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**POST MORTEM STUDIES ON THE
HUMAN ALCOHOLIC BRAIN: DNA
METHYLATION AND MOLECULAR
RESPONSES**

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To My Parents

ABSTRACT

Chronic alcoholism is a multi-factorial psychiatric disease manifested by re-occurring periods of relapse with frequent symptoms of cognitive impairments. There is no explanation to the extreme vulnerability to relapse, since this often occurs without evident molecular or biochemical changes remaining at the time of relapse. Epigenetic marks, such as DNA methylation, may represent a memory of molecular manifestations beyond evident genetic or biochemical signaling, in addition to its role as a gene activity regulator. The alcoholic genome could potentially possess an altered DNA methylation pattern by 1) DNA-methyltransferase blockage by acetaldehyde or 2) folate deficiency and aberrant methyl-donation by S-adenosyl-methionine (SAM). This thesis describes molecular and DNA methylation alterations in multiple alcoholic brain regions, with focus on a brain region important for reward related behavior and cognition – the prefrontal cortex (PFC). First, an optimal normalization for quantitative expression analysis was established by analysis of a panel of endogenous control genes. This study demonstrated the importance of brain region specific normalization genes due to differences in expression stability between brain regions. Moreover, it reveals expression differences in a number of commonly used normalizing genes between controls and alcoholics. Furthermore, components involved in programmed cell death/cell survival were analyzed to examine a possible involvement in alcoholism. Several key pro- and antiapoptotic genes and proteins were found changed in the alcoholic PFC in a direction that supports a neuronal protective adaptation rather than an active cell death. This thesis further shows how the genome of alcoholics is deprived of DNA methylation. This is demonstrated to occur globally both in humans and in a rat model of alcohol dependence, by a technique developed during the thesis work, LUMinometric Methylation Assay (LUMA), but it also occurs with gene specificity as shown in humans using a microarray based methylation technology. The results further indicate a specificity to brain regions involved in reward and cognition – PFC and the striatum. DNA hypomethylation was also shown to be functionally important to investigate since a correlation ($p = 0.00001$) between DNA hypomethylation and gene expression was found in 14 genes analyzed. Moreover, genes of expected functional groups, including alcohol metabolizing enzymes and genes regulating methylation processes were found differentially methylated, as well as the previously reported insulin signaling pathway and acetylcholine receptors. Finally, a neuronal atrophy pathway was associated to alcoholism by comparison analysis between DNA methylation microarray and previously reported gene expression microarrays. This pathway, Dentatorubral-pallidolusian atrophy (DRPLA), is responsible for an autosomal neuronal atrophy disorder with clinical symptoms of epilepsy, cerebellar ataxia, dementia and involuntary reflexes and movements - symptoms also frequently found in alcoholics.

LIST OF PUBLICATIONS

- I. **Johansson S ***, Fuchs A, Ökvist A, Karimi M, Harper C, Garrick T, Sheedy D, Hurd Y, Bakalkin G, Ekström T.J. Validation of endogenous controls for quantitative gene expression analysis: application on brain cortices of human chronic alcoholics. *Brain Research*, 2007, 1132(1):20-28.
- II. **Johansson S**, Ekström T.J, Marinova Z, Ökvist A, Sheedy D, Garrick T, Harper C, Kuzmin A, Yakovleva T and Bakalkin G *. Dysregulation of cell death machinery in the prefrontal cortex in human chronic alcoholics. *International Journal of Neuropsychopharmacology*, under revision.
- III. Karimi M, **Johansson S**, Stach D, Corcoran M, Grander D, Shalling M, Bakalkin G, Lyko F, Larsson C, Ekström T.J *. LUMA (LUMinometric Methylation Assay)--a high throughput method to the analysis of genomic DNA methylation. *Experimental Cell Research*, 2006, 312(11):1989-95.
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RELATED PUBLICATIONS

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LIST OF ABBREVIATIONS

5-HTT	5-hydroxy-tryptamine
5-HTTLPR	5-hydroxy-tryptamine transporter-linked polymorphic region
ACh receptors	nicotinic acetylcholine receptors
ACTB	Beta-actin
ALDH	Aldehyde dehydrogenase
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ATN-1	Atrophen-1
B2M	Beta-2-microglobulin
BAIAP2	Brain-specific angiogenesis inhibitor 1-associated protein 2
CGI	CpG island
ChIP	Chromatin Immunoprecipitation
CNS	Central nervous system
CpG	Cytosine-phosphate-Guanine
CREB	cyclic AMP response element-binding protein
CRH	Corticotropin releasing hormone
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DRPLA	Dentatorubral-pallidoluyisioan atrophy
GABA	Gamma-aminobutyric acid
HELP	<i>HpaII</i> tiny fragment Enrichment by Ligation-mediated PCR
INSR	Insulin receptor
LM-PCR	Ligation mediated PCR
LUMA	LUMinometric Methylation Assay
MAG1	Membrane associated guanylate kinase, WW and PDZ domain containing 2
MBP	Myelin basic protein
MC	Motor cortex
NAc	Nucleus Accumbens
NMDA	N-methyl-D-aspartic acid
PCR	Polymerase chain reaction
PFC	Prefrontal Cortex
PGK1	Phosphoglycerate kinase
PLP1	Proteolipid protein
PMI	Post mortem interval
POLR2A	RNA polymerase II
PPIA	Peptidylprolyl isomerase A
RERE	Arginine-glutamic acid dipeptide repeats
RPLP0	Ribosomal large P0
RT-PCR	Real-time PCR
SAM	S-adenosyl-methionine
TSS	Transcription start site
VTA	Ventral tegmental area

1 INTRODUCTION

The interaction between genes and the environment, in the development of physical attributes or diseases, has for a long time been a field of question marks and speculation. In the past decade, a new field of molecular research has emerged that reveals how environmental stimuli may have both instantaneous physiological effects as well as long term effects, likely transmittable over generations. This concept involves epigenetic mechanisms. Epigenetics describes any aspect other than DNA that influences the development and inheritable traits of an organism. Epigenetics originates from the term used to describe the differentiation from a totipotent state in embryonic development – epigenesis. At that time, there was no biological explanation for the phenomenon why cells born with the same genome within an organism, differentiated and developed into different cell types with unique biological responsibilities. Hence, the Greek prefix “Epi” meaning “on top of” or “in addition”, reflects the origin of the word. Epigenetics was coined by C. H Waddington in 1942, who used the word to describe how a multicellular organism uses the surrounding to produce a phenotype (Waddington, 1942).

Despite many years of studying substance use disorders such as alcoholism, no complete picture exist to how the addicted individual is sustained vulnerable to relapse many years after any withdrawal or craving symptoms have disappeared. Neither do we know why certain individuals fall into addiction while others do not. Although we do know that a correlation exist between adolescent exposure and falling into addiction later in life (McGue and Iacono, 2008), or that the genetic tendency to experience severe withdrawal is associated to avoidance of self-administration of alcohol in animals (Metten et al., 1998), or that high level of alcohol tolerance increases the susceptibility to develop alcohol dependence (Schuckit et al., 2005), or that negative emotion driven impulsivity increases alcohol susceptibility (Nigg et al., 2006; Verdejo-Garcia et al., 2007). Any one of those proposed models may be an indicator of an inheritable risk as well as a disruptor of adolescent and/or adult development and therefore a manifestation of a biological change, possibly increasing the risk for an addictive disorder. Moreover, we know that alcoholism is a complex multigenic and polygenic disorder, with a genetic contribution of at least 50%. However this alone does not unravel the pathology of this psychiatric disease. Both inheritable traits and acquired biological alterations may be epigenetically linked as a subtle cellular memory of molecular manifestations beyond evident genetic and biochemical signaling. This thesis presents the functional relevance of epigenetic research to study the interaction between environmental influences and the activity of our genes, in a direct clinical application by studying the addicted brain. Moreover, the thesis describes a novel technology to study one aspect of epigenetics, as well as epigenetic and gene activity alterations in the *post mortem* alcoholic brain.

1.1 ALCOHOLISM

Alcohol dependence is estimated to affect about 4% of the adult population and is the third leading cause of preventable death in the United States (Miller, 2008), yet only 10% to 15% of those affected receive treatment. Alcohol dependence largely varies depending on culture, region and geographic location, and it has been estimated that the lifetime prevalence of alcohol use disorder can reach up to 23% (Sher et al., 2005).

World Health Organization (WHO) has in their Global Status Report on Alcohol (2004) estimated that 76.3 million people worldwide had diagnosable alcohol use disorders (W.H.O, 2004), and that alcohol consumption is the fifth leading cause of death and disease burden worldwide. The following sections are aimed at describing the clinical manifestations of alcohol dependence in addition to discussing up to date research in a morphological, behavioral, motivational, genetic and epigenetic perspective.

1.1.1 Diagnostic criteria for alcoholism

There are several stages of responses to alcohol consumption, where the actual addictive state may arise diffusely over a long period of time, and may vary from individual to individual. The three developmental stages of addiction are: 1) acute drug effects, 2) transition from recreational use to patterns of use characteristic of addiction, i.e. uncontrollable drinking and 3) end-stage addiction. End-stage addiction is characterized by an overwhelming desire to obtain alcohol, a diminished ability to control drug seeking, and reduced pleasure from biological rewards. The diagnostic criteria for a substance use disorder is established by American Psychiatric Association in the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), and is defined by a maladaptive pattern of substance use, leading to clinically significant impairment or distress (1994). The criteria described for substance use disorder enclose physiological, social and psychological aspects (Table 1). Three or more of these criteria must be fulfilled during a twelve-month period to receive the diagnosis substance use disorder.

Table 1. Diagnostic criteria for substance dependence disorder, according to Diagnostic and Statistical Manual of Mental Disorders (DSM-IV).

A maladaptive pattern of substance abuse, leading to clinical impairment or distress, as manifested by three (or more) of the following criteria, occurring at any time in the same 12-month period:

1. Tolerance
 - a. a need for markedly increased amounts of the substance to achieve intoxication or desired effect
 - b. markedly diminished effect with continued use of the same amount of the substance
 2. Withdrawal
 - a. the characteristic withdrawal syndrome for the substance
 - b. the same (or a closely related) substance is taken to relieve or avoid withdrawal symptoms
 3. The substance is consumed in larger amounts or over a longer period of time than was originally intended
 4. There is a persistent desire or unsuccessful efforts to cut down or control substance use
 5. A great deal of time is spent on activities necessary to obtain the substance, use the substance or recover from its effects
 6. Important social, occupational or recreational activities are given up or reduced in favor of substance abuse
 7. The substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance
-

1.1.2 Etiology of alcoholism

A number of theories of alcoholism etiology have received empirical support. These models are not mutually exclusive but most likely each represent a part a complex heterogeneity that contribute to the development of the disorder. Different behavioral etiological models have been proposed including 1) positive affect regulation, which describes how drinking for positive reinforcement that is strongly associated with positive expectancies for enhancement as well as personality traits related to reward seeking (i.e. sensation seeking) mediates drinking outcome (Sher, 2003); 2) negative affect regulation, describing how tension, anxiety, stress or “self-medication” coping motivations mediate the effects of negative affect and tension-reduction expectancies on drinking outcomes; 3) pharmacological vulnerability, describes how either individuals especially sensitive to reinforcement (either positive or negative), or individuals relatively insensitive to reinforcement are at higher risk for physiological dependence; 4) deviance proneness, describes how a childhood history is attributable to a deficient socialization. Additionally, several personality models have been proposed to contribute to alcoholism including neuroticism/negative emotionality (Gratzer et al., 2004; Jackson and Sher, 2003; McCormick et al., 1998; Weitzman, 2004), impulsivity (Bennett et al., 1999; McGue et al., 1997; Trull et al., 2004), extraversion/sociability (Hill et al., 2000; Hill and Yuan, 1999), and increased risk-taking (Zuckerman and Kuhlman, 2000).

Complex heterogenic trait

The overall genetic heritability for alcoholism has been estimated from twin studies to range between 50 to 60% (Goldman et al., 2005). Many efforts have been made to identify genes responsible for alcoholism, however only a few with successful empiric replication. The most striking genetic contribution of those genes include genes coding for alcohol metabolizing enzymes (alcohol dehydrogenase ADH1B, ADH2, ADH3 and acetaldehyde dehydrogenase ALDH2) and the gamma-amino butyric acid receptor alpha 2 subunit (GABRA2) (Ding et al., 2008; Edenberg et al., 2004; Goldman et al., 2005; Lappalainen et al., 2005; Peng et al., 1999; Reich et al., 1998; Soyka et al., 2008; Thomasson et al., 1991). Alcohol dehydrogenase catalyzes the conversion of alcohol to acetaldehyde, a toxic metabolite, which is subsequently metabolized to acetate by acetaldehyde dehydrogenase. Mutations in either alcohol or acetaldehyde dehydrogenases may result in an accumulation of acetaldehyde following alcohol ingestion (Thomasson et al., 1991). This accumulation is associated with an aversive flushing reaction, similar to that caused by disulfiram (Antabus). Hence, such mutations have been proposed protective in the development of alcoholism, as found in Asian people with ADH/ALDH mutations (Muramatsu et al., 1995; Peng et al., 2007). However, the relevance of genetic contribution of ADH and ALDH has been questioned since the prevalence of this genetic variation is very rare in Caucasian ancestry, despite being a population with a high rate of alcoholism. The GABRA2 gene was identified in the collaborative study of the genetics of alcoholism (COGA), where an initial linkage study showed an association to a region on chromosome 4 (Reich et al., 1998). Fine mapping of this region identified a GABA_A receptor gene cluster linkage with EEG beta brain wave patterns, a known predisposing factor for alcoholism (Costa and Bauer, 1997; Hesselbrock et al., 1993; Porjesz et al., 2002). The GABRA2 gene showed an exclusive association to EEG beta brain wave patterns and alcoholism within the GABA_A receptor gene cluster analyzed.

Another gene of great controversy for functional consequences and its association to alcoholism is the human μ -opioid receptor gene (OPRM1) and its A118G mutation (Bond et al., 1998; Krosiak et al., 2007; Zhang et al., 2005). The role of OPRM1 A118G mutation alcohol dependent carriers is strongly supported by the fact that they experience alcohol-induced euphoria and are selectively sensitive to the therapeutic effect of the opioid receptor antagonist naltrexone (Anton et al., 2008; Arias et al., 2006; Hernandez-Avila et al., 2007; Oslin et al., 2003). Furthermore, the release of its endogenous ligand, β -endorphin, followed by activation of μ -opioid receptors in the ventral tegmental area (VTA) is believed to mediate acute rewarding effects of alcohol (Kreek et al., 2002).

In addition to the above mentioned genes associated with the development of alcohol dependence, a number of other candidate genes, mainly related to the central nervous system, have been proposed (Diamond, 1995). These include *N*-methyl-D-aspartate receptor, calcium channels, cyclic adenosine monophosphate and G proteins. In recent

years, dopamine and serotonin transport (Dick et al., 2004; Lichtermann et al., 2000; Mokrovic et al., 2008; Repo et al., 1999; Zuckerman and Kuhlman, 2000) and genes regulating enzymes important in the metabolism of dopamine and serotonin has received increasing interest (e.g. catechol-*O*-methyltransferase and monoamine oxidase) (Henderson et al., 2000; Nilsson et al., 2005; Nilsson et al., 2007; Nilsson et al., 2008; Snell et al., 2002). Dopamine associates strongly with drugs and drug-cue-induced incentive value, arousal, and euphoria (Koob and Weiss, 1992). Genes related to dopamine are therefore logical candidates to analyze. Polymorphisms of the serotonin transporter (5-HTT) gene have been associated in several studies, with personality and affective disorders (Heils et al., 1995; Ogilvie et al., 1996). Furthermore, the 5-HTTLPR polymorphism has been studied extensively in relation to affective disorders and allelic variation association to anxiety-related personality traits. Dysfunctional serotonergic neurotransmission has been implicated in alcoholism in several studies (Cloninger, 1987; Heinz et al., 2001; Lesch, 2005).

The overall conclusion from years of studying genetic inheritance of alcohol dependence is that no genes related to brain function have been firmly linked to risk of alcoholism. The inconsistent and widely spread gene associations suggest that susceptibility genes for alcoholism generally each contribute to a small proportion of the overall disease risk. This pattern of inheritance is characteristic for a complex polygenic disease. For alcohol abuse to develop fully into alcohol dependence, other factors such as environmental stimuli and persistent neuroadaptations are required.

Environmental risk factors

Environmental risk factor is a wide term with many applicable areas. In principal, there are three possible exposure settings to environmental influences for vertebrates. The first two are distal exposure (prenatal exposure) or proximal exposure (direct effects of stimuli contexts, e.g. drinking alcohol) as reviewed previously (Sher et al., 2005). Yet a third environmental exposure that recently has received increasing interest is transgenerational environmental inheritance (Champagne, 2008; Champagne and Meaney, 2007). Inheritance may not only be transmitted from parental generations to offspring through genomic information, but recent findings suggest that also epigenetic mechanisms have the capacity to mediate transmission of functional information. This is shown by studying maternal care in primates and rodents from cross-fostering studies. These data show that behavior in rearing is relying on postpartum mother-infant interaction, rather than in the DNA sequence. Furthermore, rearing behavior is transmitted to offspring together with physiological changes and behavioral response to stress by reduced hippocampal glucocorticoid receptor mRNA, elevations in adrenocorticotropin (ACTH) and corticosterone following restraint stress, and elevated hypothalamic corticotrophin releasing hormone mRNA (Caldji et al., 1998; Liu et al., 1997).

Prenatal exposure (distal) to alcohol has been demonstrated to be a specific risk factor for development of fetal alcohol syndrome and serious psychiatric disorders that are often comorbid with substance use disorders (Famy et al., 1998; O'Connor et al., 2002). Fetal alcohol syndrome often relate to a range of childhood cognitive and behavioral problems leading to antisocial behavior and alcohol dependence (Baer et al., 2003; Steinhausen et al., 1993). Two ways have been suggested to how prenatal exposure to alcohol increases the risk for substance use: 1) through a pathway related to general deviance proneness as suggested from a spectrum of antisocial behavior related to fetal alcohol syndrome and 2) through pathway related to pharmacological vulnerability (Sher et al., 2005).

Parental substance use (proximal) as a risk factor has been controversial. Genetic epidemiological studies indicate that about half of the liability to alcohol dependence is environmental (Kaprio et al., 2002; Rose et al., 2001; Viken et al., 1999). Interestingly though, is that twin data suggest that the majority of these environmental influences are unique (i.e. unshared). Moreover, there is evidence that the risk of developing alcohol abuse in children of alcoholic parents was unchanged over a five-year period regardless of whether parents were actively drinking suggesting that environmental risk factors is not accounted for alone (Chassin et al., 1999). Furthermore, parenting practices such as high warmth and behavioral control (e.g. authoritative parenting) has been demonstrated protective from negative outcomes such as antisocial behavior and the development of alcohol use disorders (Adalbjarnardottir and Rafnsson, 2001; Patock-Peckham et al., 2001).

Gene x environment interaction

There is increasing support that there is a gene x environment interaction implicated in the etiology of alcoholism, and that environmental factors or genetic predisposition alone does not underlie the dependent state. This environmental contribution may possibly be transgenerational epigenetic inheritance. One study show a role of allelic variants of the serotonin transporter gene (5-HTT) in conjunction with family function was predictive for adolescent alcohol consumption (Nilsson et al., 2005). Moreover, a genetic variant of the corticotrophin releasing hormone factor (CRHR1) gene and stressful life events was predicted interactive risk factors for alcohol use disorder as well as relapse (Blomeyer et al., 2008; Hansson et al., 2006).

1.1.3 Alterations in the alcoholic brain

Alcohol dependence is a psychiatric disease with many physiological alterations, including many organs in the body. However, the motivational and cognitive aspects of alcoholism exclusively depend on the brain physiology. Therefore, to address these aspects of the disorder, this thesis is focused on brain related molecular alterations.

In vivo morphology

It is well established that long term heavy alcohol consumption produces pathological alterations in the brain (Eckardt et al., 1986; Fadda and Rossetti, 1998; Freund, 1973; Jacobson and Lishman, 1990; Martin et al., 1986). Several techniques have been used to evaluate alcohol related brain damage *in vivo* MRI (Magnetic Resonance Imaging), CT (Computed Tomography), PET (Positron Emission Tomography), SPECT (Single Photon Emission Computed Tomography), post mortem MRI and neuropathology. There are also several modifications of older techniques such as Magnetic Resonance Diffusion Tensor Imaging (DTI), a technique to visualize white matter bundles and microstructure. The results from different studies performed on alcohol related brain morphology vary, partly due to different subject inclusion criteria and method used. *In vivo* MRI studies often report similar extent of gray matter and subjacent white matter volume deficits in the cortex (Fein et al., 2002; Jernigan et al., 1991; Pfefferbaum et al., 1992; Pfefferbaum et al., 2004), but also a significant reduction in white matter volumes has been seen in adolescents, young adults and adult alcohol use disorders using MRI and DTI (Figure 1) (De Bellis et al., 2005; Pfefferbaum et al., 2007; Rosenbloom et al., 2003). The same study provided data showing significant correlation between prefrontal cortex volume and measures of alcohol consumption. Recently, a study detected a widespread distribution of white matter deficits in both left and right hemisphere and in midsagittal sections in both men and women recovering from alcohol intoxication, although significantly greater in men than in women (Pfefferbaum et al., 2006).

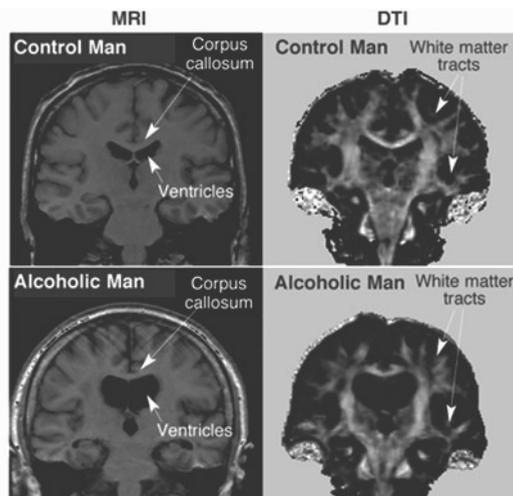


Figure 1. Images displayed in the coronal orientation from MRI and DTI studies of a 61-year-old healthy man (upper images) and a 60-year-old alcoholic man (lower images). The MRI reveals a thinner corpus callosum displaced upward by enlarged ventricles and, on the DTI, less well delineated white matter tracts in the alcoholic man compared with the healthy man (Rosenbloom et al., 2003).

Post mortem neuropathology

Post mortem neuropathological studies are often reporting greater white matter loss than gray matter loss (de la Monte, 1988; Harper et al., 1985), and demonstrate that the greatest cortical loss occurs in the frontal lobes (Harper, 1998; Kril et al., 1997; Pfefferbaum et al., 1997). Although frontal cortex might be mostly affected by alcoholism other brain regions have also been reported to be affected. The corpus callosum becomes thin (Harper and Kril, 1988), central pontine myelinolysis probably occurs in nutritionally based alcoholism (Laureno and Mark, 1995; Liamis et al., 2000; Lien et al., 1990), and there has also been reports on a reduced gray matter density within the amygdala (Fein et al., 2006). In recent years several lines of evidence have also pointed out that cerebellar and cerebellothalamocortical systems are morphologically affected (Baker et al., 1999; De Bellis et al., 2005; Sullivan, 2003; Sullivan et al., 2000; Sullivan et al., 2003). However, the cerebellar dysfunction might be a consequence of nutritional deficiency like the thiamin deficiency of Wernicke's encephalopathy and not by alcohol toxicity *per se* (Baker et al., 1999). Furthermore, morphological studies have also shown changes in structures important for the reward pathway of alcoholism such as caudate and putamen which shows enduring reduced volume in alcoholics and even nucleus accumbens which show reduction following recent alcohol exposure (Sullivan et al., 2005).

Several studies have been trying to evaluate the reason for a white matter reduction in alcoholic brain tissue. There have been documented changes in the thickness of the myelin lamellae in an experimental model (Phillips et al., 1991), disruption of white matter microstructural integrity (Pfefferbaum and Sullivan, 2002), disruption of axonal integrity (Harper, 1998), and disruption of white matter microstructure by accumulation of intracellular and extracellular fluid widespread in both hemispheres, in the corpus callosum and in the centrum semiovale (Pfefferbaum and Sullivan, 2005).

Neuroadaptations

Neuroadaptation describes how neuronal function is altered by long-lasting molecular and biochemical changes. This will result in, possibly persistent, changes in neurotransmitter signaling, synaptic plasticity and homeostasis. Neuroadaptations are changes that remain far longer than the acute alcohol effects and are also independent of changes associated with acute withdrawal effects. Following long-term alcohol use virtually all brain neurotransmitter signaling seem to be affected, making it difficult to establish which of the systems contributes the most to the transition from controlled to compulsive alcohol use. However, the alcohol dependent state is characterized by a decreased function of the reward neurocircuitry and a recruitment of antireward/stress factors, together with a hypertonic corticotrophin-releasing factor system and a hyperfunctional glutamatergic system, suggested as the most crucial cascades in the state of alcohol dependence (Vengeliene et al., 2008).

Alcohol directly modulates the activity of ion channels and receptors, subsequently leading to a cascade of synaptic events involving multiple neurotransmitters. Once the alcohol dependent state is established, an imbalance in excitatory and inhibitory neurotransmission occurs through different neurotransmitters and neuropeptides. The inhibitory neurotransmitter GABA_A receptor function is decreased after prolonged alcohol consumption, likely due to a decreased number of GABA_A receptor or from changes in the subunit composition of the receptor. On the contrary, the activity of the excitatory neurotransmitter glutamate is decreased initially by antagonistic effects of alcohol to the NMDA receptor. This will eventually result in an oversensitivity and increased glutamatergic transmission after prolonged exposure, due to an adaptation to the inhibitory effect by e.g. increase in number of NMDA receptors and/or ligand sensitivity (Figure 2)(Hoffman et al., 1990). Apart from the most studied GABA and glutamatergic systems, chronic alcohol use affects several other neurotransmitter systems and ion channels, such as the mesolimbic DAergic system, the opioid system and the endocannabinoid system in the amygdala, nucleus accumbens and VTA. In addition, glycine, 5-hydroxytryptamine 3 (serotonin) and nicotinic ACh receptors as well as L-type Ca²⁺ channels and G-protein-activated inwardly rectifying K⁺ channels are affected. Moreover, the brain stress hormone corticotrophin releasing factor (CRF) and the neuropeptide Y (NPY) seem to be highly involved (Heilig and Koob, 2007).

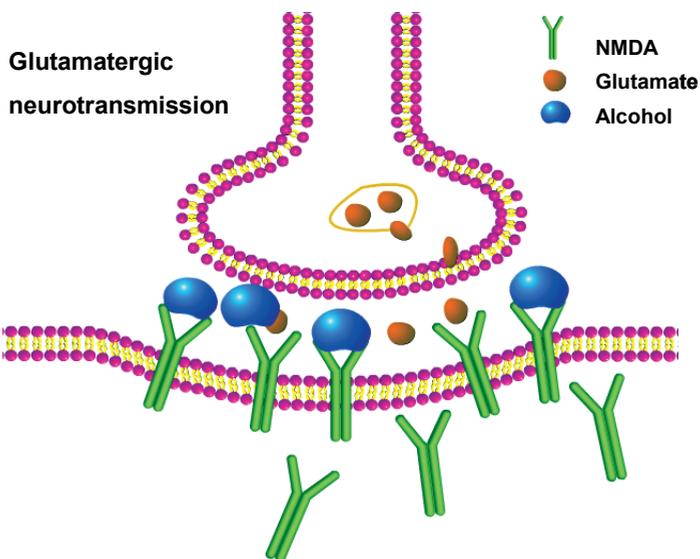


Figure 2. Excitatory glutamate neurotransmission. Alcohol has antagonistic effects on NMDA receptors in the postsynapse. During alcohol intake, the NMDA receptor is blocked by alcohol, resulting in lowered excitatory effect on the neurons. After prolonged alcohol exposure, the number of NMDA receptors increase and become more sensitive, leading to an increased glutamatergic neurotransmission, excitotoxicity and altered neuroplasticity (synaptic strength). A change in neuroplasticity could jeopardize normal learning and memory formation.

Neuroplasticity is characterized by a change in synaptic strength, and the best candidate mechanisms for this are long-term-potential (LTP) and long-term depression (LTD). They have been proposed to be critical in many forms of experience-dependent plasticity, including various forms of learning and memory (Malenka and Bear, 2004). Such mechanisms of synaptic plasticity could subsequently lead to the reorganization of neural circuitry by altering gene and protein expression in neurons that receive enhanced or diminished signals as a result of LTP or LTD. Thus, LTP and LTD have become important candidates for drug-induced alterations of neural circuit function. NMDA receptors are critically involved in the formation of LTP and LTD (Malenka and Bear, 2004) and is believed to play an important role in addiction and formation of reward circuitry (Figure 2) (reviewed in (Vanderschuren and Kalivas, 2000)). Alcohol dependence in humans is associated with persistent alterations in glutamatergic neurotransmission in the prefrontal cortex (Goldstein and Volkow, 2002; Kalivas and O'Brien, 2008). Furthermore, alcohol dependent individuals have a decreased basal activity in this region (Goldstein and Volkow, 2002), indicative of increased synaptic strength (Turrigiano and Nelson, 2004), i.e. of net synaptic potentiation. Animal studies of ethanol consumption have further demonstrated enlargements of dendritic spines and associated post synaptic densities at glutamatergic synapses (Carpenter-Hyland and Chandler, 2006; Carpenter-Hyland et al., 2004; Hendricson et al., 2007).

Suggested molecular targets of neuroadaptations in addiction related disorders have been the transcription factor cAMP response element-binding proteins (CREB) and the immediate early gene (IEG) Δ FosB (Nestler, 2004). CREB seem to play an important role in the regulation of dopaminergic transmission in the VTA, thus modulating the rewarding effects of addictive drugs (McClung and Nestler, 2003; Sakai et al., 2002). Δ FosB accumulation in striatum during acute cocaine treatment increases sensitivity and motivational properties of cocaine and has been suggested to represent a “molecular switch” that regulates the progression from acute drug responses to long lasting neuroadaptations (McClung et al., 2004). However, it is still unclear how these molecular changes in gene expression are maintained far longer than any reward-related behavior as to explain the remaining vulnerability for relapse. Not even the long-lived Δ FosB sustain accumulated that long. Epigenetics has recently been proposed to underlie such long-term changes of gene expression and subsequently biochemical alterations (Tsankova et al., 2007).

Mesocorticolimbic circuitries

The reinforcing properties of drugs of abuse, including alcohol, are strikingly powerful and conserved across species. Positive reinforcement was studied in the early 1950s with the goal to understand the control of this motivational behavior. Brain regions responsible for this control were mapped and since then it has been known that the mesocorticolimbic dopamine system, including the ventral tegmental area (VTA), dorsal striatum, nucleus accumbens (NAc), and the prefrontal cortex (PFC), is mediating pleasurable effects of drugs of abuse. The mesocorticolimbic neurons arise

from the VTA and innervate various cortical and subcortical forebrain areas (Dahlstrom and Fuxe, 1964; Ungerstedt, 1971). The subcortical areas include the ventral striatum (NAc), the bed nucleus of stria terminalis, the amygdala complex, the hippocampus, the olfactory tubercle, and the septum. The cortical projections include the PFC, the cingulate, the entorhinal cortices and the piriform.

The different brain regions in the mesocorticolimbic circuit have different functions in mediating the effects of drugs of abuse. The VTA and the ventral striatum are believed to play a critical role in rewarding effects of drugs of abuse, while the amygdala is important for regulation of negative emotional and motivational aspects of drug dependence (Grant et al., 1996). The hippocampus belongs to the traditional memory circuit and has been demonstrated important in contextual memories of drug exposure and withdrawal. PFC is processing information from motivational (VTA), emotional (amygdala) and memory (hippocampus) centres of the brain and therefore regulates the control of behavior and represents cognitive performance. Furthermore, PFC plays a key role together with NAc in the valuation of rewards and the establishment of reward-associative memories due to its innervations from VTA. In other words, PFC has an integrative function in determining valence of behavioral responses rather than ascribing positive or negative valence to individual stimuli (Bush et al., 2002; Jentsch and Taylor, 1999).

1.1.4 Dependence

Substance dependence

Over many decades' substance use disorders was considered a matter of lack-of-character. As years have past and the scientific community has dramatically developed theoretically as well as technologically, a more scientific view on dependence disorders has evolved. Initial theories proposed that the compulsion for drug taking was motivated by either the euphoric properties (positive reinforcement) or the desire to relieve withdrawal symptoms (negative reinforcement). More recently, three dominating theories have been leading the field of substance dependence research. Those include 1) incentive sensitization 2) dependence as pathology of motivation and choice and 3) hedonic allostasis.

The unifying theme between these theories is the goal to be able to explain addictive disorders in terms of dependence, relapse, craving, reward, motivation and control. Most of these studies have been performed on various substances of abuse and it should be clarified that possibly not the same molecular mechanisms occur in all types of substance use disorders. This also applies to alcohol dependence which is known to target far more systems in the body than any other drug of abuse.

Positive reinforcement by addictive drugs very much involves the mesolimbic system and the theory of incentive sensitization. This theory was proposed in 1993 (Robinson and Berridge, 1993) and suggests that repeated substance use facilitates enduring

neuroadaptive changes of the mesolimbic dopamine system and that these changes result in a behavioral state where the motivation for drug seeking is enhanced as an increased drug appetite or “wanting” (incentive sensitization). This should however be separated from the “liking” or pleasurable effects of the drug which often declines as dependence progresses. The primary candidate genes in the incentive sensitization theory have naturally been those involved in the dopaminergic neurotransmission, such as dopamine transporter (DAT1) or dopamine receptors (D1, D2) (White and Kalivas, 1998).

Another field of researchers believe that addictive disorders are a pathology of motivation and choice or the lack of control. The emotional circuit, i.e. the amygdala, the NAc and the PFC are the key players in this theory of dependence, in that a dysregulation of this motivational circuitry by repeated drug abuse will result in loss of behavioral control. Mainly the glutamatergic transmission in the PFC has proven supportive of this theory. It has been reported that self-reported craving is highly correlated to activity of the PFC in cocaine abusers (Breiter et al., 1997; Volkow et al., 1999; Volkow et al., 2003). Furthermore, inactivation of PFC in animal models prevents cue-, drug- and stress-induced reinstatement of drug-seeking (Capriles et al., 2003; McFarland et al., 2004; McLaughlin and See, 2003). Moreover, antagonism of the AMPA receptor in the NAc prevents drug- and cue-induced reinstatement of drug-seeking (Di Ciano and Everitt, 2001; Park et al., 2002). So far, focus has been on research on genes involved in modulating glutamate release and affecting postsynaptic response, in the overall aim to find explanatory models for neuroadaptations in the motivational circuit. Those genes include the inhibitory metabotropic glutamate autoreceptors mGluR2/3 as well as postsynaptic scaffolding proteins like Homer and PSD-95 (Baker et al., 2003).

Hedonic allostasis constitute the third major theory to drug addiction. Hedonic allostasis refers to the negative reinforcement developed during the progress to a dependent state (Koob and Le Moal, 2001, 2005), and reflects the shift from a reward controlled system to an anti-reward controlled system. This theory describes three stages in the “cycle of dependence”; preoccupation-anticipation, binge-intoxication and withdrawal-negative affect. During the first phases of the cycle, the substance use is primarily driven by elements associated with impulsivity and the positive reinforcing effects of drugs. As the dependent state eventually starts dominating, negative affect is gradually recruited and dependence becomes more compulsive, and the cycle is driven by negative reinforcement, i.e. taking the drug to relieve negative symptoms or withdrawal. Allostasis refers to the urge to maintain stability at the cost of shifting the set point outside of the normal homeostatic stage when physiological systems are chronically challenged. Molecular targets in finding the underlying mechanism to this “allostatic shift” have been genes involved in fear- and anxiety related behaviors as for example the corticotrophin releasing hormone (CRH) and its receptors in the amygdala complex. Studies have shown how repeated cycles of ethanol exposure and withdrawal

and the development of drug dependence in rats induces alcohol preference and stress sensitivity together with an increased expression of several CRH-related genes in the amygdala (reviewed in (Koob and Le Moal, 2008)). In the alcohol dependent state it has been shown that antagonistic treatment to CRH1 receptor attenuate behavioral manifestations of alcohol dependence (Heilig and Koob, 2007).

Alcohol dependence

In contrast to other drugs of abuse, alcohol's acute reinforcing properties are weak. As shown from animal studies, the intensity of lever pressing to obtain a single dose of the substance is hundred times higher for amphetamine and cocaine in comparison to alcohol. Addictive drugs have the power to diminish basic needs such as eating and sleeping, even to the point of dying (Bozarth and Wise, 1985). Furthermore, alcohol has both stimulatory and sedative effects and act on multiple systems in many brain regions as discussed above.

From the point of view of the major theories described above, alcohol dependence applies more as a pathology of motivation and choice, or hedonic allostasis rather than incentive sensitization due to its weak reinforcing effects. Although it should be pointed out that these theories may not be mutually exclusive in the progress of alcohol dependence. As for the involvement of motivation and choice, there are lines of support for alterations in the glutamatergic system in alcoholics, specifically in the PFC (Carpenter-Hyland and Chandler, 2006; Carpenter-Hyland et al., 2004; Goldstein and Volkow, 2002; Hendricson et al., 2007; Kalivas and O'Brien, 2008; Turrigiano and Nelson, 2004) together with evidence of therapeutic effects by acamprosate, which most likely exert its effect by silencing glutamatergic signaling (Spanagel et al., 2005). Furthermore, the NMDA antagonist memantine has been shown to reduce cue-induced craving in alcoholics (Krupitsky et al., 2007). The hedonic allostasis theory also wins support as prolonged exposure to alcohol in rodents has been shown to increase alcohol preference and stress sensitivity, as well as increased corticotrophin releasing factor (CRF) expression in the amygdala (Sommer et al., 2008). In humans, research has shown that a certain genetic variations in the CRF gene associates with higher level of binge drinking or excessive drinking in adolescents after a stressful events (Blomeyer et al., 2008; Treutlein et al., 2006). In addition, a recent study show promises for future treatment of alcoholism using a CRF1 antagonist that blocks excessive ethanol self-administration in rats (Gehlert et al., 2007).

1.2 EPIGENETICS

Epigenetics describes the mechanisms that form the properties of the genome and which can not be directly explained by the DNA primary sequence. Epigenetics is also in its purest definition heritable through cell divisions. It defines the cell's identity and memory. Epigenetic mechanisms are imposed upon the genome in a static or dynamic fashion by controlling gene activity, and induced through an exogenous stimuli. Since

the DNA sequence is simply a code for the design of proteins another level of regulation must exist to control the synthesis and level of production of proteins (Dennis, 2003; Reik and Dean, 2002; Strahl and Allis, 2000). This level of regulation constitutes the epigenetic information surrounding the DNA sequence, which is deciding if and to what extent a gene should be active depending on cell type, external stimuli, environmental factors and time in life. Physiologically, this is established as chemical attachments modifying either the cytosine base of the DNA sequence by a covalent binding of a methyl group (CH₃), or by chemical or protein attachments to the chromatin forming proteins – the histones. The histones can be modified by methyl (CH₃)-, phospho (PO₄)-, or acetyl (COCH₃) groups or by ubiquitination, SUMOylation and ADP-ribosylation. This “marking” of the genome respond in a dynamic way to external or internal stimuli and may function as a cellular memory (Bird, 2002). Epigenetic modifications are inherited from mother to daughter cell; however how this is maintained is still unknown (Bronner et al., 2007). Epigenetic modifications does not only control the activity of genes but also protect cells from transposable elements (Jaenisch and Bird, 2003; Jones and Baylin, 2002) and retroviral sequences (Bird, 2002), play a crucial role in embryonic development (Sasaki and Matsui, 2008), control the x-chromosome inactivation in females (Mohandas et al., 1981; Wolf and Migeon, 1982), and regulate genomic imprinting (Reik et al., 1987) or cellular reprogramming (Clerc and Avner, 2000). During pathological conditions either silencing or dynamic regulation of epigenetic marks may be disturbed followed by an abnormal gene activity. This thesis has been focusing on DNA methylation responses to heavy alcohol consumption; therefore the following introduction into epigenetics will describe DNA methylation and its regulation of gene activity.

1.2.1 DNA methylation

All eukaryotes, ranging from plants to humans, carry methyl (CH₃) exclusively on the cytosine residue of the DNA (Figure 3). The importance of DNA methylation is proven by the early lethality during embryonic development in DNA methyltransferase (DNMTs) deficient mice (Li et al., 1992; Okano et al., 1999). *Dnmt*-null mice have reduced level of DNA methylation, however the precise mechanism to the lethality is unknown.

The general concept has for a long time been that DNA methylation is responsible for the repressed state of gene transcription and the silencing of genes, by stably silence promoter activity (Bird and Wolffe, 1999). This knowledge has evolved from studies of DNA methylation of gene promoters on the X-chromosome, at imprinted genes and at various genes in cancers or cell lines, which imposes gene silencing that can be reversed by artificial demethylation (Bird, 2002).

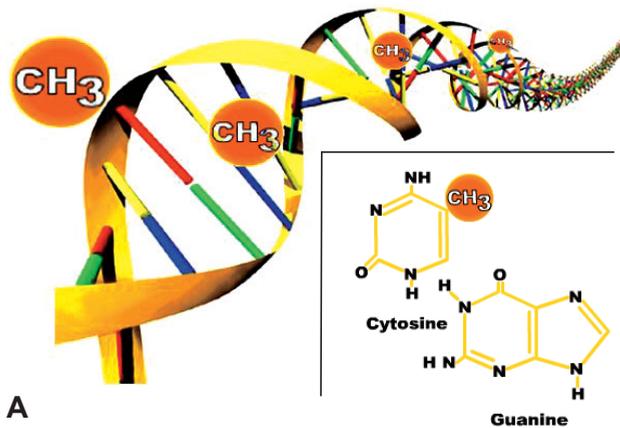


Figure 3. Exclusively cytosine residues in the DNA carry methyl-groups (CH₃). **A** | DNA strand with methylated DNA in CpG dinucleotides. **B** | A CpG dinucleotide carrying a methyl-group attached to the fifth position in the cytosine carbon ring (cytosine-phosphate-guanine).

Studying DNA methylation today is a scientific challenge. Although the function of transcriptional silencing has empirical support, the challenge still relies in the potential role of DNA methylation as a dynamic regulator of gene expression, during development and altered physiological states. The causal role of DNA methylation to regulated gene expression has been questioned, suggesting that dynamic patterns of promoter methylation provide a pretext for revisiting the possibility of a developmental role (Suzuki and Bird, 2008). This is due to the fact that few large-scale studies on DNA methylation have been able to provide functional evidence to its role. Moreover, studies on a large number of promoters show cell type specific DNA methylation. Furthermore, recent studies show novel findings of a spatial relationship between DNA methylation and genes, providing support that not only promoter methylation plays a functional role. The following introduction chapter aims at describing DNA methylation, recent findings and limits of our understanding of this epigenetic system.

DNA methylation in evolution: methylated gene bodies

Plants and animals diverged about 1.6 billion years ago, which explain obvious differences in DNA methylation seen between them. The most distinguished difference is the existence of only CpG methylation in animals (methylated cytosine 5' neighbouring a guanine), unlike the plants possessing also non-CpG methylation, CpNpG. However, also clear similarities in DNA methylation exist, which may reflect an ancestral origin in the function of DNA methylation (reviewed in (Suzuki and Bird, 2008)). Plant and invertebrate DNA methylation studies have revealed a mosaic pattern of DNA methylation in both *Arabidopsis Thaliana* plant and in the *Ciona intestinalis* (sea squirt), described as a pattern of methylated and unmethylated coexisting regions in about the same proportions. In *A. Thaliana* the genome consist of densely methylated regions to about 20% of the total genome, mainly comprising transcriptionally inactive

heterochromatin such as centromeres and pericentromeric heterochromatin as well as repetitive DNA sequences and regions (Chan et al., 2005). In *C. Intestinalis* the mosaic DNA methylation was shown to be evenly proportioned in methylated and unmethylated regions in a mosaic pattern (Simmen et al., 1999). The mammalian genome however, is dominated by evenly distributed cytosine methylation, and only a small fraction of 1-2 % is unmethylated (Bird et al., 1985; Bird, 1986; Illingworth et al., 2008). This is the reason why the mammalian genome is said to possess a global DNA methylation, unlike the invertebrate and plant genome that occupy a mosaic pattern of methylation.

In spite of the differences in DNA methylation density and pattern between plants and animals, several studies point to very likely important similarities. In both *A. Thaliana* and *C. Intestinalis* a distinct DNA methylation has been noted within transposon-free euchromatin regions (i.e. actively transcribed DNA regions), mainly within the transcribed region of genes (Suzuki et al., 2007; Zhang et al., 2006; Zilberman et al., 2007). About 33% of *A. Thaliana* genes and 60% of *C. Intestinalis* genes were occupied by dense DNA methylation in the transcribed regions, which even corresponded to the majority of DNA methylated sites in the latter species. Moreover, the 5' or 3' ends were almost exclusively absent in DNA methylation. Methylation in the transcribed regions of genes, gene body methylation, does not shut down the activity of the gene. In fact, the average expression levels were significantly higher in the gene body methylated genes as compared to promoter methylated genes and less gene body methylated genes in *A. Thaliana*. It was further found in the plant study that the genes displaying gene body methylation was moderately expressed and generalized to represent "housekeeping genes". The same observation was found in the invertebrate study where a majority of found genes were housekeeping genes. However this study also found that the most highly expressed genes were unmethylated and that gene body methylated genes in *C. Intestinalis* appeared to be the most highly conserved. Moreover, by sequencing the intergenic regions between two insect species, a comparable DNA methylation patterns was observed, with DNA methylation dense gene bodies but not at gene extremities. (Field, 2000; Hick et al., 1996; Wang et al., 2006).

In the human genome, the majority of cytosines (CpGs) in the genome are methylated, thus the logical rationale that the majority of gene bodies should be methylated. This is supported by several human studies (Eckhardt et al., 2006; Rabinowicz et al., 2003; Rakyan et al., 2004). However, due to the ubiquitous DNA methylation observed in humans, this may have two explanation models. Either, DNA methylation is targeted specifically to gene sequences or is a default state that happens to affect genes as well as most other sequences. A recent human study has provided strong support for gene body methylation as correlated to gene activity. Initially, promoter CGIs in the female inactive X-chromosome were shown to be hypermethylated to obtain the silenced state of the duplicated chromosome ($X_i = \text{inactive}$) (Mohandas et al., 1981). However, recent

data show how in fact that the active X-chromosome (X_a) is overall more methylated than X_i by looking at the whole transcribed region (Weber et al., 2005). Another study further confirmed this and showed twice as much DNA methylation on X_a compared to X_i (Hellman and Chess, 2007). To answer the question if X_a had become unusually densely methylated compared with autosomes, cell lines biallelically active in a stage prior to X-chromosome inactivation were used and revealed a similar level of DNA methylation between the two chromosomes suggesting a demethylation of X_i chromosome.

If the gene body methylation and associated gene transcription observed in the X-chromosome applies to other genes in the human genome awaits further scientific insights. However, it is likely so since many autosomal genes analyzed are heavily methylated in their transcribed region. Additionally, as in invertebrates and plants, the human 5' region often occupied with hypomethylated CGIs, frequently extends into the gene and first exons. No demethylated 3' region has been reported in humans so far. Up to date, we do not have the complete picture of what functional role gene body methylation in detail has, and if this applies throughout different species.

Human DNA methylation landscape

In the mammalian genome, DNA methylation is evenly distributed (global DNA methylation) except from certain unmethylated shorter clusters of CG-rich DNA sequences, CpG islands (CGIs) (Bird, 2002; Bird, 1986). A combination of global computational analysis of patterns of CpG depletion and direct sequencing of enriched unmethylated and methylated domains from human brain DNA confirms previous single gene findings that long adjoining methylated domains are occasionally interrupted by unmethylated regions (Rollins et al., 2006). Unmethylated clusters were enriched in the 5' regions of genes, promoters, CGIs and first exons. Moreover, this was also found in animal analysis of distribution of small DNA fragments generated by restriction endonucleases sensitive to DNA methylation. Unmethylated regions were usually found at promoters and CGIs in a 6.2-Mb segment of the mouse genome (Khulan et al., 2006). CGIs have a rich GC base composition unlike the rest of the genome, which is AT-rich. They have a length of ~1000 bp and are often associated with genes. About 56% of human genes have CGI promoters (Antequera and Bird, 1993).

A recent study has shown a “on and off” type of DNA methylation pattern by studying 1.9 million CpG sites within the human chromosomes 6, 20 and 22 (including 373 genes) in 12 different tissues (Eckhardt et al., 2006). The majority of DNA sequences studied turned out to be either relatively hypomethylated (< 30% of studied CpG sites) or hypermethylated (> 70% of studied CpG sites). This pattern suggested two alternative states of genes: active (hypomethylated) or silenced (hypermethylated). The biological rationale for this “on and off” switch in DNA methylation needs to be elucidated.

The CGIs in the human genome are usually identified by computational prediction. Most commonly, the criteria require a GC content of at least 55% and a ratio of observed to expected CpG frequency of at least 0.6. By such prediction, about half of all CGIs are found at the transcription start site (TSS) of annotated genes, the remainder located downstream of TSS or in the gene body (Illingworth et al., 2008). The biological significance of intergenic CGIs remains to be elucidated. Moreover, although a majority of CGIs in the human genome are unmethylated, a number of reports have shown methylated CGIs, ranging from 3 to 12% of the total number of CGIs analyzed (Eckhardt et al., 2006; Illingworth et al., 2008; Weber et al., 2007). Naturally, some of the genes connected to methylated CGIs belong to developmental genes, such as homeobox (HOX) genes and paired box (PAX) genes (Illingworth et al., 2008). Yet, other genes with adjacent methylated CGIs lack biological explanations. However, it is obvious that CGI methylation is a widespread phenomenon in human somatic tissue.

Recent interesting findings have suggested a CpG density association to level of transcriptional regulation. Microarray analysis of the TSS of 16 000 annotated genes revealed that CGI promoters predominantly remained unmethylated regardless of expression (Weber et al., 2005; Weber et al., 2007). Most dynamic with regards to DNA methylation were promoters with an intermediated CpG density, so called weak CpG islands. Those weak CpG islands frequently acquired DNA methylation in somatic tissue. This raises the question whether such CGIs more reflect a dynamic characteristic of gene regulation of larger DNA domains of which they are part.

Biological significance of DNA methylation

The biological significance of DNA methylation is still debated. To date, no mechanistic explanation model between DNA methylation and the transcription process has been proposed. In plants and fungus, it seems that DNA methylation is primarily a genome defence mechanism, since transposable elements are evidently specific targets and are prevented from transposition by this modification (Slotkin and Martienssen, 2007). If the genome defence mechanism theory also apply to animals has not been established. Methylation studies in the different organisms *C. Intestinalis* (Simmen et al., 1999; Suzuki et al., 2007) and the bee (Wang et al., 2006) indicate that genes, rather than transposons, are target of CpG methylation. The mammalian genome is so densely covered by CpG methylation, that is why it is not clear whether transposons are actively targeted or if they become passively methylated. However, manipulating mammalian cells to a hypomethylated genome does not increase the level of transposition, as should be the case if the defence hypothesis holds true (Wilson et al., 2007). Therefore, current data so far indicates that CpG methylation exerts its function on genes rather than elsewhere in the genome. Different DNA methylation signatures between brain regions further supports the role in biological function as there would be expected differences in gene expression as well as in protein content due to the unique roles each brain region has (Ladd-Acosta et al., 2007).

1.2.2 DNA methylation in alcoholism

Theories on altered DNA methylation

Acetaldehyde, the first oxidation product of ethanol, is usually metabolized to acetate by acetaldehyde dehydrogenases. However, if this process is saturated by accumulation of acetaldehyde by heavy alcohol load, acetaldehyde may inhibit catalyzing enzymes responsible for methylation of DNA and proteins. It is possible that this may be one of the mechanisms behind the fetal alcohol syndrome (Garro et al., 1991), since lowering of DNA methylation may greatly affect regulation of several genes important for normal development (Reik and Dean, 2002)}. Long term alcohol exposure may affect gene regulation also in the adult brain by similar mechanisms.

There are also dietary consequences of long term and heavy alcohol consumption. These include severe effects on folate metabolism since chronic alcohol consumption often is followed by thiamine and folate deficiency, due to both lowered intake of healthy food and to a deteriorated nutrition uptake in the intestine (Hamid et al., 2007). This can induce altered DNA methylation, as well as change the normal histone methylation pattern, since folate acts as a methyl donor, via S-adenosylmethionine (SAM), for both DNA methyltransferase (Halsted et al., 2002; Sokolov et al., 2003) and histone methyl transferase (Huang, 2002).

Epigenetics in the alcoholic brain

The previously described alcohol-induced alterations could lead to disturbed DNA- and histone methylation. Numerous studies have reported epigenetic changes in alcoholic tissue, mainly in blood cells and liver. Bönsch and colleagues have found that the elevated homocysteine concentration in alcoholics is associated with global DNA hypermethylation (Bonsch et al., 2004) in peripheral mononuclear cells, decreases in mRNA expression of DNA methyltransferases DNMT3a and DNMT3b (Bonsch et al., 2006), and DNA hypermethylation of the alpha synuclein promoter (Bonsch et al., 2005), a gene linked to alcohol preference and craving (Liang et al., 2003; Mash et al., 2003). Moreover, they have also found direct regulation of another gene suggested to be important for neuronal Ca²⁺ signaling: HERP (homocysteine-induced endoplasmic reticulum protein). Decreased HERP mRNA levels correlated with increased homocysteine levels (Bleich et al., 2006) and increased promoter methylation together with an association to AARE (amino acid response element) and CREB binding sites within the HERP promoter *in vitro* (Lenz et al., 2006).

Decreased DNA methylation with a concomitant decrease in DNA methyl transferase activity after ethanol exposure of pregnant rats has been reported in fetal tissues (Garro et al., 1991). There is also a report of genomic DNA hypomethylation in the rat colonic mucosa and rat liver tissue after chronic alcohol ingestion (Choi et al., 1999; Lu et al., 2000). Apparently, both global DNA hyper- and hypomethylation have been detected

as a consequence of alcohol abuse, however only hypermethylation of promoters investigated have been detected, independently of DNMT expression.

Only a few studies have documented neuronal epigenetic changes caused by ethanol. One study shows that prenatal exposure to ethanol reduces Glial Fibrillary Acidic Protein (GFAP) immunoreactivity and its mRNA levels in both astrocytes in primary culture and in brains from pups from alcohol-fed mothers (Valles et al., 1997). The fetal brains were also hypermethylated in the GFAP gene. GFAP is a cytoskeletal protein important for the morphogenesis in central nervous system (CNS). Another study detected hypomethylation of the NMDA receptor NR2B promoter and a decrease in expression of NR2B gene in adult cortex and cultured fetal cortical neurons after chronic ethanol treatment, however not after acute ethanol treatment (Marutha Ravindran and Ticku, 2004, 2005). To date, there is one publication on histone methylation, H3-K9-Me, after acute alcohol intragastric administration in rat. Results showed little (insignificant) changes in all 14 tissues analyzed (Kim and Shukla, 2006).

1.3 SCREENING FOR DNA METHYLATION

During recent years several techniques to study DNA methylation have emerged. Those include technologies to study global DNA methylation as well as gene specific DNA methylation. Gene specific DNA methylation techniques often apply microarray platforms for genome-wide detection. To be able to distinguish cytosine from methyl-cytosine, a pre-treatment procedure of the genomic DNA is required for detection. In principal, methylated DNA is separated from unmethylated DNA and subsequently analyzed in parallel. Currently, four different pre-treatments are frequently used for either global or gene specific DNA methylation detection. Those include A) digestion of genomic DNA with methylation sensitive or insensitive restriction endonucleases (E.g. HpaII, MspI, BstUI), B) chemical treatment of genomic DNA using sodium bisulphite, C) immunoprecipitation with a methyl-binding antibody (MeDIP), or D) protein precipitation with methylated DNA affinity binding proteins (e.g. MBD2B and MBD3L1) (Figure 4). Bisulphite genomic sequencing has by far the highest resolution, with single base resolution (Frommer et al., 1992). However, not until recently has it been possible in terms of accuracy, time and work effort to make a whole genome sequencing of methylation signature DNA. A new technology, 454 sequencing, has now made it possible to now sequence bisulphite treated DNA of the whole genome (Taylor et al., 2007).

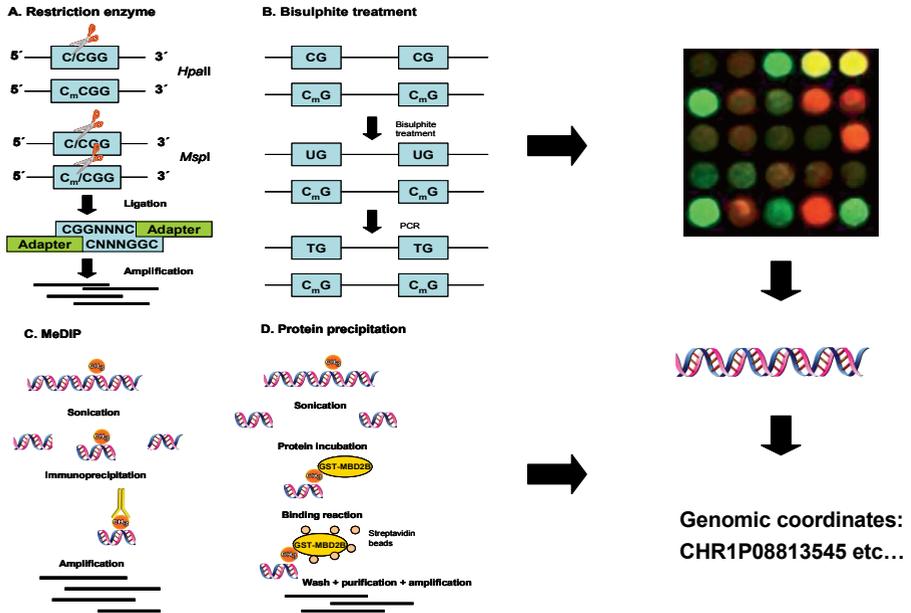


Figure 4. Genome-wide methods for studying DNA methylation. A | Genomic cleavage by methylation sensitive endonuclease restriction enzymes (e.g. *HpaII*). B | Sodium bisulphate converts unmethylated cytosine bases to uracil leaving methylated cytosines unchanged. C | An antibody specific for methylated cytosines is used to pull down methylated DNA. D | Methyl-binding proteins are used to precipitate methylated DNA. After pre-treatment of genomic DNA to separate methylated DNA from unmethylated DNA, the sample is either hybridized to a microarray or sequenced to detect level of gene specific DNA methylation.

A number of methods used to measure DNA methylation at multiple gene loci have been reported, either using methyl-specific antibodies (Methylated DNA Immunoprecipitation, MeDIP) (Weber et al., 2005), methyl-binding proteins (Differential Methylation Hybridization, DMH; Methylated-CpG Island Recovery Assay MIRA) (Rauch and Pfeifer, 2005; Yan et al., 2002), endonuclease restriction enzymes targeted for CpG sites (Restriction Landmark Genomic Scanning, RLGS; Methylation-specific digital karyotyping, MSDK, or *HpaII* tiny fragment Enrichment by Ligation-mediated PCR, HELP) (Hatada et al., 1991; Hu et al., 2005; Kawai et al., 1993; Khulan et al., 2006), or sodium bisulphite treatment (BeadArrays, Illumina) (Fan et al., 2006). All techniques have their advantages and disadvantages. Once the DNA is separated in fractions of methylated and unmethylated DNA by the above mentioned pre-treatments, the two pools are usually co-hybridized to a microarray, designed with either promoter/gene associated probes, or whole genome covered probes. If not hybridized to a microarray, the DNA can be sequenced, or analyzed by mass spectrometry or real-time PCR. A summary of microarray platforms, to screen for DNA methylation in genomic DNA, is provided in table 2.

Table 2. Summary of commonly used microarray platforms for gene loci specific DNA methylation analysis.

Company	Array type	Genes/RefSeq	Probes	Coverage	Resolution	µg DNA/WGA	Cost
Affymetrix	1. Human Tiling 2.0R 2. Human Promoter 1.0R	1. Whole genome 2. 25,500 promoters	1. 4.5 million 2. 4.5 million	1. Whole genome 2. 7.5 Kb US + 2.45 Kb DS of TSS, 59 % of CGIs	35 bp (2.5 met, 10 bp gap) ENCODE: 7 bp	3.6 µg ChIP material can be amplified by random priming PCR or other WGA (20 ng).	\$ 2225
Roche NimbleGen	1. Whole genome tile across all unique regions 2. Promoter 3. CGIs + Promoter 4. Custom promoter Tiling arrays	1. WG 2. RefSeq + USCS 3. UCSC + CGIs + RefSeq	All: 385,000 and 2,1 million (HD2)	1. Whole genome 2. 2.2 to 9 Kb US + 500-2000 bp gene 3. 800 bp US, 200 bp DS 4. 10-12.5 Kb US + 7.7-10 Kb DS	1. < 100 bp probe spacing 2. 100-200 bp spacing 3. 100-200 bp 4. 5 bp (50 mer)	200 ng-5 µg WGA, LM-PCR or T7 amplification Then even less DNA If 2.5 µg is enriched no amplification necessary.	\$ 500-1000 (re-use 3 times ~ \$170) Whole genome: \$ 475/array
Agilent Illumina	Inkjet Bead Arrays 1. Human Methylation27 2. GoldenGate Methylation Panel 3. Custom CpG panels 4. Infinium Methylation Panel	1. 27,578 CpG loci > 14,000 genes 2. 1,536 loci/sample of 807 genes	22,000	Selective	195 bp (WG) 1 bp /12 samples per array	3.5 µg 500 ng pre-bisulphite conversion	\$ 489/array \$ 225 /sample

Abbreviations: US: upstream, DS: downstream, CGI: CpG island, WGA: whole genome amplification, LM-PCR: ligated mediated PCR, ChIP: chromatin immunoprecipitation.

2 AIMS OF THESIS WORK

The overall aim of this thesis work has been to study epigenetic and molecular alterations as a consequence of alcoholism, and to elucidate the functional role of DNA methylation in relation to changes in gene activity in the alcoholic brain.

More specifically, the aims were:

- To establish a robust normalization system for quantitative analysis of gene expression in alcoholic brain tissue by analyzing a panel of endogenous control genes using a mathematical approach.
- To study molecular and biochemical alterations in the programmed cell death signaling, in the prefrontal and motor cortices of *post mortem* human chronic alcoholics.
- To develop a high-throughput technique to study global DNA methylation.
- To study global and gene specific DNA methylation alterations in the *post mortem* alcoholic brain and relate changes to gene transcription activity.

3 MATERIALS

This section will describe the material used for studying molecular and epigenetic alterations in alcoholic brain tissue. Additional material, such as cell cultures and plasmids used in paper III will not be discussed in the following section.

3.1 HUMAN ALCOHOLICS

Human brain tissue from *post mortem* chronic alcoholic individuals in the present thesis was obtained from the New South Wales Tissue Resource Center (TRC), University of Sydney, Australia (Harper et al., 2003; Harper and Matsumoto, 2005). There, a human brain bank has been established specifically from alcohol dependent individuals, with or without the Wernicke-Korsakoff syndrome. TRC can provide researchers with fresh, frozen or paraffin embedded brain sections carefully sectioned into the specific brain regions. The alcoholics used in most experiments in this thesis meet DSM-IV criteria as described in the introduction, but also additional stringent selection criteria defined by TRC. The *post mortem* tissue from alcohol dependent subjects also:

- did not have a history of multi drug abuse
- had no prolonged agonal life support
- had no history of cerebral infarction, head injury, or neurodegenerative disease (e.g. Alzheimer's disease).
- had no cases used in this study were diagnosed as Wernicke-Korsakoff syndrome
- had and age greater than 18 years
- had no developmental disorder
- had no history of other psychiatric or neurological disorder
- had a negative screen for AIDS and Hepatitis B/C
- had no obvious brain abnormalities on gross examination

During the majority of their adult lives, the alcohol consumption was greater than 80 g per day, except in one case with a daily alcohol consumption of 60 g. Control cases are matched to alcoholic cases by sex, age, race and Post Mortem Interval (PMI), and had alcohol consumption less than 10 g per day, except in one control case who consumed 35 g of alcohol per day. Mean values of demographic information is provided in Table 3. For detailed demographic information, see Table 1 in paper IV.

Table 3. Demographic data on human subjects used for analyses

Group	Controls	Alcoholics
Number	15	15
Age, year	58.80 ± 14.5 (34-82)	58.87 ± 14.0 (34-81)
Gender	Male	Male
PMI, hours	26.9 ± 16.4 (6.5-50)	31.8 ± 15.1 (8.5-60)
pH	6.46 ± 0.29 (5.88 – 6.9)	6.47 ± 0.27 (5.66 – 6.78)
Ethanol (blood)	n = 0	n = 2

Values presented as mean ± SD and range is shown in parentheses.

Abbreviations: PMI, post-mortem interval.

Control cases were patients with no neurological abnormalities or neuropathological disorders (stroke, Alzheimer's disease etc). All individuals, both alcoholics and controls in the thesis, were male Caucasians. Regions used for analysis included prefrontal cortex (superior frontal gyros, Brodmann area 9) and motor cortex, Brodmann area 4 (Figure 5). All tissues were obtained after written consent of the next of kin for each individual. The study was approved by the local ethical committee of the Karolinska Institutet, (permit 04-849/4).

A. Prefrontal cortex



B. Motor cortex

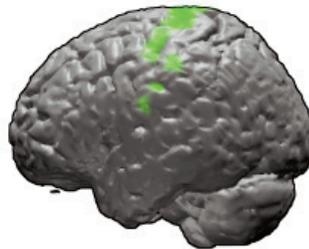


Figure 5. Human alcoholic brain regions used for molecular and DNA methylation analysis. **A** | Prefrontal cortex, superior frontal gyrus of Brodmann area 9. **B** | Motor cortex, Brodmann area 4.

3.2 RODENT MODEL OF ALCOHOL DEPENDENCE

3.2.1 Dependence model

In addition to human alcoholic subjects, an experimental rat model of alcohol dependence was used in the thesis work. A history of alcohol dependence was modeled in laboratory rats using prolonged exposure to alcohol vapor, which triggers long-lasting neural and behavioral plasticity that appear relevant for modeling human alcoholism. This type of manipulation produces persistently increased alcohol intake in genetically nonselected rats (Rimondini et al., 2002; Roberts et al., 2000). Exposure to repeated cycles of intoxication and withdrawal, which mimics the course of the clinical

condition, is most effective for inducing increased alcohol drinking (O'Dell et al., 2004; Rimondini et al., 2002). Similar to the human condition, a minimum duration of dependence is required for lasting upregulation of alcohol preference (Rimondini et al., 2003). Elevated alcohol intake in postdependent rats is sensitive to the clinically effective compound, acamprosate, while alcohol intake of nondependent rats is unaffected by the same treatment (Egli, 2005; Heyser et al., 1998; Rimondini et al., 2002; Spanagel and Zieglgansberger, 1997). Furthermore, the postdependent state is characterized by a persistently upregulated behavioral sensitivity to stress (Sommer et al., 2008; Valdez et al., 2002; Weiss et al., 2001).

3.2.2 Ethanol vapor exposure

Wistar rats (Møllegaard, Denmark) from the alcohol dependent animal model, were used in the global DNA methylation experiments (paper IV). Rats weighed 220–250 g at the beginning of the experiment and were housed 4 per cage under a reversed light/dark cycle (light of 11 A.M., light on 11 P.M.) with free access to food and water.

The exposure was as follows: 1 week of habituation to the chambers (no alcohol), 1 week of continuous exposure to 22 mg/L alcohol to adapt to the novel odor and 7 weeks of exposure to alcohol vapor adjusted to produce blood alcohol concentrations (BACs) of 150–320 mg/dL for 17 h (16:00–09:00 h) each day. Once a week during exposure, rats were weighed and blood was collected from the tail veins. Serum was assayed for alcohol (mg/dl) using an Analox system (Analox Instruments, Ltd, Lunenburg, MA, USA) according to the manufacturer's instructions. Control rats were housed under the same conditions, except for the addition of ethanol vapor to the air flow. Ethanol vapor exposure was followed by a 3-week period of abstinence in order to eliminate effects of acute withdrawal. Following completion of withdrawal period, control and exposed animals were sacrificed by decapitation, brains rapidly removed and dissected out before rapidly frozen to -70°C.

To prove that ethanol vapor exposure induced a high drinking behavior, after 3 weeks with no access to alcohol, eight randomly sampled naïve animals from the exposed and control groups were tested for voluntary alcohol consumption in a 0.2% saccharin solution. Briefly, alcohol concentration was increased as follows: day 1–3: 2% alcohol; day 4–7: 4% alcohol; day 8 onward: 6% alcohol (v/v solutions). Animals were given a continuous two-bottle free choice between alcohol-saccharin solution and saccharin solution. Consumption of each solution was measured on Monday, Wednesday and Friday at the same time. Bottle positions were alternated daily to avoid development of place preference. All animal experiments were approved by the institutional review board and conformed to the established European Community guidelines for the care and use of animals (ethics permit S84/98, Stockholm South).

4 RESULTS AND DISCUSSION

OPTIMAL NORMALIZATION FOR GENE QUANTITATIVE ANALYSIS IN HUMAN ALCOHOLIC BRAIN (PAPER I)

CONCLUSIONS: for reliable gene quantitation analysis, brain region specific endogenous control genes should be selected for normalization, since genes were found to have unique expression “stability” within each brain region. Moreover, some genes should also be avoided due to significantly different levels of expression between controls and alcoholics. The results from this study are important since they may also apply to other physiological or pathological conditions.

This initial study of the thesis aimed at finding the optimal normalizing procedure for quantitative analysis of gene expression in two brain regions of human chronic alcoholics. This was an important first step before any other quantitative analysis could be performed, in order to avoid biased results due to incorrect normalization. Frequently in gene expression studies, endogenous control genes are selected randomly, often based on previously published studies. However, different physiological conditions such as alcohol dependence might in fact alter the activity of so called “housekeeping” genes, often used as endogenous control genes. For example, transferrin receptor (*TFRC*) used for quantitation in alcoholics would be risky since the transferrin gene has been shown differentially expressed in prefrontal cortex of alcoholics previously (Liu et al., 2004). To evaluate the possibility of different expression stability, we screened the expression pattern of 16 commonly used endogenous control genes in prefrontal and motor cortices of human chronic alcoholics.

One problem arise when the expression of endogenous controls genes are evaluated since no normalization of them can be performed in order to adjust for technical differences, such as pipetting errors and cDNA synthesis efficacy. Therefore, we applied a mathematical approach by using the geNORM program (Vandesompele et al., 2002), which calculates the geometric mean, a normalization factor, of the number of genes required for a stable quantitation. “Stable” genes are those whose expression ratios are minimal across samples analyzed. Logically, the more genes used for normalization the better, since this will be approaching the true mean value across genes and samples. However, using a panel of control genes for every experiment would be costly and work intense. Thus, measuring the expression stability of several genes in an initial experiment, to evaluate which genes and the number of genes necessary for accurate normalization, is a good approach. The number of genes required may vary between physiological conditions and is determined by geNORM.

By screening 16 different genes, in prefrontal and motor cortices of human chronic alcoholics and controls, we could reveal brain region specific gene expression stability. Importin 8 (*IPO8*) and RNA polymerase II (*POLR2A*) were found to be most stably expressed across the samples in motor cortex, while beta-actin (*ACTB*) and ribosomal large P0 (*RPLP0*) represented the same in prefrontal cortex. Thus, each brain region was unique in its representation of stable reference genes for normalization. However, one should remember that the M-value rank used by geNORM in the evaluation is not quantitative and only reflects the relationship in stability between the genes analyzed. Hence, it is likely that other genes within the lower M-value range may be reliable to use, especially in combination of two or more genes for normalization. It is clear that increasing the number of control genes will improve the accuracy of normalization, as shown by the decreased V-value by addition of genes in the pairwise variation plot (Figure 1 C and D in paper I). However, this is leveled out around the addition of the 8th or 9th gene (V8/9), as added in the order most stable to least stable, indicated from the plots in Figure 1 A and B. This is indicating that the addition of genes with less stable expression will not improve the accuracy of normalization, although it will also not worsen the normalization as the V-value is kept rather unchanged. The explanation for this is probably that within-group differences, as reflected by a high M-value, are evened out by using several genes.

Our analysis could also reveal significantly different level of gene expression in both motor and prefrontal cortex for a number of genes. In the motor cortex, hypoxanthine phosphoribosyl transferase (*HPRT1*), phosphoglycerate kinase (*PGK1*) and peptidylprolyl isomerase A (*PPIA*) were shown to have an increased gene expression in the alcoholics ($p = 0.04$; 0.01 and 0.007 respectively). In the prefrontal cortex, beta-2-microglobulin (*B2M*) were decreased in the alcoholics compared to controls ($p = 0.03$).

Difference in gene expression stability, i.e. with marked within-group differences, reflects brain regional differences, while the genes differentially expressed between groups reflect a change as a result of alcohol dependence. The reason for either of these differences presented here could only be speculative. It is likely that different brain regions possess gene expression differences since they exert different functions in the brain. Genome wide expression studies have revealed brain region specific gene expression patterns, as well as common expression signatures within the brain (Ernst et al., 2007; Ladd-Acosta et al., 2007; Roth et al., 2006). Moreover, a microarray expression study of the alcoholic brain revealed expression signatures with very little in common between the brain regions analyzed (Flatscher-Bader et al., 2005). Known alcohol-responsive genes were analyzed in the VTA, NAc and the PFC. Out of the 125, 68, and 51 genes found in each region, respectively, none of those genes were overlapping between all three regions. Furthermore, only 4% were common for any two brain region, and VTA and NAc were shown to exhibit similar expression patterns in terms of function while prefrontal cortex had a unique expression pattern. Brain region specific gene expression has also been shown in the dopaminergic system of the

brain with distinct expression in crucial neurotransmission pathways (Olsen et al., 2008).

Alcohol consumption may possibly alter the gene expression of several abundant proteins by several mechanisms. One possible explanation for the difference in *PPIA* expression, encoding the protein cyclophilin A, may be its previously reported involvement in inflammation by responding to oxidative stress (Jin et al., 2004), also frequently occurring in alcoholic tissue (Albano, 2006). The *PGK1* gene has been shown to respond to hypoxic states by being a downstream regulator of hypoxia-inducible-factor-1alpha (HIF-1alpha). Alcohol has been shown to accelerate the hypoxic state in fetal tissue by attenuating the cerebral blood flow in fetuses, and thereby the oxygen delivery (Mayock et al., 2007). Thus, hypoxic state in the alcoholic brain may explain altered gene expression of *PGK1*. Furthermore, both *B2M* and *HPRT1* gene have been reported in different pathological conditions (Jordanova et al., 2003; Yamada et al., 2008) indicating their unreliability as endogenous control genes.

ANALYSIS OF THE CELL SUICIDE MACHINERY IN THE ALCOHOLIC BRAIN (PAPER II)

CONCLUSIONS: levels of gene expression and proteins of key components in apoptotic signaling found in this study, did not suggest any occurring programmed cell death in the alcoholic brain. On the contrary, our findings suggest a molecular adaptation to alcohol that may possibly counteract alcohol neurotoxicity after years of heavy alcohol consumption.

The second aim in the thesis (Paper II) was to investigate possible involvement of the apoptosis machinery in alcoholism, possibly contributing both to the addictive state and the cognitive aspects of the disease by neurodegenerative processes. Structural alterations such as reduced gray and white matter volume (de la Monte, 1988; Fadda and Rossetti, 1998; Harper et al., 1985), and reduced neuronal and glial cell density (Harper et al., 2003; Kril et al., 1997; Miguel-Hidalgo et al., 2002) may be a result of increased activity of the cell's suicide machinery as a response to alcohol neurotoxicity. Important components of cell death and cell protection were analyzed in terms of gene expression and protein content. The findings in Paper I was important for all the gene quantification analysis performed in this study.

We found a protein reduction of activated caspase-3 ($p = 0.017$) and increased levels of BCL-2 ($p = 0.073$, trend), key proteins for cell death and cell protection respectively, exclusively in the prefrontal cortex of alcoholics. This protein change may underlie a cell protective function rather than cells undergoing apoptosis. This observation was further supported on a gene expression level, where we analyzed additional genes involved in similar functions. An apoptosis-inducing factor (*AIF*), a killer molecule inducing multiple mitochondrial dysfunctions (*BID*), and a gene involved in delayed

neuron death (cyclin D1) were all found down-regulated in the prefrontal cortex of alcoholics ($p = 0.001$; $p = 0.031$, and $p = 0.010$ respectively). Again, no such change was evident in the motor cortex region. However, a gene indirectly involved in apoptotic signaling by suppressing caspase activity was also downregulated – *BIRC2* ($p = 0.002$). Moreover, other proteins analyzed such as BAX, p53 (both cell suicide proteins) and BCL-X_L (cell protection) did not reveal any change in the alcoholic brain. The findings all together suggest that specific signaling pathways of the cell death/protection systems are targeted by alcohol consumption, specifically in the prefrontal cortex. The altered proteins appear to be involved in the intrinsic apoptotic pathway or are essential for delayed cell death. The intrinsic pathway represents a response to internal imbalance, e.g. cellular stress, rather than external stimuli such as toxins, cytokines, hormones and growth factors. Release factors for the intrinsic pathway might be nuclear receptors binding glucocorticoids, radiation, heat, aberrant nutrition balance, viral infection and hypoxia (Robbins). Both nutrition deficiency and hypoxia have been reported in alcoholic tissue (Hamid et al., 2007; Kim et al., 2007; Mayock et al., 2007).

The findings may suggest several things. First, no apparent cell death activity is ongoing in the *post mortem* tissue analyzed. Second, this may indicate cellular and molecular adaptations after many years of heavy alcohol drinking and/or attempts to retain cellular homeostasis. Third, it may also reflect a cellular recovery during abstinent periods, which may well be the case at the time of death in the alcoholic individuals analyzed. This is a fact very difficult to control. Furthermore, the findings may indicate inherited molecular differences that may partly underlie etiological manifestations of alcoholism. Finally, it may also reflect *post mortem* processes of cell death.

DEVELOPMENT OF A NOVEL TECHNIQUE TO STUDY GLOBAL DNA METHYLATION IN THE ALCOHOLIC BRAIN (PAPER III)

CONCLUSIONS: by using endonuclease restriction enzyme cleavage of genomic DNA in combination with a polymerase extension step by Pyrosequencing™, we developed a high-throughput method to measure global DNA methylation – Luminometric Methylation Assay (LUMA).

The next aim of the thesis (Paper III) was to develop a sensitive technique to globally study DNA methylation under various normal and pathological conditions, including the alcoholic brain, as an initial approach of the epigenetic analysis in chronic alcoholics. However, available techniques either required large amounts of DNA, were work intense or involved work with isotopes. Therefore, we introduced the pyrosequencing platform in combination with methylation sensitive restriction enzyme cleavage, an approach which enabled high-throughput analysis with minimal DNA

amount requirements as well as usage of an internal control independent of CpG methylation. This was crucial, since limitation of tissue material is always a concern when working with human material.

Although global DNA methylation methods do not give detailed information about genomic positions and genes affected, important information can still be generated. As demonstrated in a related publication by us, “Impact of inflammation on epigenetic DNA methylation - a novel risk factor for cardiovascular disease? ”, the level of global DNA hypermethylation in chronic kidney disease patients is significantly correlated to the level of inflammation and survival prognosis (Stenvinkel and Ekstrom, 2008; Stenvinkel et al., 2007). This illustrates how global DNA methylation may be used in early diagnosis and as a prognostic factor.

The LUMA procedure includes two experimental parts. First, 200-500 ng of genomic DNA is enzymatically cleaved in two separate reactions, one including the methylation sensitive restriction endonuclease *HpaII* (reaction A), and the other its isoschizomer enzyme *MspI* (reaction B). Both enzymes have the target sequence CCGG and leave a 5'-CG overhang, however if the internal cytosine is methylated, C_mCGG, *HpaII* will not cut. Each reaction also includes the internal control, *EcoRI*, with the recognition sequence GAATTC, leaving a 5'-AATT overhang completely independent of CpG methylation. The DNA is incubated for 4 hours at 37° C to ensure complete digestion. The second part involves detection of nucleotide incorporation into the genomic overhangs, 5'-CG and 5'-AATT. The pyrosequencing instrument (PSQ™MA) is programmed to add dNTPs in four consecutive steps including Step 1: dATP; Step 2: mixture of dGTP + dCTP; Step 3: dTTP; and Step 4: mixture of dGTP + dCTP (Figure 6). Following each step of nucleotides added, inorganic pyrophosphate (PPi) is released and converted to ATP by ATP-sulfurylase and adenosine-5'-phosphosulfate. ATP then drives the conversion from luciferin to oxyluciferin by luciferase which generates a light that is proportional to the amount of PPi and thus dNTPs incorporated into the cut DNA. This is followed by peak height measurements as detected by a charge couple device (CCD) camera. Peak heights as well, are directly proportional to the level of dNTP incorporation. Step 1 and step 3 of nucleotide addition reflect the level of *EcoRI* cleavage, and similarly, step 2 reflects *HpaII* or *MspI* cleavage, from each A and B reaction respectively. Hence, by calculating $(HpaII/EcoRI)/(MspI/EcoRI)$, the level of global DNA methylation will be estimated.

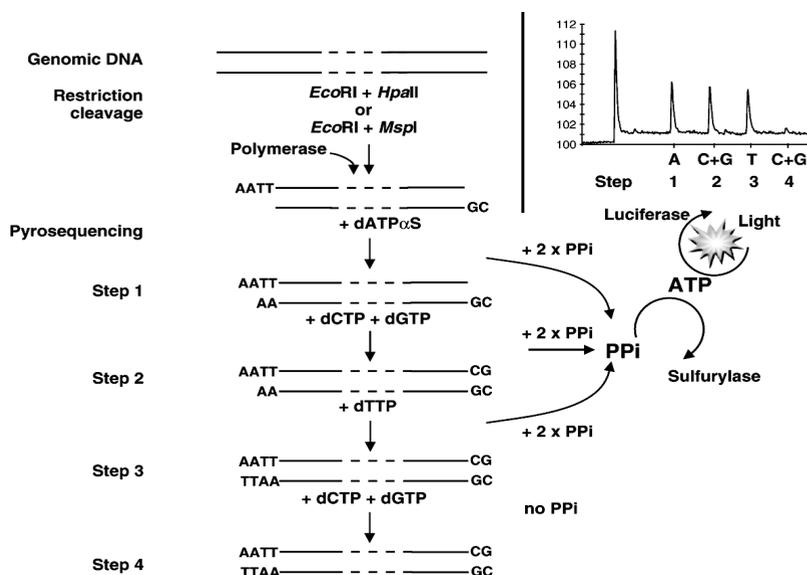


Figure 6. Experimental process of the LUMA assay developed by our lab and described in paper III in the thesis. Genomic DNA is cleaved by C_m CGG methylation sensitive and insensitive restriction enzymes (*HpaII* and *MspI*) together with an internal reference enzyme, *EcoRI*. DNA overhangs created by each enzyme are filled with dNTPs in consecutive steps using the Pyrosequencing™ technology. Peak heights detected by a CCD camera are directly proportional to the number of nucleotides incorporated. *HpaII* and *MspI* cleavage (C + G) is compared to *EcoRI* (A and T) cleavage by calculating the ratio ($HpaII/EcoRI$)/($MspI/EcoRI$). This will reflect the level of global DNA methylation.

By using serial dilutions of pBlueScript plasmid (125 ng to 2000 ng) we could show a high method sensitivity using different DNA concentrations by a good linearity between peak height and DNA concentration. Moreover, DNA methylation ranging from 0 % to 100 % using different proportions of unmethylated and methylated lambda DNA showed a good inverse correlation between the level of methylation and *HpaII/MspI* ratio ($R^2 = 0.98$), further indicating high accuracy. In addition, we could show a linear correlation between *HpaII/MspI* ratios and *HpaII/EcoRI* ratios, indicating that *MspI* is not necessary to measure level of DNA methylation. This notion was applied in further analysis of human alcoholics (Paper IV), by only performing the *HpaII/EcoRI* reaction, to minimize the usage of DNA.

ANALYSIS OF GLOBAL AND GENE SPECIFIC DNA METHYLATION IN THE ALCOHOLIC BRAIN IN RELATION TO AFFECTED BIOLOGICAL FUNCTIONS (PAPER IV)

CONCLUSIONS: the alcoholic brain is overall deprived in CpG methylation, with specificity to brain regions important for reward and cognition. In paper IV we can show this by both global – using the human alcoholic autopsy material and in rats with

a history of alcohol dependence – as well as in gene specific analysis in the human samples. We further show how DNA hypomethylation significantly correlates with gene expression in a number of genes demonstrating a functional role of DNA methylation. In addition, functional annotation analysis revealed expected groups affected by altered DNA methylation, such as acetaldehyde dehydrogenases, DNA- and protein methylation regulators and previously reported insulin signaling and acetylcholine receptor involvement. This implicates the importance to study DNA methylation in aspects of biological function. Finally, a novel pathway possibly involved in alcoholism pathology, DRPLA, was found by filtering analysis. Interestingly, this pathway is highly involved in a neurodegenerative disorder, Pallidoluysian atrophy, characterized by clinical features very similar to alcoholism – epilepsy, cerebellar ataxia, dementia and involuntary reflexes and movements.

The final aim of the thesis (Paper IV) was to investigate epigenetic alterations in the alcoholic brain by studying the interrelationship between DNA methylation and molecular changes. This was performed by employing previous conclusions about normalization (Paper I) and the development of LUMA (Paper III), in addition to a gene specific methylation assay (HELP). The hypothesis was that heavy alcohol consumption, by a direct inhibition of DNA methyltransferases through acetaldehyde, and/or through a folate deficiency and reduced S-adenosyl-methionine (SAM) activity, may result in an aberrant DNA methylation. Altered DNA methylation could possibly be a genome-wide and/or a gene-specific phenomenon, involving the whole brain or targeted brain regions. To find an answer to this question, global DNA methylation was assessed using LUMA developed during the thesis work, in addition to a methylation gene specific microarray assay (*HpaII* tiny fragment Enrichment by Ligation-mediated PCR, HELP). Moreover, two brain regions were used from *post mortem* chronic alcoholics in addition to multiple brain regions from a rat model of alcohol dependence. To find out about the functional relevance of studying DNA methylation, as discussed in the light of current knowledge in the introduction, we compared gene specific methylation data to gene expression activity of a number of genes by RT-PCR analysis.

Our first finding was that the alcoholic brain indeed was aberrantly methylated as hypothesized. As suspected, heavy alcohol consumption seem to decrease DNA methylation as was seen in both humans and rats. On a global level, the human prefrontal cortex and the rat striatum showed a lower level of DNA methylation compared to controls ($p = 0.045$ and $p = 0.07$, trend). However, human motor cortex and rat occipital and parietal cortex did not reveal any significant reduction in global DNA methylation. Although, when the human tissue was continuously analyzed by gene specificity, a majority of gene loci were hypomethylated in both prefrontal and motor cortices, indicating a hypomethylation in both brain regions by this technique. We think the explanation for this is reflected by technical differences interrogating partly different genomic sequences. Since both techniques employ the enzymatic cleavage by *HpaII* and *MspI* endonucleases, sensitive and non-sensitive to DNA

methylation respectively, one would argue that they should yield the same results. However, an amplification process in the HELP assay distinguishes it from LUMA by only representing a fraction of the *HpaII* and *MspI* sites measured by LUMA. Moreover, results generated from HELP will primarily represent annotated genes due to the probe design, whereas LUMA may reflect methylation changes in other regions such as repetitive elements and intronic regions. In conclusion, alcoholism seem to decrease DNA methylation overall throughout the genome, however with a general specificity to brain regions involved in reward and cognition (prefrontal cortex and striatum). This may indicate a greater vulnerability of those regions and reflect actual biological changes as a consequence of alcohol dependence, as those regions play a major role in the development of addictive disorders.

Our second finding involved a strong functional association to DNA methylation. The level of DNA methylation was inversely correlated to level of gene transcription activity in 14 genes analyzed ($r = 0.40$, $p = 0.00001$). Specifically, the myelin basic protein (*MBP*) was found hypermethylated with a subsequent decreased mRNA expression ($p = 0.04$). This finding is supported by previous findings of *MBP* reduction in chronic alcoholics (Lewohl et al., 2005; Liu et al., 2004; Mayfield et al., 2002) in addition to a white matter loss, mainly in the prefrontal cortex (de la Monte, 1988; Harper, 1998). Proteolipid protein (*PLPI*), another important protein for the myelin sheet, was also found to have decreased gene expression; however no significant DNA methylation change was evident. This was further noted when all 14 genes were analyzed separately, as not all genes had a strong correlation between transcriptional activity and DNA methylation. Altogether, this supports the previous general perception that DNA hypomethylation is positively regulating gene transcription, at least in part. The data also suggest that other transcriptional regulators, such as histone modifications, probably co-regulate gene activity. A cross-talk between DNA methylation and histone modifications has been proposed in other studies after demonstration of e.g. co-reactivity of DNA methyltransferases and histone deacetylases (as reviewed in (Vaissiere et al., 2008)). Another explanation model is that DNA methylation changes may not directly alter the activity of a gene. Rather, it may reflect a “molecular memory” with long term effects over cell divisions or generations. This may represent an underlying mechanism to why addicts remain vulnerable to relapse years after any withdrawal symptoms or addictive behaviors have disappeared.

Another finding with functional importance was the significant change in DNA methylation in the group of genes metabolizing alcohol, alcohol- and aldehyde dehydrogenases, indicating a possible up-regulation of gene activity as a consequence of increased alcohol metabolism. Moreover, many genes regulating methylation processes of DNA and of proteins and histones were also found differentially methylated, which would be a reasonable finding in a DNA methylation aberrant system. Using DAVID tool for functional annotations, other groups previously reported involved in alcoholism or other types of addiction were found, including

insulin signaling, actin cytoskeleton, axon guidance (Table 4 or S3 in paper IV) or acetylcholine receptor activity (Table S4 in paper IV)(Davis and de Fiebre, 2006; Li et al., 2008). Both actin cytoskeleton and axon guidance include the MAPK signaling pathway, frequently found involved in addictive disorders (Depaz et al., 2005; Liesi, 1997; Lindsley et al., 2006; McClung et al., 2004).

Finally, our analysis could reveal a novel pathway affected by both altered DNA methylation as well as changed gene activity, in addition to other functional pathways with altered DNA methylation, previously reported involved in addictive disorders (e.g. insulin signaling) (Table 4). This pathway, Dentatorubral-pallidoluysian atrophy (DRPLA), is responsible for another disorder with severe neuronal atrophy followed by clinical symptoms of epilepsy, cerebellar ataxia, dementia and involuntary reflexes and movements, symptoms also frequently found in heavy alcoholics (Figure 7). This pathway was exclusively filtered out when the DNA methylation data was compared to previously published microarray gene expression data (Liu et al., 2004; Mayfield et al., 2002), and include the key proteins *ATN-1* (atrophin-1), *RERE* (arginine-glutamic acid dipeptide repeats), insulin receptor (*INSR*) and BAI1-associated protein 2 (*BAIAP2*). It is possible that this signaling pathway is involved in alcoholism causing a neuronal atrophy, which may underlie morphological findings of decreased brain volume and neuronal density. In paper II in the thesis, we did not find any support for an active cell death machinery. However, it is possible that alternative apoptotic signaling pathways, other than the intrinsic and extrinsic, are turned on as a result of alcohol abuse. Possible myelin loss, as indicated in a reduction of *MBP* and *PLP1* in this study, further supports the association to pallidoluysian atrophy, since myelin loss is thought to be responsible for the dementia seen in this disorder.

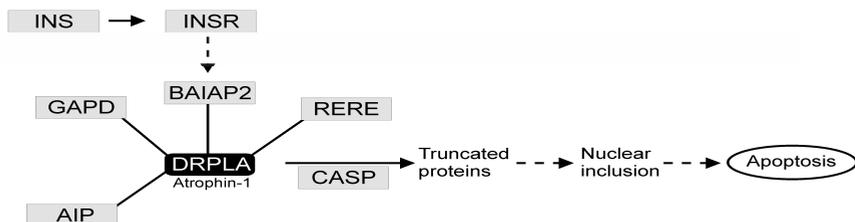


Figure 7. The Dentatorubral-pallidoluysian atrophy (DRPLA) pathway. The DRPLA pathway was found to have an altered DNA methylation in alcoholics. Activity changes of this pathway in another disorder result in neuronal atrophy followed by clinical symptoms of cerebellar ataxia, dementia, epilepsy and involuntary movements.

Table 4. Functional pathways found significantly different in DNA methylation in the prefrontal cortex of chronic alcoholics. The Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 (<http://david.abcc.ncifcrf.gov/>) was used for analysis.

Category	Term	Count	%	P-value	List Total	Pop Hits	Pop Total	Fold Change
KEGG_PATHWAY	Regulation of actin cytoskeleton	31	1.87%	0.003	354	214	4214	1.72
KEGG_PATHWAY	Cytokine-cytokine receptor interaction	29	1.75%	0.083	354	256	4214	1.34
KEGG_PATHWAY	Axon guidance	19	1.15%	0.025	354	132	4214	1.71
KEGG_PATHWAY	Insulin signaling pathway	18	1.09%	0.053	354	134	4214	1.60
KEGG_PATHWAY	Phosphatidylinositol signaling system	15	0.91%	0.004	354	76	4214	2.35
KEGG_PATHWAY	Glycerophospholipid metabolism	11	0.67%	0.06	354	69	4214	1.90
KEGG_PATHWAY	Adherens junction	11	0.67%	0.08	354	74	4214	1.77
KEGG_PATHWAY	Inositol phosphate metabolism	9	0.54%	0.06	354	51	4214	2.10
KEGG_PATHWAY	Benzoate degradation via CoA ligation	7	0.42%	0.03	354	28	4214	2.98

5 CONCLUDING REMARKS

Epigenetic marks, such as DNA methylation and histone modifications, are believed to control the activity of our genes by mediating environmental influences, and thus regulating an organism's physiological state. In addition, epigenetic marks may also represent a memory in molecular manifestations beyond evident genetic or biochemical signaling, that may be linked to both inherited and acquired biological alterations. Addiction, such as alcoholism or other substance abuse disorders provide a direct application to study such mechanisms since clinical symptoms, like the extreme vulnerability to relapse, remain long after any molecular changes have disappeared. Therefore, in this thesis, molecular and DNA methylation responses to heavy alcohol consumption have been studied, with specific focus on the brain. Potentially, the alcoholic genome could possess an aberrant DNA methylation pattern since the first oxidation product of ethanol, acetaldehyde, blocks DNA-methyltransferases. Moreover, folate deficiency, often found in alcoholics, can induce altered DNA methylation since folate is closely interconnected with the metabolism of the methyl donor S-adenosyl-methionine (SAM). In summary, this may contribute to altered epigenetic and molecular activity in the brain.

This thesis describes how alcoholism results in a genome with an overall loss of DNA methylation (Paper IV). This was shown on a global level as well as on a gene specific level. The human prefrontal cortex (PFC) and the striatum of rats with a history of alcohol dependence was revealed to possess a global DNA hypomethylation, by using a technique developed during the thesis work – Luminometric Methylation Assay (LUMA) (Paper III). Both PFC and striatum play important roles in reward functions due to their involvement in the dopaminergic system, and thus their importance for the reinforcing effects of addictive compounds. Moreover, PFC is processing emotional information from the amygdala, motivational information from the ventral tegmental area, and memory information from the hippocampus, and thus is crucial for cognitive performance. Cognitive dysfunction is often described in alcoholics such as dementia and difficulties in problem-solving. Aberrant DNA methylation in those brain regions may represent either 1) a direct shift in gene expression activity resulting in a changed physiological state and possibly a molecular adaptation, or 2) a “molecular memory” representing an epigenomic adaptation to alcohol abuse, both of which may underlie cognitive and etiological aspects of addiction.

Functional relevance to study DNA methylation by e.g. regulation of gene activity was supported by the expression analysis of a number of genes using a normalization approach established initially in the thesis work (Paper I). This analysis was showing a significant correlation between DNA hypomethylation and level of mRNA expression (Paper IV). However, not all genes demonstrated a strong correlation, indicating either 1) regulation and/or co-regulation of other transcriptional regulators, or 2) an epigenetic

mark as a result of alcohol abuse, that does not directly influence the activity of genes, i.e. an epigenomic memory. The latter may hypothetically predispose to later effects triggered by environmental factors. The study provided additional support to a functional importance by the observation that many genes within alcohol metabolism processes were hypomethylated and with potentially increased activity. Those included a number of alcohol and acetaldehyde dehydrogenases. Moreover, genes of proteins regulating DNA, histone and protein methylation activities had decreased levels of DNA methylation, a non-surprising observation if the methylation system *per se* is challenged by environmental pressure. In addition, previously reported functional groups or signaling pathways including insulin signaling, actin cytoskeleton, axon guidance and acetylcholine receptors were also found differentially methylated.

Finally, different cell death signaling pathways were directly studied (Paper III) or indirectly discovered (Paper IV). The initial hypothesis was that programmed cell death, including the intrinsic and/or extrinsic pathway, could be activated in the alcoholic brain, which could possibly underlie structural changes in brain morphology and neuronal density, ultimately resulting in cognitive dysfunction. However, we did not find support for this hypothesis since a key protein in cell death, activated caspase-3 was decreased, and a cell survival key protein, Bcl-2 was increased (Paper III). Moreover, several other components of the intrinsic apoptotic signaling or delayed cell death had decreased level of mRNA expression (*PDCD8*, *BID* and cyclin D1 and *BIRC2*). The results suggest a cellular protective adaptation or inherited molecular differences in alcoholics rather than a cell death process.

On the contrary, supporting cell death activation was the novel finding of a neuronal atrophy signaling pathway in the DNA methylation experiments (Paper IV). This pathway was exclusively filtered out by comparing all differentially methylated genes to differentially expressed genes reported in previous microarray studies. This pathway, Dentatorubral-pallidoluysian atrophy (DRPLA), is responsible for an autosomal neuronal atrophy disorder with clinical symptoms of epilepsy, cerebellar ataxia, dementia and involuntary reflexes and movements - symptoms also frequently found in alcoholics. Key proteins of this pathway, *RERE* (arginine-glutamic acid dipeptide repeats), atrophin-1, *BAIAP2* (BAI1-associated protein 2), and insulin receptor, were hypomethylated and/or had differential gene expression in the alcoholic prefrontal cortex. Moreover, a key protein composing the myelin sheet, MBP, was found hypermethylated and with decreased gene expression, suggesting a demyelination in alcoholics, an observation also found in pallidoluysian atrophy patients and believed to be responsible for the dementia. Altogether, this suggests that alcoholism alters apoptotic signaling pathways, however with different specified biological consequences.

6 FUTURE PERSPECTIVES

This thesis describes studies in a new field of research – epigenetics, and moreover, in a psychiatric illness that has a complex etiology involving many biological aspects such as genetics, behavior, motivation and environmental influences.

Therefore, additional studies, improved technologies and interdisciplinary research are necessary to unravel the true explanation model to addictive behavior and cognitive symptoms found in alcoholics. More specifically, ongoing and future studies related to the work in this thesis are the following:

- Continued confirmation analysis of the DRPLA pathway, including bisulphite pyrosequencing of targeted regions of the *RERE*, *BALAP2* and *ATN-1* genes and gene expression analysis by RT-PCR in the same individuals used in the DNA methylation analysis.
- Perform gene expression analysis of alcohol and acetaldehyde dehydrogenases or other functional groups of genes with aberrant DNA methylation to further establish the direct role of DNA methylation on gene activity.
- Perform studies on other transcriptional regulators, such as histone modifications, in co-regulatory assays to find better understanding for the epigenetic control of gene activity.
- Perform *in vitro* analysis with ethanol and nutritional deprivation in neuron cultures to find evidence for theoretical hypotheses and possibly mechanistic explanations for altered DNA methylation.

7 POPULÄRVETENSKAPLIGT PERSPEKTIV

Alkoholism är en multifaktoriell psykiatrisk sjukdom, som drabbar i genomsnitt 4% av världens befolkning. Dessa inkluderar genetiska faktorer, som uppskattas vara ca 50% och utgörs av flera olika geners påverkan, beteende och personlighetsrelaterade orsaker samt miljömässig påverkan. Trots årtionden av intensiv forskning har det varit svårt att finna exakta förklaringsmodeller till alkoholism, likväl som till andra beroendesjukdomar. Karakteristiska kliniska fynd, såsom kognitiv nedsättning i form av t.ex. demens, eller den extrema återfallsrisken, har ännu inte kunnat förklarats vetenskapligt. Flera olika hypoteser och förklaringsmodeller har lagts fram där det finns bevis för molekyllära förändringar i nervcellssynapser och förändring av hjärnans signalsubstanser, såsom glutamat, GABA eller dopamin, som i samma ordning reglerar stimulering, inhibering samt belöning in hjärnan. Tre huvudsakliga teorier dominerar forskningsfältet för närvarande och dessa är 1) bestående förändringar i dopaminsystemet som resulterar i en ökad motivation of drivkraft att använda drogen (incentive sensitization), 2) patologiska förändringar av motivationsdrivna handlingar eller brist av kontroll bestående av framför allt förändringar i glutamattransmission i hjärnans belöningssystem (dependence of pathology of motivation and choice), eller 3) negativ förstärkning, d.v.s. där ett återanvändande av drogen syftar till att återställa en kronisk fysiologisk obalans, ofta av stressrelaterade substanser såsom kortikotropin (hedonic allostasis). Dock har inga studier ännu kunnat se kvarstående molekyllära förändringar flera år efter att missbruket avslutats, trots den kvarvarande höga risken för återfall. Sårbarheten för återfall kan kanske förklaras av ett så kallat ”molekyllär minne”, där permanenta förändringar, adaptationer, inte direkt yttrar sig i biokemiska förändringar utan snarare predisponerar individen för yttre påverkan vid ett senare tillfälle.

Ett sådant ”molekyllärt minne” skulle kunna förklaras utav epigenetiska mekanismer. Epigenetik beskriver hur geners aktivitet regleras av interna (hormoner e.t.c.) eller externa (miljöpåverkan) influenser, utan att för den skull förändra den primära DNA sekvensen. Denna reglering sker ofta momentant men kan potentiellt alltså även manifesteras som ett minne, även om detta ännu inte är bevisat. Fysiologiskt yttrar sig epigenetik som kemiska markörer som dynamiskt binder eller avlägsnar sig från DNAt eller det protein som komprimerar DNAt, histoner. En sådan kemisk markör är metyl (CH₃), som specifikt kan binda till en av de fyra byggstenar DNAt är uppbyggt av – cytosin. Detta kallas DNA metylering. Ett tydligt exempel på DNA metyleringens roll är den inaktiva X-kromosomen hos kvinnor, som är nedtystad utav DNA metylering för att undvika dubbel aktivitet hos de båda X-kromosomerna. DNA metylering styr även andra geners aktivitet genom dess metyleringsdensitet i reglerande genregioner. Generellt sett har aktiva gener lite metylering medan inaktiva gener ofta har högre grad metylering i dess startregioner, s.k. promotor.

I denna avhandling har vi studerat DNA metylerings- och genaktivitetsförändringar i den alkoholiserade hjärnan - både hos människa och i en råttmodell som representerar ett alkoholberoende. DNA metylering skulle teoretiskt sätt kunna vara förändrat då den första nedbrytningsprodukten av alkohol, acetaldehyd, inhiberar det enzym som adderar metylgrupper till DNAt samt att en folatbrist, vanlig hos alkoholister, skulle kunna förändra DNA metyleringen därför att folat är viktig vid metyldonationsprocessen. Vi kunde bekräfta vår hypotes då vi kan se att DNAt i den alkoholiserade hjärnan har en förminskad mängd metylering globalt sett (gener såväl som DNA regioner utanför kodande gener) hos både människa och hos råtta, specifikt i hjärnregioner som är viktiga för belöning och kognition, prefrontal cortex och striatum (artikel IV). Detta mättes med hjälp av en teknik som utvecklades i avhandlingen – LUMA (arbete III). Denna minskning kunde även påvisas när vi studerade den mänskliga hjärnan utifrån en genspecifik metod som analyserar DNA metylering hos över 12 000 av våra gener. Genom genspecifik analys kunde vi även jämföra DNA metyleringen hos respektive gen med dess aktivitet och kunde se en korrelering mellan dessa två (artikel IV). Ju mer metylerat, desto mindre aktivitet, vilket vi kunde se hos 14 olika gener som vi analyserade utifrån en normaliseringsstrategi som utvecklades initialt i avhandlingen (artikel I). Förändrad DNA metylering kunde ses specifikt i grupper av gener som har betydelse för alkoholmissbruk. Bland annat viktiga enzymer vars funktion är att metabolisera alkohol i kroppen, eller i insulin- eller acetylkolin-signalerings som tidigare visats vara involverade i alkoholism och annat drogmissbruk.

Slutligen studerades programmerad celledöd, en naturlig process vid patologiska cellulära förändringar, i den alkoholiserade hjärnan för att utröna en eventuell inblandning i kognitionsnedsättning eller beroendeuppkomst. Nivåer och aktivitet av proteiner och gener viktiga för en sådan process indikerade inte någon aktiv cell död (artikel II). Snarare visade resultaten på motsatsen, vilket antyder en cellulär adaptation som motverkar en nervcellsöd, något som indikerar en anpassning i hjärnan till ett kroniskt alkoholintag alternativt en genetisk förändring hos alkoholiserade individer. Däremot fann vi en signalväg som hade avvikande metyleringsmönster och som ej tidigare rapporterats inom alkoholmissbruk, kallad DRPLA (artikel IV). Denna signalväg har visats vara dysfunktionell i en annan allvarlig sjukdom med nervcellsöd till följd, Pallidulysian atrophy, och som resulterar i symptom väldigt lika alkoholistens: demens, epilepsi, ataxi (ryckig gång) och ofrivilliga reflex- och muskelrörelser.

Sammanfattningsvis kan denna avhandling påvisa vikten av att studera epigenetiska mekanismer såsom DNA metylering på grund av dess viktiga biologiska funktion, för att komplettera genetisk, biokemisk och beteendevetenskaplig forskning i kartläggningen av bakomliggande orsaker och ärftlighet av beroendesjukdomar och andra patologiska tillstånd.

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