human brain involved in cognitive control of addiction. Regulation of PDYN was found to be brain area specific, apparently affected by the genetic and epigenetic factors, and possibly dependent on the internal properties of the gene such as its ability to form non-canonical DNA secondary structures.

Malik Muntaz Taqi, Uppsala University, Department of Pharmaceutical Biosciences, Box 591, SE-751 24 Uppsala, Sweden.

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To my parents
List of papers

This thesis is based on the following papers, which are referred in the text by their Roman numerals:


All papers are written by first author in association with co-authors. In paper II and III first authors are responsible for all analysis and major part in laboratory work. In paper I, I have been involved in biochemical analysis. In paper IV; I performed PAGE experiments, Sebastian Wärmländer conducted NMR and CD spectra while Roman Zubarev did mass spectroscopy for oligonucleotides.

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Papers not included in this thesis


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Abbreviations

**ADH 1B**
alcohol dehydrogenase 1B

**ALDH 2**
aldehyde dehydrogenase 2

**AP-1**
activator protein 1

**BDNF**
brain-derived neurotrophic factor

**BSA**
bovine serum albumin

**cAMP**
cyclic adenosine monophosphate

**CD**
circular dichroism

**cDNA**
complementary DNA

**ChIP**
chromatin immunoprecipitation

**CHRM 2**
cholinergic receptor muscarinic 2

**COGA**
Collaborative Study on the Genetics of Alcoholism

**C-PD**
*PDYN* C allele

**Cq**
amplification cycles

**CREB**
cAMP response element-binding protein

**C_T**
critical threshold

**CTCF**
cccctc-binding factor

**DA**
dopamine

**dl-PFC**
dorsolateral prefrontal cortex

**DNase I**
deoxyribonuclease I

**DNMT**
DNA methyltransferase

**DOR**
δ-opioid receptors

**DRE**
downstream regulatory element

**DREAM**
DRE antagonist modulator

**DTT**
dithiothreitol

**EMSA**
electromobility shift assay

**EOS**
endogenous opioid system

**FL**
full-length

**GABRA 2**
gamma-amino butyric acid receptor alpha 2

**GABRG 3**
gamma-amino butyric acid receptor alpha 3

**gDNA**
genomic DNA

**HMG**
high mobility group

**KOR**
k-opioid receptors

**MC**
motor cortex

**MOR**
μ-opioid receptors

**NAc**
nucleus accumbens

**ncRNAs**
non coding RNAs
NE neoendorphin
NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells
NMR nuclear magnetic resonance
nor-BNI norbinaltorphimine
OPRD 1 opioid receptor delta 1
OFC orbitofrontal cortex
OPRK 1 opioid receptor kappa 1
OPRM 1 opioid receptor mu 1
PAGE polyacrylamide gel electrophoresis
PCA principal component analysis
PENK proenkephalin
PDYN prodynorphin
PET positron emission tomography
POMC proopiomelanocortin
qRT-PCR quantitative reverse transcriptase-PCR
RFB rat fetal brain
RQI RNA Quality Indicator
SCA 23 spinocerebellar ataxia 23
SD standard deviation
SDS sodium dodecylsulfate
SEM standard error of mean
SN substantia nigra
SNP single nucleotide polymorphism
mSNP methylation-associated SNP
ssDNA single stranded DNA
Ta-BF T allele DNA binding factor
TAS2R 16 taste receptor type 2, member 16
T-PD PDYN T allele
TSS transcription start site
USF upstream stimulating factor
VNTR variable number tandem repeat
YY1 yin-yang 1
5mC 5-methylcytosine
5hmC 5-hydromethylcytosine
1. Introduction

The opioid peptide precursors are encoded by three genes: proopiomelanocortin (POMC), proenkephalin (PENK), and prodynorphin (PDYN) that give rise to multiple opioid peptides including β-endorphin, Met- and Leu-enkephalins, dynorphins and neoendorphins (NE). The opioid peptides act through G-protein-coupled μ-, δ- and κ-opioid receptors (MOR, DOR and KOR). Dynorphins bind to the KOR with higher affinity than to the MOR and DOR, and therefore may function as endogenous ligands for the KOR in the central nervous system (1-3).

Several lines of evidence demonstrate an important role of the endogenous opioid system (EOS) in alcohol dependence. Molecular changes in the EOS induced by addictive drugs and alcohol may underlie neuroplastic adaptations relevant for transition to addiction (4-6). The EOS regulates reward and is involved in cognitive control of substance addiction (6-11). Pharmacological and genetic manipulations with the opioid receptors alter alcohol consumption in animals (4,5,12-18). In clinics, the non-selective opioid antagonist naltrexone reduces alcohol drinking and relapse rates in subgroups of alcoholics (11,19-22). Polymorphisms in the MOR-encoding gene, OPRM1, KOR-encoding gene, OPRK1, and the PDYN gene are associated with alcoholism (23-28).

Besides regulation of neurotransmission in reward circuits, the EOS may be involved in specific cognitive processes relevant for control of addictive behaviors including craving, decision-making and impulsivity (6-11). In alcoholics, effects of naltrexone in response to alcohol cues, and during decision-making is associated with the orbitofrontal cortex (OFC) (7), while MOR binding in the dorsolateral prefrontal cortex (dl-PFC) is functionally related to alcohol craving (8). Processes relevant for motivated behaviors, pursuing of reward, and risk taking, including the development of substance use disorders are related to functions of the EOS (9). Several impulse-control disorders including pathological gambling and binge eating may be relieved by naltrexone suggesting a role of endogenous opioids in impulsivity [reviewed in (29,30)]. Thus, effects of alcohol on neurocognitive processes controlling drug/alcohol seeking and taking behaviors may be mediated in part through the EOS, and that molecular dysregulation of this system may contribute to disruption of these processes which may ultimately lead to the alcohol dependence.
1.1. The EOS in alcohol dependence

1.1.1. Animal studies

Analysis of neurochemical adaptation suggests that the EOS has an important role in the control of alcohol drinking behaviors. It has been demonstrated that acute alcohol administration increases POMC and PENK mRNA and peptides in discrete brain regions in rodents (31-34). In vitro studies demonstrated that alcohol stimulates β-endorphin secretion from the hypothalamus (32,35-37). Acute alcohol exposure experiments support the notion that opioid peptides may mediate the reinforcing effects of alcohol (38). In contrast, chronic alcohol administration decreases POMC gene expression (39), desensitizes β-endorphin release (40-42) and decreases MOR number and affinity (43). Thus, downregulation of the EOS by chronic alcohol administration may contribute to withdrawal and abstinence state, and promote alcohol consumption via negative reinforcement (44,45).

Evidence for a role of the EOS in the regulation of alcohol consumption has been obtained in pharmacological experiments with opioid antagonists, and genetically modified animals. Naloxone, naltrexone and nalmefene, which are nonselective opioid antagonists, have been shown to decrease alcohol consumption in rodents (12,13,46-52). Antagonists which are selective for the MOR and DOR decrease alcohol self-administration in rodents under several experimental conditions (53-55). The use of selective opioid antagonists allowed to establish that both the β-endorphin and enkephalin systems are involved in the maintenance of alcohol consumption (56).

Mice with targeted deletions in the EOS genes have been extensively used in analysis of alcohol consumption behaviors. MOR knockout mice do not self-administer alcohol (57), whereas DOR knockout mice showed a greater preference for alcohol and consumed more alcohol than their wild type counterparts (58). The elevated alcohol consumption by DOR knockout mice could be due to a compensatory increase in MOR activity in the absence of the DOR (59). Mice with a targeted disruption of the POMC gene, therefore lacking β-endorphin peptide, ligand for MOR, also drink less alcohol than their wild-type littermates (60,61).

1.1.2. The dynorphin/KOR system in drug addiction

The role of the dynorphin/KOR system in alcohol dependence could be better understood if in parallel we consider the adaptations in this system associated with drug addiction. Both acute and repeated psychostimulant administration increase Pdyn expression in reward-related regions in animal models, similar to what is observed in human (62,63). In rodents, administration of a single or repeated injection of cocaine or amphetamine produces robust elevations in Pdyn mRNA (64-66) and dynorphins immunoreactivity (62) in
the nucleus accumbens (NAc) and/or dorsal striatum. Cocaine self-administration increases dorsolateral and dorsomedial striatal $Pdyn$ expression to a similar extent in yoked self-administering rats (63). In primates, both acute and chronic high dose cocaine self-administration increase $Pdyn$ expression in the rostral caudate and putamen (67). This suggests that $Pdyn$ expression elevations are due to pharmacological effects of cocaine rather than drug taking behaviors per se.

Acute nicotine injection produces a dose-dependent increase in $Pdyn$ mRNA and dynorphins immunoreactivity in the striatum (68). $Pdyn$ expression elevations are long-lasting in striatum whereas peptide immunoreactivity is transiently increased. Like other substances of abuse, systemic nicotine administration elevates extracellular NAc dopamine (DA) levels to produce its rewarding effects (69) while KOR activation in NAc decreases DA elevated levels (4). Moreover, the ability of systemic U50,488 to decrease NAc DA overflow is exacerbated in nicotine-dependent adult rats relative to controls. These findings suggest that nicotine exposure produce changes in the dynorphin/KOR system which may modulate DA neurotransmission that may lead to enhanced negative emotional state and reduced nicotine rewarding effects. Alterations in KOR systems produced by chronic nicotine exposure play an important role in mediating nicotine withdrawal because nor-BNI pretreatment blocks somatic signs of spontaneous withdrawal in nicotine-dependent adult rats. Conversely, U50,488 administration potentiates somatic withdrawal signs. Collectively, these results suggest that the dynorphin/KOR system alterations produced by chronic nicotine enhance KOR signaling during nicotine withdrawal that may result in dysphoria and physical signs of withdrawal.

Chronic morphine administration increases dynorphin A and B immunoreactivity in the NAc, hypothalamus, and hippocampus of rats (70-73). Chronic morphine also increases $Pdyn$ expression in the locus coeruleus (74), which is interesting because this brain area has been implicated in mediating several behavioral effects of opiates (75). Both systemic and intra-substantia nigra (SN) morphine administration increases SN extracellular dynorphin B levels (76). Given that a large proportion of dynorphin inputs to the SN arise from the dorsal striatum (77), activation of MOR in the SN may disinhibit DA neurons (78,79) resulting in positive modulation of the direct pathway of the basal ganglia, which would enhance SN dynorphin levels.

The dynorphin/KOR system has been implicated in mediating the enhanced sensitivity to the reinforcing effects of opiates and other drugs of abuse in rodents. KOR agonists decrease opiate self administration (80,81), an effect associated with decreased NAc DA release (81). In caudal striatum dynorphin A- and B-immunoreactivity is increased in heroin-experienced rats when expecting heroin as compared to saline treated animals (82). This suggests that the dynorphin/KOR system may be altered by expectancy of drug availability rather than by direct opiate exposure. KOR activation by
endogenous or exogenous ligands decreases the severity of opiate withdrawal. Nor-BNI treatment increases somatic signs of opiate withdrawal and the conditioned effects of morphine withdrawal (83). The somatic opioid withdrawal signs are at least partly mediated by an increased release of nor-epinephrine in the brain (84,85). KOR agonists may block opioid withdrawal by decreasing the opioid withdrawal mediated release of nor-epinephrine. The KOR is coupled to an N-type calcium channel and activation of the KOR inhibits N-type calcium currents (86). Drugs that block the N-type calcium channels have been shown to block nor-epinephrine release from sympathetic neurons (87). This is in line with the observation that dynorphin A (1-13) and the KOR agonist ethylketocyclazocine decrease the potassium evoked release of \[^{3}H\] noradrenaline from guinea pig cortical slices (88). In addition, the KOR agonist U50,488 has been shown to inhibit nor-epinephrine release from guinea pig brain slices (89). Future studies are needed to investigate if the KOR agonists prevent nor-epinephrine release in the brain associated with opioid withdrawal (90,91). Moreover, naloxone-precipitated morphine withdrawal decreases DA overflow in the NAc; an effect that is more robust in nor-BNI treated rats relative to vehicle controls (83), suggesting that mesolimbic DA responses may be associated with enhanced expression of somatic and conditioned withdrawal.

This suggests that \(P_{dyn}\) mRNA and its peptides levels are elevated by both acute and chronic exposure to drugs of abuse. Activation of the dynorphin/KOR system may counteract the positive reinforcing effects of MOR which may lead to drug craving and may serve as critical factor in the development and maintenance of drug dependence.

1.1.3. The dynorphin/KOR system in alcohol dependence

The hypothesis that dynorphin opioid peptides and their cognate KOR play a role in alcohol dependence is supported by several studies (4,5,18). Chronic alcohol intake has been reported to upregulate dynorphin levels in the brain regions associated with motivation and reward in rats (92). Dynorphin levels differ in strains of rats exhibiting high and low alcohol preference; alcohol preferring rats have lower levels of dynorphin in the NAc (93). Increased dynorphin levels may enhance negative reinforcing effects of alcohol which may lead to reduced alcohol preference.

Pharmacological experiments demonstrated that alcohol increases the MOR mediated positive reinforcing effect (94), which may lead to enhanced alcohol intake. Conversely, the increased dynorphin/KOR system activity may induce negative reinforcing effects and inhibits high alcohol consumption (56). The blockade of the KOR may enhance alcohol self-administration while KOR agonists have been found to attenuate alcohol consumption possibly through modulating the DA release (95-97). Selective KOR agonists U50,488 and bremazocine attenuate alcohol intake in rats (98,99). In mice,
the acute stimulation of brain-derived neurotrophic factor (BDNF) receptor decreases alcohol intake; while norbinaltorphimine (nor-BNI), a selective KOR antagonist, inhibits BDNF effect on alcohol consumption (100). Additionally, KOR agonist treatment prior to the experiment reduced alcohol preference in rats, an effect that was reversed by the simultaneous administration of the KOR antagonist MR-2266-BS (101).

Recent studies on alcohol dependent animals demonstrate contrary outcome compared to previous studies on the dynorphin/KOR system in non-dependent animals. Intracerebroventricular administration of the selective KOR antagonist nor-BNI has been shown to reduce escalated alcohol self-administration in dependent animals, suggesting that the dynorphin/KOR system functions are altered in alcohol dependence. These alterations may alleviate the negative emotional states associated with alcohol withdrawal and dependence (102). These data suggest a modulatory role of dynorphins over alcohol intake in the dependent state, in which the dynorphin/KOR system functions to increase alcohol intake.

Although studies with KOR deficient mice are limited, few analyses revealed a lower alcohol intake accompanied by a decreased consumption of saccharine in saccharine preference tests in KOR deficient mice (103). Similarly, mice lacking the Pdyn gene showed lower preference for alcohol and consumed lower amounts of alcohol in a two-bottle choice test (104). However, this effect was observed only in female but not male mice, and its interpretation was complicated due to strong reduction of saccharin preference in mice of both sexes, and the absence of differences between wild type and Pdyn knockout mice in several alcohol behavioral tests (loss of righting reflex, acute alcohol withdrawal, alcohol -induced conditioned place preference and conditioned taste aversion).

Altogether, animal experiments suggest that the EOS has an important role in the development of alcohol dependence. The main findings are that MOR modulates the positive reinforcing effects of alcohol (58,94,105,106) while these effects are counteracted by KOR activation (107). Disbalance of these two opposing opioid systems by chronic alcohol intake may represent a critical factor in the development and maintenance of alcohol dependence.

1.2. The EOS in human alcoholics

1.2.1. Clinical studies

Naltrexone, a nonselective opioid antagonist was approved by American Food and Drug Administration in 1994 for the treatment of alcoholism. Two initial trials demonstrated that naltrexone treatment results in fewer drinking days and lower rates of relapse (19,20) therefore this compound was advanced as one of the more promising pharmacological interventions for
alcohol dependence (108). Naltrexone has been shown to reduce either the priming or rewarding effect of alcohol (109-112).

More recent trials have generally demonstrated beneficial effects of naltrexone on heavy drinking rates, particularly among those who are compliant with the medication (113-115). Naltrexone has also been found to reduce drinks per drinking day (19,113), to reduce craving (20) and to enhance resistance (reduce urge and impulse) to drink (113,116). Treatment response to naltrexone, however, is not uniform; while some patients seem to benefit from this pharmacotherapy, others do not (117,118). It is unclear what subtypes of the opioid receptors are targeted by naltrexone in the brain of alcoholics, and actions of which endogenous opioid peptides are blocked by this antagonist.

1.2.2. Imaging studies

Neuroimaging studies have enhanced our understanding about the adaptations in the EOS relevant for alcohol reinforcement and addictive behavior. A link between the EOS and alcohol craving has been demonstrated using positron emission tomography (PET) with $^{11}$C-labeled carfentanil, a radioligand that binds specifically and reversibly to the MOR (119).

In alcohol dependent subjects change in MOR availability has been suggested to be associated with increased alcohol craving and relapse (120). PET study has demonstrated that lower MOR binding potential in dl-PFC, anterior frontal cortex and parietal cortex was correlated with multiple behavioral measures, including high alcohol craving, mood and withdrawal severity in alcoholics undergoing withdrawal (8). These findings suggest a functional relationship between alcohol craving and MOR binding potential in dl-PFC, anterior frontal cortex and parietal cortex of alcoholics undergoing withdrawal. These findings support an important role of the EOS, particularly MOR in alcohol dependence.

Impulsivity, a heterogeneous characteristic consisting of multiple dimensions that include sensation seeking, lack of planning, lack of persistence and urgency has received substantial attention because of its association with risky behaviors. Studies have demonstrated that impulsivity is involved in the development of addiction in humans; opiate addicts are more impulsive than non addicts (121-123). PET study has revealed that high impulsiveness and low deliberation scores were associated with significantly higher regional MOR concentrations and greater stress-induced EOS activation. Effects were evident in the dl-PFC, OFC, anterior cingulate, thalamus, NAc, and basolateral amygdala, regions involved in motivational behaviors and drugs abuse (9). Thus, this study demonstrates that in humans impulsivity and deliberation, behavioral facets relevant to motivational behaviors, the pursuit of reward, and risk taking, including the development of substance abuse are related to the functions of the EOS.
In summary, neuroimaging studies demonstrated that the EOS in dl-PFC, anterior frontal cortex and parietal cortex, OFC, anterior cingulate, thalamus, NAc, and basolateral amygdala, brain areas responsible for reinforcement, decision-making, motivation and impulse control is involved in the addicted behavior. Importantly, alterations in the EOS correlate with craving and relapse behavior (8,9).

1.2.3. Genetics of alcohol dependence

Alcohol dependence is a complex genetic disorder. Both twin and adoption studies demonstrate a strong heritable component involved in the risk for alcoholism (124-128). The lack of pattern of inheritance suggests that multiple genes contribute to the risk for alcoholism. One of the largest datasets that have provided successful results in identifying genes that influence the risk of alcohol dependence is the Collaborative Study on the Genetics of Alcoholism (COGA) (129). Several genes including gamma-amino butyric acid receptor alpha 2 ($GABRA\,2$) and gamma-amino butyric acid receptor gamma 3 ($GABRG\,3$); cholinergic receptor muscarinic 2 ($CHRM\,2$) and taste receptor type 2, member 16 ($TAS2R\,16$) have already been identified by COGA studies as associated with alcohol dependence (130-133). Although no one gene is likely to play an overwhelming role in increasing susceptibility, there are variations in two genes, alcohol dehydrogenase 1B ($ADH\,1B$) and aldehyde dehydrogenase 2 ($ALDH\,2$), that play protective roles by affecting alcohol metabolism. Particular coding variations in these genes are associated with strong (but not complete) protection against alcoholism in the Asian populations in which they are primarily found (134-136).

Mounting evidence has implicated the genetic variations in the EOS coding genes as a central player in the etiology of alcohol dependence. The MOR1 encoding gene, $OPRM\,1$, contains several single nucleotide polymorphisms (SNPs) in exon I. One of these, A118G (rs1799971), has been associated with alcohol dependence in diverse ethnic populations including Japanese, Korean and European descendants (24,137-142). Another study in which 13 SNPs throughout the $OPRM\,1$ gene were examined in case control studies with European-American and Russian populations and found that two haplotype blocks identified in intron 1 and 3 of $OPRM\,1$ gene were associated with alcohol and/or drug dependence (142). A few studies have examined whether genetic variations in delta DOR1 encoding gene, $OPRD\,1$, have association with alcohol dependence. Two coding variants in $OPRD\,1$, G80T (rs1042114) and T921C (rs2234918), are mainly studied for their associated with alcohol and/or drug dependence but there was no evidence of association with the two polymorphisms in alcohol-dependent Taiwanese Hans (143), or alcohol-dependent Germans (144). These findings about the association of genetic variations in $OPRD\,1$ with alcohol dependence are in line with the outcome of a family-based comprehensive study in which 219
alcoholics from European-American families were analyzed for associations between alcohol dependence and multiple genetic variations in $OPRM\,1$, $OPRD\,1$, $PENK$ or $POMC$ genes including SNPs and haplotypes. They found no significant association between genetic variations in $OPRM\,1$, $OPRD\,1$, $PENK$ or $POMC$ and alcohol dependence (27). Contradiction in the association of genetic variations in $OPRM\,1$ and alcohol dependence could be explained by the different criteria of subject selection in different studies. Taken together these findings suggest either modest or no support for the idea that variations in $OPRM\,1$, $OPRD\,1$, $PENK$ and $POMC$ are associated with alcohol dependence.

Several lines of evidence propose a central role of genetic variations present in $PDYN$ and $OPRK\,1$ genes in the vulnerability to develop alcohol dependence. A family-based study using sample of 1860 European-American individuals from 219 alcoholic families analyzed associations between alcohol dependence and multiple SNPs in the promoter, exon 4 and 3'-UTR of the $PDYN$ gene (25). The strong association in $PDYN$ was evident for SNPs in the 3'-UTR, exons 3 and 4, as well as SNPs at the 5'-promoter and upstream region. Analyses of haplotypes provided additional evidence for association of $PDYN$ with alcohol dependence. A haplotype block of six SNPs associated with alcohol dependence was found in the 3’-UTR (25). Several of $PDYN$ SNPs associated with alcohol dependence also demonstrate association with cocaine/alcohol codependence (145), opioid dependence (146), or episodic memory in elderly people (147).

The human $PDYN$ gene has 68-bp nucleotide variable number tandem repeat (VNTR) polymorphism in promoter region. Allele and genotype frequencies of these repeats varied in people with different ethnic background, and have been proposed to be associated with cocaine addiction in human (148,149). In African Americans either three or four VNTR repeats are shown to be associated with cocaine dependence and cocaine/alcohol codependence (150,151).

Genetic polymorphisms in $OPRK\,1$ which encodes the KOR are also associated with the risk for alcohol dependence (25,26) and opiate addiction (152,153). Thirteen SNPs were genotyped across $OPRK\,1$ in alcohol-dependent families to analyze $OPRK\,1$ variants associated with increased risk for alcohol dependence. The strongest association in $OPRK\,1$ was for five SNPs in intron 2. Haplotype analyses in $OPRK\,1$ did not yield further support for association for alcohol dependence (25). However a haplotype of eight $OPRK\,1$ SNPs was identified in Americans of Spanish origin with significant difference in frequencies between opiate addicts and controls (153). An independent study revealed an association of the SNP rs1051660 with opiate addiction in a European American population (152). A high frequency 830-bp insertion/deletion was found 1389-bp upstream of the transcription start site of $OPRK\,1$. The presence of an 830-bp insert, rather than its deletion, was associated with alcohol dependence in European-Americans.
Association of variations in two genes coding for both receptor and its ligands with alcohol dependence suggested a critical role of dynorphin-KOR mediated interneuronal communication in this pathological state.

1.2.4. PDYN transcripts

The PDYN gene has a complex pattern of transcription in the human brain where multiple transcription initiation sites that give rise to seven mRNA types with different 5’-ends were detected (154). Canonical form of PDYN mRNA coding the full-length protein (FL1-PDYN mRNA) consists of 3 introns and 4 exons. The second FL form (FL2) with a novel first exon (2’), three new 5’-truncated transcripts, T1-T3, a splice variant, Sp1, in the coding region of exon 4, which contains the dynorphin-encoding sequences, and splice variant, Sp2, which lacks exons 2 and 3 were described.

The FL1-PDYN mRNA showed a classic PDYN mRNA expression pattern, predominantly in limbic-related structures such as the ventral striatum, dorsal striatum patch compartment, accessory basal and cortical amygdala, dentate gyrus of the hippocampus, and entorhinal cortex. In contrast, FL2-mRNA was more limited in its distribution with selective expression predominantly in, for example, the claustrum and supraoptic hypothalamus. Even when present in the same brain structure (e.g. basal nucleus of Meynert and hypothalamus) the two PDYN mRNA forms were differentially expressed in discrete subnuclei (154). In contrast to the human PDYN gene, PDYN transcripts which code for the FL proteins have been identified in other species such as the mouse, rat, guinea pig, and amphibians (154). Thus, PDYN expression in different human brain regions is characterized by high variability.

Human PDYN exon 4, which encodes neuropeptides, has a weak promoter activity that may contribute to the intragenic initiation of transcription of T1 and T2 PDYN mRNA translated into two N-terminally truncated PDYNs with molecular size of 12 and 6 kDa, respectively. T1 and T2 proteins lack a signal peptide and are primarily located in the cell nucleus and cytosol, respectively, suggesting their non-opioid functions (154). Taken together, expression of several PDYN transcripts in various brain areas at different levels suggests a complex brain specific pattern of PDYN gene expression which may define human pathological conditions such as alcohol dependence.

It became apparent that a significant portion of the transcriptome, non-coding RNAs (ncRNAs), has little or no protein-coding capacity. These ncRNAs could be involved in the regulation of the expression of neighboring protein-coding genes possibly through epigenetic mechanisms (155,156). A gene encoding a long ncRNAs, AK090681; 60272 nucleotides long, with 4 non-coding exons and 3 introns, is transcribed from the opposite strand of human PDYN (157). The AK090681 ncRNA may impact the PDYN expression suggesting additional mechanism for PDYN gene regulation.
1.2.5. PDYN regulation

Regulation of PDYN transcription has not been extensively studied in the chromosomal context. Recent technological advances make it feasible to map the genome-wide interactions of transcription factors with their target DNA sequences. One example is the chromatin immunoprecipitation (ChIP) assay, which in combination with either microarray hybridization (ChIP-chip) or sequencing (ChIP-seq) allows to determine the genomic association of DNA binding proteins with their targets and to analyze gene regulation at the genome wide scale. Genomic studies have demonstrated that PDYN gene could be targeted by several transcription factors (Figure 1) including CCCTC-binding factor (CTCF). CTCF contains eleven zinc fingers, binds to a wide range of DNA sequences and has diverse regulatory functions, including transcriptional activation/repression, insulation and imprinting. CTCF may mediate intra- and interchromosomal contacts through the formation of loops between two CTCF-bound insulators (158). The formation of these loops could either activate (159) or repress gene transcription (158,160) by facilitating or inhibiting the enhancer or inhibitor interactions with promoter. Two strong peaks of CTCF bound to its response elements, overlapping with exon 3 and 4 of antisense AK090681 RNA, have been found to frame the PDYN gene in several cell lines (Figure 1). The CTCF peaks located downstream and upstream of PDYN also overlap with strong c-Myc and NRSF/REST peaks respectively (Figure 1). These findings suggest that interactions of these three transcription factors may be critical for the independent PDYN regulation. The PDYN transcription unit may be separated by CTCF-mediated chromatin looping from other genes in this area including AK090681 located on the complementary strand. Genomic studies also demonstrated that upstream stimulating factors 1/2 (USF1/2) and c-Myc (161-164) (Figure 1) could target PDYN.

Several transcription factors including activator protein 1 (AP-1) family protein FosB, cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), downstream regulatory element (DRE) antagonist modulator (DREAM), yin-yang1 (YY1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) have also been implicated in the regulation of PDYN gene in early studies (165-174) (Figure 2). Expression of the human PDYN gene is also regulated by the DREAM, the calcium-binding protein that inhibits gene transcription (168,169). DREAM-deficient mice demonstrate increased Pdyn expression (175). Another transcription factor, CREB has been reported to regulate expression of the Pdyn gene in rodents, and has implication in addiction (165). The rat Pdyn gene contains three consensus CRE sites and one nonconventional CRE site involved in Pdyn expression in vivo and in vitro (166,167). Furthermore, animal studies proposed that the persistent activation of ΔFosB (C-terminus truncated FosB protein), the AP-1 constituent may be a common pathway for addictive dis-
orders, and that *Pdyn* is one of the transcriptional targets for this factor (171). However, no study has demonstrated yet whether CREB and AP-1 with ΔFosB as a constituent may regulate the human *PDYN* gene expression.

**Figure 1.** Image was obtained from The UCSC Genome Browser on Human 2006 (NCBI36/hg18) Assembly. Peaks show the interactions of CTCF, c-Myc and NRSF/REST with the *PDYN* domain in GM12878 and K562 cells. USF1 and USF2 are also shown to interact with *PDYN* gene in cells. Peaks correspond to segments bound by the transcription factors (CTCF, c-Myc, NRSF/REST, USF 1 and USF2) are identified by ChIP-chip or ChIP-seq assay.
A human specific VNTR polymorphism is present in the promoter of human PDYN gene that may regulate its transcription (176,177). AP-1 binding elements in the VNTR have been analyzed in functional and genetic association studies. Cell-specific effects of the number of VNTR (rs35286281) repeats on the transcriptional activity of the PDYN promoter have been reported; in SK-N-SH and H69 cells, three or four repeats led to lower PDYN gene expression than one or two repeats, while the opposite effect was found in HEK293 and NG108-15 cells (177,178).

**Figure 2.** PDYN gene contains several transcription start sites (TSS; 5 are shown) and a VNTR in the 5’- region. Exons/introns structure is shown for FL-1 mRNA. Localization of seven PDYN pathogenic mutations causing neurodegenerative disorder spinocerebellar ataxia 23 (SCA 23) (179) within short segment that include dynorphin A and B segment is shown by small vertical lines. DNase I hypersensitivity peak overlapping with dynorphin A segment is shown. Location for three CpG-SNPs which show high association with alcohol dependence (27) are indicated by arrow (narrow) and SNP number. Putative binding sites for JUND/FOSB, NF-κB, c-Myc/USF2, YY1 and Ta-BF are shown by bold arrow. Transcription factors that presumably interact with CpG-SNPs *in vitro* studies.

Considering a role of persistent adaptations in PDYN expression for the development and maintenance of substance dependence, it is important to understand transcriptional and epigenetic mechanisms of regulation of this gene in the brain. No cellular model for analysis of these processes has been yet developed. In cell lines PDYN was found to be expressed at the negligibly low levels that are substantially lower than those in the brain and cerebellum. This residual expression of PDYN was not responsive to pharmacological treatments or genetic manipulations that are often used for activation or inhibition of other neuronal genes (unpublished observations). Therefore, mechanisms of PDYN regulation should be addressed *in vivo* using a combination of genetic and biochemical approaches.
1.3. Epigenetic mechanisms in alcoholism

Epigenetics is typically defined as the study of heritable changes in gene expression that are not due to changes in DNA sequence (180). Hence, identical DNA sequences with different epigenetic profile can result in differential gene expression in the different tissues/ organs of the individuals. Epigenetic profile seems to be time and tissue specific and can be quite diverse even within a tissue or individual. According to the functional definition, epigenetic changes represent alteration in gene expression that self-perpetuating in absence of the original signal that caused them (181). Environmental factors, including addictive drugs and alcohol, can modulate gene expression by inducing alteration in epigenetic marks such as DNA methylation and histones modifications. Epigenetic changes in the brain have been associated with a range of neurobiological processes including brain development, synaptic plasticity, learning and memory, and pathological changes such as drug addiction (182-184).

1.3.1. DNA methylation

DNA methylation is dynamic epigenetic modification implicated in numerous biological processes including gene transcription. At the same time DNA methylation is thought to be one of the most stable epigenetic modifications. This modification mainly occurs in cytosines that precede guanines, usually referred to as CpGs dinucleotides, to yield 5-methylcytosine (\(5mC\)) (185). Recent analysis of human methylomes demonstrated that in embryonic stem cells a notable proportion (~25%) of methylcytosine residues were not in the CpG context. Non-CpG methylation was enriched within exons and introns (186).

DNA methylation patterns are thought to be established and maintained by three DNA methyl transferases (DNMT). Mammalian DNA methyltransferase enzymes fit into two general classes based on their preferred DNA substrate. The de novo DNMT3a and DNMT3b are mainly responsible for introducing cytosine methylation at previously unmethylated CpG sites, whereas the maintenance DNMT1 copies pre-existing methylation patterns onto the new DNA strand during DNA replication (187-189).

Recently a novel epigenetic DNA modification, 5-hydroxymethylcytosine (\(5hmC\)) has also been identified. \(5hmC\) has been shown to be found predominantly within gene bodies as well as non-promoter regions. \(5hmC\) is highly abundant in the brain, suggesting a role in epigenetic control of neuronal function (190). A recent study has demonstrated that in addition to DNMTs, Tet enzymes may also regulate DNA methylation by modifying \(5mC\) and have been proposed to promote DNA demethylation in multiple ways (191). By converting \(5mC\) to \(5hmC\), Tet enzyme diminishes DNA methylation. Moreover, because \(5hmC\) is not recognized by DNMT1, its presence would pro-
mote passive demethylation. Finally, $\mathrm{^{5}mC}$ might be actively removed by a DNA repair system and replaced by unmodified cytosine.

DNA methylation generally serves as repressor for gene transcription (192-194). There are two general mechanisms by which DNA methylation inhibits gene expression; first, modification of cytosine bases can inhibit the association of specific DNA-binding factors with their DNA target sequences (195). Second, proteins that recognize methyl-CpG can elicit the repressive potential on the methylated DNA regions (193,196).

DNA methylation in the gene body has been reported to be positively associated with gene expression. In the highly expressed genes, a pattern of low methylation in the promoter region and high methylation in the rest of the gene body was observed (197). Consistently, hypomethylation of gene body has been associated with weakly expressed or silenced genes (198). Non-CpG methylation has been found to be positively correlated with gene expression (186).

DNA methylation has been implicated in a diverse range of cellular functions including tissue-specific gene expression, cell differentiation, genomic imprinting, X chromosome inactivation and regulation of chromatin structure (199). DNA methylation is governed by a complex interplay of genetic and environmental factors (200). Common complex diseases may involve phenotypic variants with both genetic variation and environmentally triggered epigenetic change that modulates the effects of DNA sequence variation. The common disease genetic and epigenetic hypothesis argues that in addition to genetic variation, epigenetics provides an added layer of variation that might mediate the relationship between genotype and internal and external environmental factors (201). This epigenetic component could help to explain the marked increase in common diseases with age, as well as the frequent discordance of diseases such as bipolar disorder between monozygotic twins. Given that epigenetics is at the heart of phenotypic variation in health and disease, it seems likely that understanding and manipulating the epigenome holds enormous promise for preventing and treating common human illnesses. Epigenetics also offers an important window to understanding the role of the environment's interactions with the genome in causing disease, and in modulating those interactions to improve human health.

Methylation has been shown to be altered by abused substances. In humans, total DNA methylation was higher in genomic DNA of peripheral blood cell from alcoholics than controls (202,203) with a concomitant decrease in the expression of DNMT-3a and DNMT-3b (204). This observation suggests that interaction between genetic and environmental factors induces long lasting epigenetic changes that may alter gene expression and vulnerability to develop disease including alcohol dependence.

The HabMap database identified 2,252,113 C/T and G/A SNPs in the autosomal chromosomes. Of those, 34% are located within a CpG dinucleotide and named as CpG-SNPs (205). Sequences containing CpG dinucleo-
tides are 6.7-fold more abundant at the polymorphic sites than expected (206). SNPs could serve as functional cis-regulatory variant by forming or disrupting a CpG, which methylation is therefore allele-specific, and could be linked to allele-specific gene expression.

It has been proposed that genetic, epigenetic and environmental factors may influence the risk for the development of diseases through their effects on gene transcription. We hypothesized that mechanistically these effects may be integrated through regulation of methylation of CpG-SNPs associated with a disorder, hence affecting expression of high or low risk allele (Figure 3). Thus in addition to two alleles postulated by genetic hypothesis of disease, two epi-alleles of methylated and unmethylated cytosine in CpG-SNPs may have differential effects on disease vulnerability by engaging epigenetic mechanisms. It is also worth considering the possibility that, in some cases disease-associated epigenetic variation could arise prior to disease onset but may not be causative for the disease per se.

**Figure 3.** The genetic, epigenetic and environmental factors may influence the risk of a disease through their effect on gene transcription. Mechanistically, these effects may be integrated through regulation of methylation of CpG dinucleotides overlapping with SNPs, where C-variant is either associated with a disorder or a non-risk genotype.

A role of DNA methylation in alcohol dependence has not yet been established. No data on methylation of plastic genes critical for the development and maintenance of the dependence, and on global, genome wide methylation changes have been published for human or animal brain. Several studies described changes in methylation of several genes and total DNA in blood, gametes and embryonic DNA taken from dependent subjects (202,207-209).
Epigenetic mechanisms by which alcohol exposure could alter DNA methylation that consequently leads to alteration in the pattern of gene expression have not been yet systematically addressed. The studies are needed to map the alcohol induced changes in methylation status of individual CpG on the genome-wide scale, and to determine the functional link between alcohol induced changes in methylation of specific CpGs and alteration in expression of genes at the genome wide level and those known to contribute to the development and maintenance of addictive state.

1.3.2. Histone modification

There are 4 core histones H2A, H2B, H3, and H4 and a linker histone H1 in eukaryotic cells. Histones are arranged in an octamer structure in the nucleosome that is composed of H3-H4 tetramers with H2A-H2B dimer surrounding them. N-terminal tails of histone proteins can be modified by six different pathways i.e., acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation, and sumoylation. These modifications are specifically associated with activation or inhibition of gene transcription.

Methylation of H3K9, H3K20 and H3K27 is associated with gene silencing, whereas that of H3K4, H3K36 and H3K79 correlates with activated genes (210,211). Histone methylations are mediated by histone methyltransferases where the methyl group is donated by S-adenosyl-methionine.

Several cellular and animal studies analyzed effects of alcohol exposure on histone modifications. Alcohol causes selective H3K9 acetylation in primary culture of rat hepatocytes (212). This was also observed in hepatic stellate cells (213) and in rats acutely treated in vivo with alcohol (214). H3K9 acetylation was positively correlated with transcriptional activation of gene expression (215). Acute alcohol exposure was associated with decreased activity of histone deacetylases and increased histone acetylation in rat brain. While withdrawal from chronic alcohol treatment had the opposite effect by increasing histone deacetylases activity and decreasing histone acetylation, histone acetylation was also correlated with changes in gene expression (216).

PDYN gene expression has been correlated with histone modifications in cellular study (217). Activatory chromatin marks, H3K4me3 and H3K9 acetylation levels in PDYN gene promoter were increased while PDYN expression was inhibited following the exposure to alcohol. Inhibition of PDYN transcription likely occurred due to alcohol and/or acetaldehyde toxicity and has a limited biologic relevance because acute and chronic treatment of animals and human alcoholism results in activation of transcription of this gene or produces no changes in brain areas involved in addictive processes (4,218).

In conclusion, the relative lack of mechanistic insight beyond besides correlative observations on changes in DNA methylation and histone modifica-
tions, does not yet allow drawing a picture of the impact of chromatin remodelling on changes in gene transcription associated with human neuropsychiatric disorders and neuropsychiatric diseases in general. The complexity of DNA methylation and histone modifications has not yet been well addressed in the context of alcoholism. Analysis of these epigenetic events together with genetic polymorphisms that may affect allele specific expression of the epigenetic signature and that may be associated with the disease will be critical in gaining an insight into the mechanisms underlying alcohol dependence.

1.4. Non-canonical DNA secondary structures in gene transcription

DNA can adopt several structural conformations which are largely, although not exclusively, controlled by the pattern of hydrogen bonding. Up to now ten non-B form, non-canonical DNA secondary structures are recognized. These forms include intrinsic local variations, for example, changes in twist angle, a stably curved DNA, A-form DNA; inducible local and global secondary structures, e.g., melting, Z-form DNA, cruciforms, triplex DNA, super helices, loops and recently described slipped-stranded DNA (219,220). These variant structures can influence the interaction of DNA with proteins and consequently stimulate or repress processes that are governed by proteins such as transcription, repair, recombination or replication. One example is supercoiling, which is known to regulate genetic expression in prokaryotes (221,222) and eukaryotes (223-226). Therefore, DNA structure may possess regulatory potential on protein binding and, thus, on protein function.

Thermodynamically, the formation of non-canonical secondary structures is unfavorable in fully paired double-stranded DNA. However, during actively ongoing transcription or replication, DNA strands are locally separated. Under such conditions, the energetic constraints that impede secondary structure formation in fully paired DNA will be obviated, thereby promoting the formation of secondary DNA structures by intrastrand pairing. Indeed, active replication and transcription are accompanied by an increased formation of unusual DNA structures that have been detected in vivo (227-230). Formation of such structures has been associated with regulation of transcription, replication and recombination (231-233).

One example is provided by the p53 tumor repressor protein. Specific binding of p53 is greatly enhanced when the cognate DNA binding sites are present in a non-linear stem-loop conformation (234). For eukaryotes, hairpins or cruciforms have also been implicated in control of transcription. One family of proteins that binds DNA secondary structures are the high mobility group (HMG) chromosomal proteins. These are a family of double stranded
DNA- and single stranded DNA (ssDNA)-binding proteins extractable from nuclei and chromatin, and, like histones, they are thought to have a role in chromosomal structure, function, and transcription (235). HMG proteins induce formation of the deoxyribonuclease I (DNase I) sensitive sites in transcriptionally active chromatin. HMG I was shown (i) to bind to regions containing inverted repeats, (ii) to protect these regions from S1 nuclease digestion, and (iii) to remove a transcriptional block in the region of DNA containing a putative cruciform structure (236). More recently, a putative cruciform structure in the positive regulatory domain II of interferon-β has been described as a high affinity site for HMG I, which may regulate transcription of this gene (237).

DNA methylation in the promoter region serves as repressor for gene transcription (192-194), either $5\text{m}C$ can inhibit the interaction of DNA-binding factors with their DNA target sequences (195) or $5\text{m}C$-binding protein such as methyl CpG binding protein 2 can bind and elicit the repressive potential on the methylated DNA regions (193,196). In early studies, DNA methylation was described to increase the stability of a non-canonical DNA secondary structure, in particular the formation of Z-DNA in short double stranded DNA segments, that may have a global effect on the superhelical tension of an entire chromosomal domain and influence stabilities of supercoil-dependent DNA structures and the activities of protein-binding sites or promoters (238,239).

The neuropeptide encoding sequences are relatively short (15-90 nucleotides), and present in several repeats in a gene. Thus, *PENK* has 6 copies of Met-enkephalin sequence. Neuropeptide-encoding sequences but not intervening fragments are highly conserved between species (240) at both amino acid and nucleotide levels. In these features neuropeptide-encoding sequences are similar to DNA regulatory elements that serve as recognition sites for proteins regulating gene transcription, chromatin remodeling and DNA recombination. This hypothesis is in line with the view that exon sequences may participate in regulatory events involving formation of nucleosome, transcription elongation, and RNA splicing. This is supported by the observations that the segment of *PDYN* exon 4, which codes for $\alpha$-NE, dynorphin A and dynorphin B, also contains several transcription start sites and splicing sites for *PDYN* mRNAs. Thus, *Sp1* and *Sp2* *PDYN* mRNAs are spliced in this region while three truncated *PDYN* transcripts T1-T3 are initiated within exon 4. T1 transcript is initiated upstream of the $\alpha$-NE encoding sequence, T2 between $\alpha$-NE and dynorphin A encoding sequences, and T3 at the termination codon. Transcription of T1-T3 *PDYN* mRNAs has been proposed to be initiated by an intragenic promoter located in exon 4 (154).

The hypothesis on regulatory role of the *PDYN* exon 4 neuropeptide domain is supported by the presence of SNP (rs6045819; T>C; C risk allele) significantly associated with alcohol dependence, cocaine dependence and
alcohol and cocaine codependence (25,145) in this region. The C, risk allele of this SNP may form non-canonical E-box (CACGAG) that may represent a target for E-box binding transcription factors.

*PDYN* has two CpG rich domains or short CpG islands located in the promoter region and exon 4, respectively. The exon 4 CpG rich area overlaps with the dynorphin-encoding sequences. Our study demonstrated that methylation pattern of this domain is conserved between human individuals, while strongly differs between human tissues and cultured human cell lines; and brain and peripheral human tissues (241). The differences were attributed to differential methylation of two individual CpGs out of 13 CpGs present in this domain. One of these CpGs is located in the codon preceding the dynorphin A-encoding sequence while the second between α-NE and dynorphin A-encoding sequences. Methylation of these two CpG sites was 18 ± 9% in cell lines and 90 ± 7.1% in brain tissues, and was completely abolished in DNMT1/3b deficient cells. Full-length *PDYN* mRNA and CpG methylation did not correlate suggesting a novel epigenetic function for these CpGs in regulation of DNA conformation/chromatin remodeling, which are possibly not involved in transcription of canonical *PDYN* mRNA. As proposed previously exon 4 may be involved in *PDYN* splicing, and one possibility is that gene body methylation is relevant for tissue specific mRNA splicing.

Early analysis of transcription factors identified binding sites for the multifunctional transcription factor YY1 in exon 4 (172,173). This factor may directly bind to the Leu-enkephalin-encoding sequences in exon 4 and modulate reporter gene transcription from these sites. YY1 is known to interact directly with RNA polymerase II, and may initiate transcription from the TATA-less promoters including that in the *PDYN* exon 4 (242). Besides YY1-binding sites, several κB elements are present in the neuropeptide-encoding domain of exon 4, suggesting NF-κB-dependent regulation of *PDYN* transcription (174). DNA binding sites for YY1 and other factors have been found within coding regions of several genes (243-245), so the potential for such regulation would not be unique for *PDYN*. In addition to YY1 and NF-κB, USF1 and USF2 (162) and c-Myc (163,164) may target *PDYN* exon 4 region that may contribute to the regulation of *PDYN* transcription (Figure 2). However, interaction of all these factors with the *PDYN* exon 4 in the brain cells should be reevaluated using recently developed advanced methods including ChIP assays.

Another salient feature of the *PDYN* exon 4 is the presence of DNase I hypersensitivity peak overlapping with dynorphin A segment (Figure 2). DNase I, an endonuclease, acts on single-stranded DNA, double-stranded DNA, and chromatin. Short regions of chromatin cleavage by DNase I with high sensitivity are called DNase I hypersensitive sites. In a hypersensitive site, the nucleosomal structure is not organized in the usual fashion, which results in many fold increase in sensitivity to enzyme attack than in bulk
chromatin. Hypersensitive sites which are generated as results of the binding of transcription factors that displace histone octamer are often present in an active gene as these sites are associated with the open chromatin, which facilitates the interaction of transcription factors to their cognate binding sites to modulate gene expression. DNase I hypersensitive sites may be involved in vivo in the formation of non-canonical DNA secondary structure by facilitating the presence of ssDNA which is more prone to form non-canonical DNA secondary structure (246).

The critical observation supporting the notion on the regulatory role of dynorphin-encoding sequences came from the recent discovery of PDYN mutations that cause dominant genetic neurodegenerative disease SCA 23 (179). Three of four identified mutations are located in the dynorphin A-encoding sequence suggesting this sequence as a mutational hotspot. This is further supported by more recent identification of four new SCA 23 causative mutations that all reside within the dynorphin A- and B-encoding segment (manuscript in preparation). High frequency of the dynorphin A mutations may reflect the importance of physiological or pathophysiological role of the peptide products dynorphins encoded by this sequence; selection scheme based on clinical phenotype; or the enhanced mutability of this DNA segment (179).

Mutations may result from chemical alteration of nucleotides, for example deamination of 5mC to thymine (247,248). A base misincorporation by DNA-polymerase during DNA replication followed by unsuccessful repair represents another cause of mutations (249). ssDNA arising in the course of gene transcription or DNA replication may form non-canonical DNA structures that contain mismatched nucleotides which are recognized as damage by the DNA repair proteins (246,250-252). Reparation of these mismatches may lead to introduction of mutations in short DNA segments, hotspot, associated with human disorders (253-255).

Altogether, these considerations suggest that PDYN exon 4 has a potential for formation of non-canonical DNA secondary structure(s). We may propose that PDYN transcription can be regulated at two levels; first, by the binding of transcription factors to their consensus binding sites in double-stranded DNA present in the promoter/enhancer. Second, the formation of non-canonical DNA secondary structure(s) in exon 4 may either facilitate or abrogate the binding of regulatory proteins to DNA and, therefore, may modulate PDYN transcription at the level of transcriptional elongation, splicing or initiation of transcription from the alternative transcription start sites.
2. Hypothesis and aims of the thesis

Our hypothesis is that adaptations in the dynorphin/KOR system play a critical role in the development of alcohol dependence. These adaptations may depend on the genetic, epigenetic and environmental factors. *PDYN* regulation in the human brain is apparently complex and may involve (a) several transcription factors interacting with regulatory elements of this gene and (b) non-canonical mechanisms such as formation of non-B DNA structures by dynorphin-encoding sequences in the gene.

Aims of the study:

- To examine whether the EOS undergoes molecular adaptations in the brain of human alcoholics (Paper I).

- To evaluate whether methylation of *PDYN* CpG-SNPs associated with alcohol dependence is altered in the brain of human alcoholics, whether this alteration is associated with changes in gene expression, and whether there is DNA binding factor(s) that differentially binds to methylated and unmethylated variants of CpG-SNPs (Paper II).

- To examine whether the *PDYN* promoter SNP (rs1997794) associated with alcohol dependence may have an impact on *PDYN* expression in human brain, and whether variants of this SNP may be differentially targeted by transcription factor(s) involved in regulation of *PDYN* transcription (Paper III).

- To analyze whether the dynorphin-encoding sequences that represent the hotspot of human pathogenic mutations may serve as regulatory elements controlling gene transcription through the formation of non-canonical DNA secondary structures, and whether this formation is affected by methylation of cytosine at the CpG sites and human pathogenic mutations (Paper IV).
3. Material and methods

3.1. Human samples/case selection

Tissues were collected at the New South Wales Tissue Resource Centre, University of Sydney, Australia (256). Analysis included 14 chronic alcoholics and 14 controls. All subjects were males of the European descent. Alcohol dependent subjects met criteria for Diagnostic and Statistical Manual for Mental Disorders, 4th edition and National Health and Medical Research Council/World Health Organization criteria, and consumed greater than 80 g of alcohol per day for the majority of their adult lives. Controls had either abstained from alcohol completely or were social drinkers, who consumed less than 20 g of alcohol per day on average. Control cases were matched to alcoholic cases by sex, age, race and postmortem interval. Cases with a history of polydrug abuse (with evidence that the individual abused other drugs such as cocaine or heroin) or with medical complications such as liver cirrhosis and the Wernicke–Korsakoff syndrome or alcoholic cases with concomitant diseases were excluded. Cases with a prolonged agonal life support or cases with a history of cerebral infarction, head injury or neurodegenerative diseases (e.g., Alzheimer's disease) were also excluded. The main body of the population was smokers including 83% of alcoholics and 75% of control subjects. Samples were taken by qualified pathologists under full ethical clearance from the Sydney South West Area Health Service Human Ethics Committee (X03-0074). Informed written consent was obtained from the next of kin.

DNA-binding proteins were analyzed in extracts prepared from postmortem human brain tissues collected at Department of Forensic Medicine, Karolinska Institute, Stockholm, Sweden, with the consent of relatives. The study was approved by the local ethical committee of the Karolinska Institute.

3.2. Preparation of nuclear extract

Nuclear extracts from human brain dl-PFC, NAc and Sprague-Dawley GD-20 rat fetal brain (RFB) were prepared using a protocol (174), adapted from Dignam et al. (257). Briefly, tissues were homogenized with pestle B in Dignam's buffer A, supplemented with protease inhibitors. The homogenate
was centrifuged for 5 min at 4,500 x g, the pellet was extracted in buffer C supplemented with 0.2% Nonidet P-40 and protease inhibitors and was centrifuged twice at 20,000 x g for 10 min. The resulting supernatant was designated as the "nuclear" extract and kept at -80°C until studied. DC assay (Bio-Rad, Hercules, CA) was used for measuring protein concentrations.

3.3. Electromobility shift assay

The electromobility shift assay (EMSA) was performed essentially as described previously (258). Nuclear extracts (dl-PFC: 25 µg; RFB: 5 µg) were added to the binding mixture (20 mM HEPES, pH 7.5; 50 mM NaCl, 1 mM Na-EDTA, 37.5% glycerol, and 1.5 mM dithiothreitol (DTT), with 20 µg bovine serum albumin (BSA; Roche Diagnostics, Mannheim, Germany), 0.3 µg poly(dI–dC)–poly(dI–dC) and 90,000 cpm 32P–labeled oligonucleotide in 20 µl reaction medium, incubated for 20 min at room temperature, and resolved on a 5% native polyacrylamide gel in 0.5×TGE (25 mM Tris-HCl, 0.19 M glycine, 1 mM EDTA, pH 8.5) buffer. After the electrophoresis, gels were fixed in 15% alcohol containing 5% acetic acid for 15 min, dried, and analyzed by autoradiography or by Phosphoimager BAS 1500 (Fuji Film, Kanagawa, Japan) using Fuji Film Image Gauge software for quantification.

3.4. SDS-PAGE molecular mass determination

Analysis of molecular mass of DNA-binding protein in RFB extract was performed as described elsewhere (259). Briefly, RFB nuclear extract (125 µg) was denatured in sodium dodecylsulfate (SDS) loading buffer for 5 min at 95°C, and subjected to polyacrylamide gel electrophoresis (PAGE) on 10% SDS-polyacrylamide Tricine gel. The gel strips with resolved proteins were sliced uniformly into molecular mass intervals. Gel slices were crushed into 1.5 volumes of renaturation buffer (3% Triton X-100, 20 mM HEPES, 100 mM NaCl, 5 mg/ml BSA, 3 mM ZnCl2, 3 mM MgCl2, 2 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine-HCl) and incubated overnight at 4°C. The polyacrylamide was pelleted by centrifugation, and the supernatant was then assayed for DNA binding activity by EMSA. Molecular mass standards (Amersham International, Amersham, UK) were used to determine the molecular mass intervals of the excised gel slices.

3.5. Cell culturing and transfection

Human HeLa carcinoma cells were grown in Iscove’s modified Dulbecco’s, supplemented with 10% Fetal Bovine Serum in a 37°C incubator with 5%
CO2. Transfections with plasmid were carried out using Lipofectamine™ Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Mock-transfected cells were used as negative controls.

3.6. Immunoblotting

Protein extracts for immunoblotting were prepared by homogenization of tissue powder/pelleted cells in SDS buffer, and sonication. DC assay (Bio-Rad, Hercules, CA) was used for determination of protein concentration. Proteins were resolved by SDS-PAGE on 10% Tricine gels and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Blots were probed with rabbit polyclonal anti-FosB antibody (cat. # sc-48, Santa Cruz, CA; may identify human protein) at 1:200; and incubated with the peroxidase-conjugated secondary antibody (cat. # 170-6515, Bio-Rad) at 1:25000. Blots were developed in Amersham’s Enhanced Chemiluminescence System (Amersham, Little Chalfont, UK). Films were digitized using a scanner.

3.7. DNA purification and genotyping

DNA was purified from human brain samples using Wizard Genomic DNA Purification kit (Promega, Madison, WI). Genotypes were analyzed by pyrosequencing; primers were designed with Pyrosequencing Assay Design Software v1.0.6 (Biotage, Uppsala, Sweden). Reverse primers were 5’-biotinylated for forward sequencing. Amplification was performed using the following conditions: initial denaturation for 3 min at 94°C; 50 cycles of denaturing for 30 sec at 94°C, annealing for 30 sec at 63°C and elongation for 30 sec at 72°C; then 5 min at 72°C and hold at 4°C.

The PCR products were sequenced by pyrosequencing using the PyroMark ID System (Biotage, Uppsala, Sweden). Briefly, sample preparation was carried out using Vacuum Prep Tool according to standard procedures. The biotinylated strand of the target region was immobilized to 3 µl Streptavidin Sepharose™ HP beads (GE Healthcare, formerly Amersham Biosciences) which are retained on the filter plate, while the nonbiotinylated strands are washed off under denaturing conditions. 2 µl of sequencing primer (5 µM) was annealed to the template strand for 2 min at 80°C. In the pyrosequencing reaction, the incorporation of nucleotides complementary to a template strand is monitored bioluminometrically. All equipment was handled to standard procedures (Biotage, Uppsala, Sweden). The intensity of the bioluminometric responses are directly proportional to the amount of incorporated nucleotides and are termed as pyrograms, a representation of the
complete synthesis reaction. Genotypes were identified by the PyroMark ID 1.0 software (Biotage, Uppsala, Sweden), SNP mode.

3.8. RNA quality control and gene expression analysis by qRT-PCR

RNA preparation was performed using RNeasy Lipid Tissue Mini Kit (QIAGEN, Maryland, USA). Total RNA was quantified using microspectrophotometry by Nanodrop® (Nanodrop Technologies, Inc., USA). RNA Quality Indicator (RQI) was measured using Bio-Rad Experion (Bio-Rad Laboratories, Hercules, CA) with Eukaryote Total RNA StdSens assay according to manufacturer's protocol. RNA preparations with RQI values above 5.0 are generally considered as suitable for quantitative reverse transcriptase-PCR (qRT-PCR) (260,261). Average RQI in the analyzed set of samples was 7.28 ± 1.55 (mean ± SD) (89% of samples have RQI higher than 5.0) demonstrating high quality of isolated RNA. Notably, random primed reverse transcription used for preparation of complementary DNA (cDNA) allows samples with some degree of RNA fragmentation to become eligible (262).

RNeasy Lipid Tissue Mini Kit used in the study exploits integrated QIAzol and RNeasy silica-membrane that efficiently removes DNA contaminants without treatment with DNAase. DNAase treatment was omitted from the protocol in order to circumvent degradation of low abundant PDYN and other opioid mRNAs by residual RNAase activity in enzyme preparations. Most important, TaqMan® experimental design with intron overlapping primer set used in the study excluded interference of contaminating gDNA with qRT-PCR (263).

Primers were designed with “Vector NTI advance 11” software (Invitrogen, Carlsbad, CA). All primer pairs had an amplification efficiency of more than 90% for each pair of primers. “No-template control” reactions were run in parallel to demonstrate absence of signal within each experiment. No amplification was evident in samples devoid of template cDNA [amplification cycles (Cq) = 40]. Melting curve analysis was performed for each run to confirm the specificity of amplification and lack of primer dimers. To ensure correct amplification, PCR-products were separated on agarose gel and sequenced in both directions.

qPCR was performed on CFX96™ Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA). Reaction mixture consisted of cDNA (corresponding to 125 ng transcribed RNA), 2 × iQ SYBR Green Supermix (Bio-Rad Laboratories) and 0.25 μM each of forward and reverse primers. The following conditions were applied for the two steps RT qPCR reaction: 95°C for 3 min followed by 40 cycles at 95°C for 10s, annealing temperature
for 61.5°C for 30s. After PCR, a melting curve was recorded by increasing the temperature from 65 to 95°C and plotting the first negative derivative (-dF/dT) of the fluorescence vs temperature to determine the melting temperature of the PCR products. The fluorescence of individual samples was measured by the CFX96 Real-Time Detection System at the end of every cycle.

cDNA synthesis was performed with the High Capacity cDNA Archive kit (Applied Biosystems, Foster city, CA). For the optimization procedure of cDNA synthesis, different RNA input amounts were tested, and optimal amount of 250 ng of RNA was used for cDNA synthesis. Random hexamer primers were used for the cDNA synthesis, carried with 10U of MultiScribe reverse transcriptase in total volume 100 µl for 10 min at 25°C followed by 2 h at 37°C. cDNA samples were aliquoted and stored at -20°C.

Gene expression levels were analyzed by a TaqMan® Low Density Gene Arrays (Applied Biosystems). To the customized 384-well micro fluidic card containing probes and primers for each gene, cDNA and TaqMan® Universal PCR Master Mix (Applied Biosystems) was added in a final concentration of 65 pg cDNA per sample and gene in 1 µl well volume. Every sample was run in triplicate on the same array for each gene. The PCR amplification was performed at 50°C for 2 min, 94.5°C for 10 min and 40 cycles of 97°C for 30 sec followed by 59.7°C for 1 min. To measure the quantity of a given RNA species, the Cq were monitored by the Applied Biosystems 7900HT Fast Real-Time PCR System. Each mRNA expression was calculated by relative quantification using a normalization factor (geometric mean of two reference genes selected by geNORM program (http://medgen.ugent.be/genorm/) (264), and the qBASE program for internal and external calibration and easy care of large RT-PCR datasets (http://medgen.ugent.be/qbase/). Using the previously developed approach for analysis of reference genes (265), the beta-actin and ribosomal large P0 genes for dl-PFC, the peptidylprolyl isomerase A and phosphoglycerate kinase 1 genes for OFC and polymerase (RNA) II (DNA directed) polypeptide A (POLR2A) and ubiquitin C (UBC) genes for the hippocampus were chosen for normalization.

3.9. Dynorphin radioimmuno assay
The procedure was described elsewhere (266). Briefly, hot 1M acetic acid was added to frozen tissues powder, samples were boiled for 5 min, ultraso- nicated for 2 min, boiled again for 5 min and centrifuged for 15 min at 20,000 × g. Tissue extracts were run through a SP-Sephadex ion exchange C-25 column, peptides were gradually eluted with 1.6 M pyridine/1.6 M formic acid buffer, lyophilized overnight, diluted in CH$_3$OH: 0.1 M HCl and stored at -20°C. Samples were incubated with $^{125}$I-labeled peptide and prima-
ry antiserum with final dilutions, dynorphin A 1: 120,000, dynorphin B 1: 200,000 overnight at 4°C, and then with the sheep anti-rabbit antiserum (Pharmacia Decanting Suspension, Uppsala, Sweden) for 1 h at 4°C, centrifuged for 10 min at 12,000 × g, and pellet was used for counting on 1470 Wallac Wizard gamma-counter.

3.10. Chromatin immunoprecipitation assay

Binding of JUND and FOSB proteins to the PDYN promoter region containing SNP rs1997794, was analyzed by ChIP assay using postmortem human specimens of the caudate nucleus. Briefly, 50 mg of powdered tissue from four subjects were pooled together and cross-linked in 1% formaldehyde solution. Cell nuclei were isolated and treated with micrococcal nuclease followed by brief sonication on Branson Sonifier to cut DNA into small fragments (1000-1500 bp). After incubation with anti-FosB (Sc-48x; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-JunD (Sc-74x; Santa Cruz Biotechnology, CA) antibodies or control IgG (12-370, Millipore), protein G magnetic beads were added to the reaction mixture. After incubation at 4°C for 4 h, the beads with immunoprecipitated chromatin were washed twice with low salt ChIP buffer (50 mM NaCl and 0.1% SDS), once with high salt ChIP buffer (150 mM NaCl and 250 mM LiCl), and then twice with TE buffer (1mM EDTA-Na and Tris-HCl, 10 mM, pH 8). Chromatin was eluted with elution buffer (0.1 M NaHCO3 and 1% SDS), DNA was decross-linked by incubation in 200mM NaCl overnight at 65°C then treated with proteinase K at 55°C for 1.5 h. Magnetic beads were captured, and DNA was purified by phenol/chloroform extraction, precipitated with alcohol, and dissolved in 10 mM Tris-HCl, pH 8.0.

The fragment of interest was quantified by real time SYBR Green PCR using PDYN promoter SNP forward -184 primer (5’- CCTGTCACGAAGACCTGGAAGCCT -3’) and PDYN promoter SNP reverse -138 primer (5’- GGAAGTGGCTGAGAGCAGTC -3’) at 50°C for 2 min, 94.5°C for 10 min and 40 cycles of 97°C for 30 sec followed by 60.3°C for 1 min. Enrichment was determined by critical threshold (C\_T) measurements (changes in fluorescence per PCR cycle number at a given threshold). The genomic DNA was diluted to give a standard curve, which were constructed with a 10-fold dilution series ranging from 10^7 to 10^2 copies for each pair of primers. The concentrations of “IP” DNA, and “Input” DNA in copies was calculated by plotting the C\_T values of each sample on the standard curve, using the Bio-Rad CFX96 software. The percentage of binding of DNA binding factor was calculated using following formula:

\[
%\text{Binding}_x = \frac{[\text{"IP" } \text{DNA}]_x}{[\text{"Input" } \text{DNA}]_x} \times 100%
\]
Where [“IP” DNA] is a concentration of genomic DNA which is immuno-precipitated with antibody/IgG, while [“Input” DNA] is concentration of non-precipitated DNA according to standard curve; $x$ is a binding for a particular pair of primers.

3.11. Mass spectrometry of oligonucleotides

Premier qTOF mass spectrometer (Waters, USA) using negative-mode electrospray ionization was employed to study the homogeneity of methylated oligonucleotide samples. The oligonucleotide samples were dissolved to concentration of 8 g/L in 1:1 (v/v) water-acetonitrile solution to which 3% piperidine was added for assisting deprotonation and salt adduct removal. The mass spectrum was accumulated for 5 minutes and data were integrated. Neutral mass deconvolution was performed using commercial software (Waters, USA).

3.12. Circular dichroism spectroscopy

A Chirascan circular dichroism (CD) unit (Applied Photophysics, Surrey UK) was used to record oligonucleotide melting profiles between 5 °C and 60 °C. A 2 mm quartz cuvette was used to hold 400 μl samples of oligonucleotides in the concentration range 20-30 μM. The samples were dissolved in 10 mM sodium phosphate buffer at pH 7.3, and melting profiles were recorded at 275 nm at a rate of 0.2 °C/min. Before the measurements all samples were boiled at 95 °C for 3-5 min.

3.13. Nuclear magnetic resonance spectroscopy

A Bruker Avance 500 MHz nuclear magnetic resonance (NMR) spectrometer equipped with a cryogenic probehead was used to record 1D spectra at 4 °C and at 37 °C of unlabelled oligonucleotides dissolved in 10 mM sodium phosphate buffer containing 90/10 H₂O/D₂O at pH 7.3. The sample concentrations were in the range 100-300 μM, with 30 μM tetramethylsilane added for reference purposes. Before the measurements all samples were boiled at 95 °C for 3-5 min.
4. Results and discussion

4.1. The impact of alcohol on the EOS genes expression (paper I)

The EOS plays a critical role in addictive processes. Molecular dysregulations in this system may be specific for different stages of addiction cycle and neurocircuitries involved, and therefore may differentially contribute to the initiation and maintenance of addiction. We analyzed the expression of EOS genes including *OPRM 1*, *OPRD 1* and *OPRK 1* opioid receptor and *POMC*, *PENK* and *PDYN* opioid peptide precursor genes in the dl-PFC (Brodmann area 9), hippocampus (dentate gyrus) and OFC (Brodmann area 47), areas involved in cognitive control of impulsivity and the reoccupation/anticipation stage of addiction cycle, in human alcohol dependent and control subjects. The motor cortex (MC; Brodmann area 4), not involved in alcohol dependence was included as control for regional specificity.

*POMC* expressed at low levels was excluded from further analysis. *PDYN* and *OPRK 1* mRNAs in the dl-PFC and OFC, respectively, were significantly higher in alcoholics compared to controls (Figure 4 a, b). No significant differences in expression of other four genes in these two brain regions and all five genes in the hippocampus and MC were evident between the two groups. We also examined whether dynorphins A and B were altered in alcoholics by radioimmunoassay. The levels of dynorphins A and B were significantly increased in the dl-PFC and the hippocampus in alcoholics (Figure 4 a, c). No significant differences were found in the MC. Analysis of Spearman rank correlations identified significant (P <0.05) correlations between *PDYN* mRNA and dynorphins (all subjects: *PDYN* mRNA - dynorphin B in the dl-PFC (r = 0.54) and MC (r = 0.61); alcoholics: *PDYN* mRNA - dynorphin B (r = 0.73) in dl-PFC). Dynorphins acting through KOR may control release of glutamate and other neurotransmitters in the cortical areas and hippocampus (4). Cycles of alcohol consumption and withdrawal may dysregulate hippocampal and cortical neurotransmission by targeting the dynorphin/KOR system and that may induce a shift to a new pathological neurotransmission pattern.

The present findings suggest that long-term heavy alcohol consumption leads to activation of the selective EOS component, the dynorphin/KOR system in discrete brain loci. This may contribute to neurocognitive dysfunctions relevant for craving and disrupted inhibitory control.
Figure 4. The EOS including PENK, PDYN, OPRM 1, OPRD 1, OPRK 1 mRNA and dynorphins in the dl-PFC (a), OPRK 1 mRNA in the OFC (b) and dynorphins in the hippocampus (c) was upregulated in human alcoholics. Levels in alcoholics (n = 14) relative to controls (n = 14) are presented as the mean ± S.E.M. in relative units for EOS mRNAs and dynorphin A and B. * P < 0.05; ** P < 0.01; *** P < 0.001, alcoholics versus controls; two-way ANOVA followed by post hoc Student’s t-test.
4.2. Methylated CpG-SNPs and PDYN gene expression (paper II)

Interactions between genetic, epigenetic and environmental factors affecting gene expression may influence the risk for neuropsychiatric diseases. Mechanistically, these interactions may be mediated through alterations in methylation of SNPs that are associated with a disorder and form CpG dinucleotides. In this study, we analyzed methylation of PDYN CpG-SNPs that are associated with alcohol dependence, in the brains of human alcoholics.

Three (rs1997794; rs6045819 and rs2235749) out of five PDYN SNPs that are associated with alcoholism with high significance, form a CpG dinucleotide. The methylation-associated SNP (mSNP) in the PDYN promoter (rs1997794; the risk G allele forms CpG) was methylated in the dl-PFC of control (n = 10; mean ± SD, 15 ± 10%) and alcohol dependent subjects (n = 10; 23% ± 11%; Student’s t-test, P = 0.11) at low levels.

Methylation of the exon 4 mSNP (rs6045819; the risk C allele forms CpG; controls: n = 3, mean 79%; alcoholics: n = 2, mean 66%) may be functional. However, the limited number of subjects precluded further analysis.

The C variant of the exon 4, 3’-UTR mSNP (rs2235749) is the non-risk, major allele. Its methylation was higher in the dl-PFC of alcoholics (66 ± 4%) compared with controls (61 ± 4%; P = 0.001). Immunohistochemical analysis with anti-PDYN antibodies demonstrated that approximately 15 % of cells in the dl-PFC express PDYN (unpublished observations). Therefore, the 5% increase in methylation levels in alcoholics when all DNA molecules are taken as 100%, may correspond to de novo methylation of both alleles in 33.3%, or one allele in 66.7% of PDYN expressing pyramidal neuronal cells. No differences (P = 0.45) were evident in the MC, an area not involved in alcohol dependence.

Data on methylation of 3’-UTR mSNP were analyzed by two-way ANOVA with group (controls and alcoholics) and region (dl-PFC and MC) as independent between-group factors. A significant group effect (F(1.40) = 4.7, P < 0.05), a significant region effect (F(1.40) = 7.2, P = 0.01) and a significant group × region interaction (F(1.40) = 10.56, P < 0.01) were revealed (Figure 5). A post hoc Student’s t-test showed significant differences in the methylation status in the dl-PFC for pooled CC and CT genotypes (P = 0.001) between controls and alcoholics, and for each genotype separately (CC genotype: P < 0.05; CT genotype: P < 0.02).

In the dl-PFC, methylation of the 3’-UTR mSNP was significantly correlated with dynorphin A (P < 0.02; r = 0.49) and B (P < 0.03; r = 0.44), but not with PDYN mRNA (CC and CT genotypes were pooled). The CT genotype showed significant or trend correlations between i) methylation and PDYN mRNA (P < 0.06; r = 0.64); ii) methylation and dynorphin A (P < 0.04; r = 0.68); and methylation and dynorphin B (P < 0.08; r = 0.61).
The increased 3’-UTR mSNP methylation may affect PDYN expression in subjects with the non-risk C allele and hypothetically the elevated dynorphins may contribute to susceptibility to develop alcohol dependence in these individuals. Two-way ANOVA was undertaken to test this speculation, which failed to assess whether the risk allele/genotype is associated with increased PDYN expression, likely due to the low number of subjects with the risk, T allele (n = 5).

Figure 5. In the dl-PFC, methylation of the 3’-UTR SNP was significantly increased in alcoholics.

To assess whether there is molecular mechanism of selective recognition of unmethylated and methylated C allele, and the risk, T allele of the 3’-UTR mSNP, we used EMSA. EMSA with nuclear extract of human dl-PFC and rat embryonic brain, later is enriched in transcription factors, identified a T allele DNA binding factor (Ta-BF) demonstrating high, intermediate and low affinity for the T allele, and methylated and unmethylated C alleles, respectively. The T allele forms an E-box (CATATG), a DNA-target for E-box binding transcription factors. Ta-BF did not interact with two canonical E-box oligonucleotides, and antibodies against c-Myc and USF2, dominant E-box binding proteins identified in human dl-PFC, and against NeuroD, which targets an E-box variant formed by the T allele, did not affect Ta-BF–DNA binding. Therefore, Ta-BF was not an E-box binding protein. EMSA of proteins that had been renatured after separation with SDS-PAGE identified Ta-BF as a 63 kDa factor. A positive correlation between PDYN expression and 3’-UTR mSNP methylation may be explained if Ta-BF binding to the methylated C allele results in transcriptional activation.

These findings suggest that the genetic, epigenetic and environmental factors associated with a risk for alcohol dependence may mechanistically converge on the PDYN 3’-UTR CpG–SNP and that the resulting methylation
signals may be translated into disease predisposition via alterations in PDYN transcription by such factors as Ta-BF.

4.3. The AP-1 interaction with PDYN promoter SNP (paper III)

Here we reanalyzed the impact of PDYN’s promoter SNP (rs1997794: T>C) variants on the PDYN expression. The Pearson’s χ² test did not reveal significant differences of the genotypic and allelic association of this SNP with alcohol-dependence likely due to a small number of subjects analyzed. Two-way factorial ANOVA revealed significant effect of alcohol-dependence in dl-PFC (F (1.19) =7.03, P = 0.016) and a significant interaction between alcohol dependence and genotype in the MC (F (2.19) =7.61, P = 0.004). Post hoc test revealed a significant elevation by 1.7 fold of PDYN mRNA in alcoholics compared to controls in the dl-PFC (t 23=-2.78, P = 0.01) that has been reported (in paper I). No other significant differences were found.

To identify variables which have the largest spread in the combined data set and which account for largest part of variance, we performed a principal component analysis (PCA). The PCA identified two factors with eigenvalues > 1 that explained 62% of the variance. Factor 1 (F1; 37%) had high loadings with alcoholism (0.77) and PDYN mRNA levels in the dl-PFC (0.85) and MC (0.60). Factor 2 (F2; 25%) had high loadings with genotype (0.87) and PDYN mRNA expression in the hippocampus (-0.70). High loadings indicate strong linear correlation between the factor and parameters. The PCA suggests that variations in PDYN expression in the dl-PFC are related to alcohol-dependence, whereas those in the hippocampus depend on the PDYN genotype.

The T, low risk allele of the PDYN promoter SNP resides within a TGACACA sequence previously identified as a non-canonical AP-1-binding element (267-270). Analysis of AP-1 interaction with the C and T alleles of the non-canonical PDYN AP-1 site was performed by EMSA. Incubation of the oligonucleotide with canonical AP-1 binding element used as a labeled probe with nuclear extract prepared from human NAc, produced retarded complexes. The upper complex was specific for AP-1; its formation was blocked by wild-type AP-1 oligonucleotide but not mutant AP-1 oligonucleotide. The PDYN T allele oligonucleotide (T-PD) but not its C (C-PD) and methylated C allele (mC-PD) used as unlabeled competitors, inhibited formation of the labeled probe - AP-1 complex.

To analyze composition of AP-1 complex in human NAc, effects of antibodies against AP-1 constituents c-Fos, FosB and JunD on AP-1 complex formation were studied by EMSA. Anti-JunD-antibody alone and in combination with anti-c-Fos- or anti-FosB antibodies supershifted virtually all
AP-1 complex. Anti-c-Fos antibody did not affect the AP-1 complex formation whereas anti-FosB-antibody substantially depleted it. The latter antibodies may interact with both FOSB and ΔFOSB, the C-terminally truncated FOSB variant implicated in drug addiction in rodents (271). Interestingly that western blot analysis using the same antibodies identified the 46 kDa FOSB as a dominant form of the protein in the human NAc and OFC, while two ΔFOSB forms (27 and 37 kDa) were present at negligible levels compared to those of the 46 kDa FOSB. FOSB and ΔFOSB proteins ectopically expressed in HeLa cells transfected with respective plasmids were used as positive controls in the western blot experiments. Thus, AP-1 complex in the human NAc may predominantly consist of JUND homodimer and JUND-FOSB heterodimer, that both may target non-canonical AP-1 binding element in PDYN promoter.

The previous study implied that the T, low risk allele of the PDYN promoter SNP is associated with lower PDYN expression compared to the C allele in human brain cortical areas (272). In the absence of significant differences between genotypes in PDYN expression, our data nevertheless show tendency for association of lower PDYN mRNA levels with the T allele compared to the C allele consistent with the previous analysis (272). This tendency is observed in control subjects but not in alcoholics.

These results suggest that impact of the PDYN promoter SNP associated with alcohol dependence on expression of this gene in the human brain is region specific, and that non-canonical AP-1 binding site formed by the T, low risk allele may be targeted by JUND and FOSB proteins. In general terms, this study supports the notion that the effects of genetic variations associated with a disease on gene expression may be mediated through alteration in affinity for transcription factors that bind to DNA elements in which SNPs reside.

4.4. Impact of CpG methylation and human pathogenic mutations on conformational polymorphism (Paper IV)

ssDNA is characterized by high conformational flexibility that allows these molecules to adopt a variety of conformations and form non-canonical DNA secondary structures. We evaluated whether cytosine methylation at CpG sites, in addition to its role in gene expression and mutagenesis (Figure 6), and human pathogenic SCA 23 mutations may affect conformational flexibility of short ssDNA molecules using native PAGE, NMR and CD. A set of 37-mer ssDNA oligonucleotides with either two or four CpG sites or one SCA 23 mutation in each molecule was used in the analysis.

We analyzed the dynorphin-oligonucleotides by native PAGE at 4°C and 37°C and compared their mobilities with DNA markers. The α-NE-
oligonucleotide did not differ in mobility from the 37-mer oligonucleotide marker at both temperatures. Dynorphin A-encoding oligonucleotide show rapid migration on a gel than antisense oligonucleotide (dynorphin A (AS)-oligonucleotide and 37-nt marker, which showed similar mobilities at 4°C. At 37°C the mobility of all these oligonucleotides was similar.

The dynorphin A-oligonucleotide methylated at the CpG-1 site showed two bands with different mobilities, apparently from two different conformers in equilibrium with each other: one of them had mobility similar to that of unmethylated oligonucleotide while the other one moved slower. Methylation at CpG-2 produced no significant changes. Di-methylation at the CpG-1 and CpG-2 or at CpG-1 and CpG-3 resulted in the mobility pattern observed for single CpG-1 methylation, with the difference that the slower moving conformer became dominant. Methylation of the α-NE-oligonucleotide at the CpG site homologous in position to the CpG-1 in the dynorphin A-oligonucleotide produced no obvious effects. The methylated dynorphin A-oligonucleotides did not differ in mobility from the unmethylated sequence at 37°C.

The SCA 23 mutation M2 but not M1 or M3 resulted in substantial decrease in mobility of dynorphin A-oligonucleotide at 4°C, while the three mutants showed more similar migration pattern at 37°C. The M4-oligonucleotide showed elevated mobility at 4°C and 37°C compared to the 37-nt DNA marker. The M5-oligonucleotide did not differ from the control marker at both temperatures.

The migrations patterns on native gels did not depend on the presence or absence of mono- and bivalent cations and duration of incubation (10 or 30 min, or 18 h) before loading on the gel. In contrast to the results observed under native gel conditions, all analyzed 37-mer oligonucleotides demonstrated virtually identical mobility on the denaturing gels at both temperatures.

Taken together, the principal finding of this study was increased mobility of dynorphin A-oligonucleotide on native PAGE at 4°C in comparison with control oligonucleotides. This suggests formation of a compact structure by molecules of dynorphin A-oligonucleotide allowing their rapid migration on the gel. NMR and CD spectroscopy supported this notion. The second finding was that cytosine methylation either at one or two CpG sites of the dynorphin A-oligonucleotide resulted in the formation of an additional conformer existing in the equilibrium with the first one at 4°C. The third finding was that three human point mutations in dynorphin A-encoding sequence, which cause the dominant genetic neurodegenerative disease SCA 23 (179) (Figure 2) and two other point mutations prevented the dynorphin A-oligonucleotide to form its basic conformation at 4°C. Results obtained native PAGE, NMR and CD spectroscopy generally correlated.

The formation of secondary structure may affect DNA-protein interactions and consequently stimulate or repress processes that are governed by
proteins such as transcription, repair, recombination or replication. One example is supercoiling, which is known to regulate genetic expression in prokaryotes (221,222) and eukaryotes (223-226). Our results suggest that cytosine methylation may affect conformational flexibility of ssDNA molecules and therefore their propensity to form specific secondary structures.

Figure 6. Conformational polymorphisms may provide mechanistic background for the formation of non-canonical DNA structures that (a) allow high rate of mutations (mutational hotspots) and (b) regulate gene transcription, RNA splicing and DNA recombination. We hypothesize that in addition to the effects on gene transcription and mutagenesis, methylation and demethylation of cytosine in CpG dinucleotides may influence ssDNA conformations.

It is tempting to speculate that the ability of cytosine methylation to interfere with conformational flexibility of ssDNA segments in chromatin context may be relevant for regulation of processes where double stranded DNA is unwinded to ssDNA, such as gene transcription, DNA replication and DNA recombination.
5. Conclusions

This thesis reports the findings that support the hypothesis on a critical role of adaptations in the dynorphin/KOR system in the human brain in alcohol dependence. These adaptations may differ between brain areas depending on mechanisms of PDYN regulation involved, their sensitivity to alcohol, and interactions between the genetic, epigenetic and environmental factors including alcohol.

- The dynorphin/KOR system including PDYN mRNA and dynorphins in the dl-PFC, dynorphins in the hippocampus, and OPRK 1 mRNA in the OFC, is upregulated in human alcoholics. These alterations apparently represent the most consistent adaptive changes in the EOS in the brain of human alcoholics, and may contribute to the impairment of cognitive control of addictive behaviors.
- Three PDYN SNPs that demonstrate the most significant association with alcoholism and form CpG sites were methylated in human brain at different levels. Methylation of the C, non-risk variant of the PDYN 3’-UTR SNP (rs2235749 SNP: C>T) was significantly increased in the dl-PFC but not MC of alcohol dependent subjects.
- Methylation of the PDYN 3’-UTR SNP positively and significantly correlated with PDYN mRNA and dynorphin levels suggesting a functional link between the methylation and gene expression.
- The T allele specific binding factor (Ta-BF) with differential binding affinity for the T allele and methylated and unmethylated C alleles of the 3’-UTR SNP has been identified in the human brain. The Ta-BF may be involved in regulation of PDYN expression through binding to the T allele or methylated 3’-UTR mSNP C allele.
- The T, low risk allele of PDYN promoter SNP (rs1997794: T>C), which forms a non-canonical AP-1-binding element, apparently represents the target site for JUND and FOSB proteins. The T to C transition abrogated AP-1 binding. AP-1 complex predominantly consists of JUND homodimers and/or JUND/FOSB heterodimers in the dl-PFC of human brain.
- The principal component analysis suggested that PDYN expression in the dl-PFC may be related to alcoholism, whereas in the hippocampus may depend on the genotype. The impact of genetic
variations on *PDYN* transcription may be relevant for diverse adaptive responses of this gene to alcohol.

- The dynorphin A-encoding sequence in *PDYN* has potential to form non-canonical DNA secondary structure. This may contribute to transcriptional regulation of the gene and increased rate of pathogenic mutations in this gene region. Methylation of cytosine at the CpG sites and human pathogenic mutations could modify non-canonical structure formation by the dynorphin A-encoding sequence.

- *PDYN* regulation in the human brain is complex and may involve several transcription factors such as JUND, FOSB and Ta-BF, methylation of CpG/SNPs, and formation of non-canonical DNA structures that all may differentially contribute to canonical and alternative initiation of transcription and mRNA splicing. Impairment of *PDYN* regulatory mechanisms may define human pathological conditions including substance dependence.
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References


120. Mann, K., Agartz, I., Harper, C., Shoa, S., Rawlings, R.R., Momenan, R., Hommer, D.W., Pfefferbaum, A., Sullivan, E.V.,
Evidence of common and specific genetic effects: association of the muscarinic acetylcholine receptor M2 (CHRM2) gene with alcohol dependence and major depressive syndrome. Hum Mol Genet, 13, 1903-1911.


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