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VARIATION AT CANDIDATE GENE LOCI AND THEIR FUNCTIONAL IMPORTANCE IN RODENT MODELS OF ALCOHOL DEPENDENCE

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A world where anything is possible……..

To my parents

ABSTRACT

As genetic research has progressed, psychiatric disorders, including the addictions have emerged as moderately heritable. For alcoholism, the overall heritability has been estimated from twin studies to range between 50 to 60%. In spite of efforts to identify candidate genes for alcoholism, thus far only very few genes have been consistently replicated. This thesis work was aimed at identifying and characterizing novel candidate genes using genetic rat models of alcoholism.

An initial study applied oligonucleotide DNA based microarrays to identify genes that are differentially expressed in key brain regions for addiction (i.e. nucleus accumbens, amygdala, hippocampus and medial prefrontal cortex) of alcohol-preferring AA rats compared to non-preferring ANA rats. The main finding of this study was differential expression of a cluster of genes involved in the mitogen-activated protein kinase signaling pathways and the G-protein coupled receptor regulator, beta-arrestin 2 (ARRB2).

In the next study we followed up on the latter finding. Elevated expression and genetic variation of the ARRB2 gene was found in the striatum and the hippocampus of AA rats compared to the ANA line. The increased expression was associated with a 7-marker haplotype in complete linkage dysequilibrium, which segregated fully between the lines, and was unique to the preferring line. These findings were functionally validated using mice lacking ARRB2, which displayed both reduced voluntary alcohol consumption and alcohol induced psychomotor stimulation. The results suggest that ARRB2 modulates acute responses to alcohol and is an important mediator of alcohol reward.

Next, gene expression in the medial prefrontal cortex of AA and ANA rats was assessed. Microarray analysis revealed that glutathione S-transferase alpha 4 (GSTA4) expression levels were increased in the AA line compared to the ANA line. The results suggested that ANA rats seem to be impaired in metabolic pathways that may limit their capacity to metabolize alcohol, compromise their defense against reactive oxygen species and reduce longevity.

Finally, corticotrophin releasing hormone receptor subtype 1 (CRHR1) expression was analyzed in search of genes underlying the phenotype of alcohol-preferring msP rats. Increased CRHR1 mRNA levels were observed in several brain regions of msP rats, including the amygdala, compared to non-preferring Wistar rats. This finding was accompanied by genetic variation at the CRHR1 locus and increased sensitivity to reinstatement of alcohol following environmental stress. Taken together, the results presented here shows the combined use of genetic models and high throughput screening is a viable approach for identifying novel candidate genes for alcoholism. In addition, CRHR1 finding also suggests that this strategy can be used to discover genes that exert their influence in a gene x environment dependent manner.

LIST OF PUBLICATIONS

I. Arlinde C, Sommer W, **Björk K**, Reimers M, Hyytia P, Kiianmaa K, Heilig M: A cluster of differentially expressed signal transduction genes identified by microarray analysis in a rat genetic model of alcoholism. Pharmacogenomics Journal 2004;4:208-218.

II. **Björk K**, Rimondini R, Hansson AC, Terasmaa A, Hyytiä P, Heilig M, Sommer, WH: A role of Beta-arrestin 2 in mediating the rewarding effects of alcohol. FASEB J. *In press*

III. **Björk K**, Saarikoski ST, Arlinde C, Kovanen L, Osei-Hyiaman D, Ubaldi M, Reimers M, Hyytia P, Heilig M, Sommer WH: Glutathione-S-transferase expression in the brain: possible role in alcohol preference and longevity. FASEB J. 2006 Sep;20(11):1826-35.

IV. Hansson AC, Cippitelli A, Sommer WH, Fedeli A, **Björk K**, Soverchia L, Terasmaa A, Massi M, Heilig M, Ciccocioppo R: Variation at the rat Crhr1 locus and sensitivity to relapse into alcohol seeking induced by environmental stress. Proc Natl Acad Sci U S A. 2006 Oct 10;103(41):15236-41.

CONTENTS

LIST OF ABBREVIATIONS

DSM-IV	Diagnostic and statistical manual of mental disorders IV
G × E	Gene x environment
ADH1B	Alcohol dehydrogenase 1B
ALDH2	Aldehyde dehydrogenase 2
VTA	Ventral tegmental area
GABA	Gamma-amino-butyric acid
MAOA	Monoamine oxidase A
5-HTT	5-Hydroxy-tryptophan transporter
CRHR1	Corticotrophin releasing hormone receptor, subtype 1
CREB	cAMP responsive element binding protein
MAPK	Mitogen-activated protein kinase
AA	Alko, alcohol
ANA	Alko, non-alcohol
msP	Marchegian sardinian preferring
SSRI	Selective serotonin reuptake inhibitors
CRH	Corticotrophin releasing hormone
AMPA	Alpha-amino-3-hydroxy-5-Methyl-4-isoxazole Propionic Acid
QTL	Quantitave trait locus
NMDA	N-methyl-D-aspartate
Wt	Wild-type
SNP	Single nucleotide polymorphism
ARRB2	Beta-arrestin 2
GSTA4	Glutathione S-transferase alpha 4
NR3A	NMDA receptor subunit 3A

1 INTRODUCTION

1.1 ALCOHOLISM

Alcohol dependence, or alcoholism, is a major public health challenge and accounts for 4% of the global health burden (1). In the United States alone, approximately 9.7 million adults fulfill the criteria for a diagnosis of alcohol dependence according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) annually (2). The following introduction is aimed at providing a basic insight into the behavioral, motivational and genetic aspects of alcoholism. Different approaches for identifying candidate genes for alcoholism and dominating theories on substance disorders are also discussed.

1.1.1 Diagnostic criteria for alcoholism

An established tool for the diagnosis of substance dependence, including alcoholism, is the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), which is issued by the American Psychiatric Association. The criteria described therein encompass physiological, social and psychological aspects of substance dependence (Table 1.) It is important to note that defined in this manner, substance dependence is distinct from physical dependence on a given substance, and is conceptually more closely related to the concept of an addiction. Substance dependence and addiction are therefore used interchangeably in this text.

There are two important implications of how the DSM-IV is constructed. First, the application of a uniform set of diagnostic criteria to different substance use disorders suggests that there is a core of elements that is shared between those disorders. Second, the idea that three or more of these criteria need to be fulfilled during a twelve-month period to yield a diagnosis takes into account that individual differences in presentation and different stages of disorder exist. In other words, two persons with different sets of DSM-IV criteria can still be receiving the same diagnosis.

Table 1. Diagnostic criteria for substance dependence according to 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
2. Withdrawal
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.

The two first criteria, tolerance development and withdrawal symptoms describe well-defined physiological symptoms. Tolerance is defined as a 50% or greater increase of drug intake necessary to achieve the same effect compared to the time of initial drug intake. Withdrawal is characterized by drug-specific physiological symptoms that occur after cessation of drug intake. Examples of alcohol withdrawal are increased anxiety and increased sympathetic nervous system activity, manifested as increased blood pressure and heart rate. The third criterion, loss of control over intake, is a paramount feature of substance dependence. It adheres to the observation that individuals suffering from substance dependence repeatedly consume excessive amounts of the drug compared to what was initially intended. The fourth criterion is most likely associated with the loss of control, in that the individual attempts to reduce drug use after realizing that the situation is out of hand. Criteria five and six can be seen as measures of social function, so that, as the process of dependence progresses, increasing amount of time is spent to acquire the drug and consume it. As a function of this progression,

normal everyday activities which were previously considered as important are sacrificed in favor of drug-related activities. The final criterion underscores a central point in the modern view of substance dependence. A substantial number of individuals with drug dependence disorders continue their drug use despite an explicit desire to quit and even if they are aware of its adverse consequences. This suggests that addictive disorders are not a product of poor character or lack of self-discipline and shows why moralistic treatment approaches are not viable.

1.1.2 Alcoholism – a disorder of the brain

Alcoholism is a disorder that affects numerous physiological systems. This thesis is however solely focused on motivational aspects of alcoholism. The reinforcing properties of drugs of abuse are remarkably powerful and conserved across species. In an early study Olds et al mapped brain regions important for positive reinforcement (3). These and other studies pointed toward the importance of the mesolimbic dopamine system in mediating the pleasurable effects of drugs of abuse. Since then, additional brain areas have been suggested to be vital for developing and maintaining dependence, most notably the amygdala and the prefrontal cortex. Grant et al showed that drug craving is correlated to activation of the amygdala in cocaine abusers, suggesting that regions that process fearful or negative emotions might be important for this aspect of dependence (4). Finally, a consistent feature of dependence disorders is the inability to resist drug use in a situation where the drug is present. The prefrontal cortex has been shown to play an important role for behavioral inhibition and, dysregulation of this region has been proposed to be crucial for relapse to drug-seeking (5).

1.2 MOLECULAR FACTORS IN ALCOHOLISM

As genetic research has progressed, psychiatric disorders, including the addictions, have emerged as moderately heritable. For alcoholism, the overall heritability has been estimated from twin studies to range between 50 to 60% with most of the disease risk being attributed to non-shared environmental factors (6). In spite of efforts to identify candidate genes for alcoholism, very few genes have so far been consistently replicated. Findings so far include genes coding for alcohol metabolizing enzymes and the gamma-amino butyric acid receptor alpha 2 subunit (GABRA2) (6-10). The latter gene was identified as a part of the collaborative study of the

genetics of alcoholism (COGA) initiative. An initial linkage study showed modest linkage peak in a region on chromosome 4 (11). Fine mapping of this region provided evidence for linkage of the $GABA_A$ receptor gene cluster with EEG beta brain wave patterns, a known predisposing factor for alcoholism (12-14). In an effort to identify the specific candidate gene, Edenberg et al investigated 69 single nucleotide polymorphisms (SNPs), dispersed within the $GABA_A$ receptor gene cluster (8). The GABRA2 gene was found to be associated with both EEG beta brain wave patterns and alcoholism. In contrast no other members of the gene cluster showed an association.

The GABRA2 finding suggests that susceptibility genes for alcoholism in general each contribute to a small proportion of the overall disease risk. This pattern of inheritance is typical for a complex polygenic disease. Identification of additional susceptibility genes for alcoholism would not only provide information about the underlying genetic components driving addiction, but may also provide novel targets for pharmacotherapy.

In principle there are three ways in which molecular factors can influence the development of alcoholism including, 1) direct gene effects, 2) gene × environment (G × E) interactions and 3) neuroadaptations (see Figure 1). Direct gene effects and G × E interactions can be viewed upon as pre-existing susceptibility factors that render the individual increasingly vulnerable to developing dependence or to relapse into drug-seeking following abstinence. Neuroadaptations, on the other hand, are long-lasting changes in neural function, that can be caused by repeated drug use and be the result of changes in gene expression patterns in the brain. The candidate genes presented in this thesis are susceptibility factors, presumably exerting their effects according to the two former mechanisms. However, since the concept of neuroadaptive processes is considered a key mechanism for development of dependence and an integral part of current theories on addictive disorders it will be discussed here in detail. The three mechanisms presented herein are not mutually exclusive and are likely to act in concert to facilitate progression into dependence. In addition, it is conceivable that genetic susceptibility factors and neuroadaptive processes can act independently to generate the same phenotypes (a phenomenon referred to as *phenocopies*).

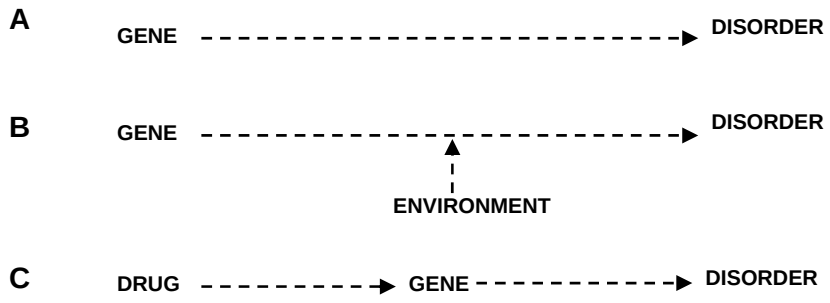

Figure 1. Molecular factors in alcoholism. Schematic representation of ways in which molecular factors can contribute to the risk for developing alcoholism. A) Direct gene effects, for example innate factors affecting rewarding effects of alcohol. B) G × E interactions, in which genetic variation confers susceptibility for development in a certain environmental context. C) Neuroadaptations that arise after repeated drug use and presumably serves to maintain dependence.

1.2.1 Direct gene effects

Direct gene effects on susceptibility for alcoholism can be illustrated by genes involved in alcohol metabolism or in mediating its acute rewarding effects. The most striking example of such gene effects are provided by alcohol dehydrogenase 1B (ADH1B) and aldehyde dehydrogenase 2 (ALDH2). ADH1B catalyzes the conversion of alcohol to acetaldehyde, a toxic metabolite, which is subsequently metabolized to acetate by ALDH2. Two functional mutations have been identified at the human ADH1B and ALDH2 loci, referred to as the His47Arg and Glu487Lys alleles, respectively. Carrying either of these mutations, results in an accumulation of acetaldehyde following alcohol ingestion (7). The increased levels of acetaldehyde are associated with an aversive flushing reaction, similar to that caused by disulfiram (Antabus). The His47Arg and Glu487Lys mutations have been shown to yield a protective effect on the risk of developing alcoholism in eastern Asian populations, where these alleles are abundant (6;15). It should however also be noted that this mutation might also have deleterious effects. The mutations do not seem to increase overall survival rates, suggesting the detrimental pleiotropic actions of these loci (16;17).

A further example of how genetic components might directly influence the risk for developing alcoholism is provided by the A118G mutation in the human mu-opioid receptor gene (OPRM1). This mutation is located in the N-terminal part of the protein, affecting a possible glycosylation site (18). There has been great controversy with regard to the functional consequences of this mutation (18-20). There are, however, studies pointing to a gain of function, i.e. increased receptor signaling, as a result of augmented affinity of the receptor to its endogenous ligand, beta-endorphin (18). In addition to functional studies, the OPRM1 A118G polymorphism has been subject to several genetic studies. The variant G allele has been shown to be associated with both heroin and alcohol dependence in some (21;22) but not in other studies (23).

Beta-endorphin release and subsequent activation of mu-opioid receptors in the ventral tegmental area (VTA) is thought to mediate the acute rewarding effects of alcohol (24). Mu-opioid receptor stimulation facilitates dopamine release in the mesolimbic dopamine pathway through disinhibition of gamma-aminobutyric acid (GABA) interneurons in the VTA (25-27). An increase in mu-receptor signaling in individuals carrying the G variant might therefore result in an elevated sensation of reward and render these individuals increasingly vulnerable to developing alcoholism.

Although the functional consequences of the OPRM1 118G variant and its association with a diagnosis of alcoholism remains controversial and may vary in different ethnic populations, its role is strongly supported by the observations that alcohol dependent OPRM1 118G carriers experience increased alcohol-induced euphoria and are selectively sensitive to the therapeutic effect of the opioid receptor antagonist naltrexone (23;28-30) (see 4.5.1).

Analogous to the alcohol metabolizing enzymes described above it is probable that the A118G variant has pleiotropic effects. A mutation with deleterious consequences, i.e. susceptibility for addictive disorders, such as the A118G variant, is highly unlikely to be selected for and maintained in a population unless it is associated with additional beneficial effects. In a recent study, Barr et al points to a possible beneficial effect of the A118G polymorphism. The authors suggest that an orthologue of the human A118G mutation might affect attachment behavior in infant rhesus macaques. Infant

macaques carrying the variant allele displayed higher levels of attachment behavior during early infancy, increased distress after maternal separation, and increased maternal contact after being reunited with their mother (31).

The OPRM1 polymorphism is most likely subject to balanced selection. This is a form of natural selection which, in contrast to directional selection, selects for multiple genetic variants to maintain the fitness of a specific population. The A118G polymorphism might initially have been selected for its effects on attachment behavior or altered stress response and aggressive behavior (31-33). Throughout history new, negative selection pressures may have been introduced on this allele. Multiple selection pressures might in turn result in the maintaining of both alleles in the population, in a ratio that is dependent on the strength of each selection pressure.

1.2.2 Gene × Environment interactions

G × E interactions can be defined as effects on a person's health following exposure to environmental stimuli that vary contingent upon the genotype of that person. In a pioneering study, Caspi et al reported the first case of a possible G × E interaction which determined the outcome of a psychiatric disorder. They demonstrated that a functional polymorphism at the monoamine oxidase A (MAOA) locus moderates the long term effects of child abuse. Maltreated children whose genotype conferred low levels of MAOA were found to be more prone to develop conduct disorder, antisocial personality and adult violent crime compared to children whose genotype conferred high levels of MAOA (34). Numerous similar studies have also investigated, amongst other things, the moderation of polymorphisms in the serotonin transporter (5-HTT) and the corticotrophin releasing hormone receptor, subtype 1 (CRHR1) on depression following stressful life events (35;36). Growing evidence suggests that G × E interactions also play an important role in alcoholism. Blomeyer et al recently reported that an interaction of a genetic variant of the CRHR1 gene and stressful life events predicts heavy adolescent alcohol use (37). Interestingly, two of the candidate genes presented in this thesis also seems to exert their effect in a G × E dependent manner (see 4.4 and 4.5).

1.2.3 Neuroadaptations

Neuroadaptations are defined here as long-lasting molecular changes that occur following chronic intake of addictive drugs. Some of these neuroadaptations are likely responsible for behavioral changes in response to chronic exposure to the drug. It is important to note that the neuroadaptations are long-term changes that persist far longer than acute drug effects and are also independent of the changes that are associated with immediate withdrawal. A primary criterion for neuroadaptations relevant for dependence disorders should be that they persist as long as long-term behavioral manifestations (i.e. susceptibility for relapse). In search of such mechanisms, the transcription factor, cAMP responsive binding protein (CREB) and the immediate early gene (IEG) ΔFosB have received considerable attention (38). CREB seems to play an important role in regulating dopaminergic neurons in the VTA and modulating the rewarding effects of addictive drugs, including cocaine (39;40). ΔFosB is an IEG with an unusually long half life, a feature that results in the accumulation of ΔFosB in the striatum following acute treatment with cocaine (41). Accumulation of ΔFosB 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 (42).

Neuroadaptations have also been reported after chronic alcohol treatment (43;44). By subjecting rats to repeated cycles of alcohol intoxication and withdrawal, Rimondini et al showed that after exposure, rats increased their alcohol preference. These long-lasting behavioral changes were accompanied by alterations of gene expression patterns in the amygdala and the prefrontal cortex which included glutamatergic, endocannabinoid, monoaminergic and mitogen-activated protein kinase (MAPK) pathways (44).

It is still unclear how such persistent changes in gene expression are maintained. Even the most long-lasting molecular changes previously described have not been found to persist as long as some of the behavioral manifestations of drug abuse (45). Epigenetic gene regulation has recently been proposed as a mechanism underlying long-term changes (46). The term epigenetics refers to stable chromatin and DNA modifications that do not

involve changes in the underlying DNA sequence of the organism. Histone acetylation and promoter methylation are two important examples of epigenetic gene regulation. Interestingly, a recent study by Kumar et al reported that acute and chronic cocaine treatment affects histone acetylation of numerous promoters including those for c-Fos and cyclin dependent kinase 5 (CDK5). Whether this is a universal mechanism for other addictive drugs remains to be elucidated (47).

1.3 GENETIC MODELS OF ALCOHOLISM

The development of several rodent lines with differential alcohol preference through selective breeding has been a successful approach to study excessive alcohol consumption and its underlying genetic factors (48) (see Table 2.). The utility of these lines is based on the assumption that in the high-drinking line, the selection pressure gradually leads to enrichment of alleles promoting alcohol drinking, while the alleles accumulated in the low-drinking line have opposite effects. However, two inherent features of the selection procedure need to be noted when studying dependence related phenotypes in these lines, firstly: co-segregation of non-selected traits and secondly, a narrowed reaction norm. The first phenomenon can result from random segregation of alleles that are neutral to the selection pressure. In populations with a relatively small number of breeders, those alleles tend to be either fixed or lost with time. Because of this effect and pleiotropic actions of the loci under selection, selected line pairs will not only show differences relevant to the phenotype being selected for, but also in various other phenotypic traits. The term behavioral reaction norm refers to the amount of change in a quantitative phenotypic trait displayed in response to a certain amount of environmental challenge or pressure. Wild or non-selected outbred populations of experimental animals have usually a broad behavioral reaction norm. Selective breeding often leads to a narrowing of the reaction norm; such that the animals may be “locked into” a particular phenotype. The reduced variability in reaction norm is most likely correlated with increased genetic homogeneity (49).

Table 2. Rat lines selected for high alcohol preference

Line	Breeder	Generations	Intake 10% EtOH (g/kg/day)	Important co-segregated traits	Ref.
UChB	Uni Santiago Chile	>78	4-7		(50)
AA	NPHI Helsinki Finland	>95	5-6	Low anxiety, high impulsivity	(49)
P	Indiana Uni USA	> 50	5-8	High anxiety	(51)
HAD	Indiana Uni USA	>35	9.5		(52)
sP	Uni Cagliari Italy	>64	6-7	High anxiety	(53)
msP	Uni Camerino Italy	>35	7-8	High anxiety	(54)
WHP	IPN Warszawa Poland	>27	4-8		(55)

1.3.1 The AA and ANA lines

The alko, alcohol (AA) and the alko, non-alcohol (ANA) rat lines are among the best-established selection-based models. These lines have been bidirectionally bred for high (AA) and low alcohol consumption (ANA) for over 90 generations (49). In addition to alcohol preference, AA rats display decreased anxiety and a high degree of impulsivity (56). Increased life-span is another interesting trait that has co-segregated with alcohol preference even though the trait is probably not related to this phenotype per se (57). Initial molecular studies focused on the role of classical neurotransmitter systems in determining the phenotype in these lines. However, little evidence has been found that supports an important role for either dopaminergic, cholinergic or serotonergic systems for alcohol preference

(58;59). In contrast, AA rats display region-specific differences in both opioid peptide and receptor levels compared to ANA rats (60;61). AA rats also self-administer significantly higher levels of opiate agonists (62). Taken together these results indicate that the alcohol-preferring phenotype of the AA rats may in part be due to a dysregulation of the opiate system. Recent studies have also pointed to a possible role for the endocannabinoid system for alcohol preference in these lines. Hansson et al showed that efficacy of the endogenous enzymes responsible for degradation of endocannabinoids, i.e. fatty acid amino hydroxylase and monoacylglycerol lipase, is impaired in the prefrontal cortex of AA rats (63). Despite these findings, a large proportion of the genetic factors underlying differential alcohol self-administration between these lines remain largely unknown.

1.3.2 Marchigian Sardinian preferring rats

Marchigian Sardinian preferring (msP) rats have been selected for high alcohol preference for more than 50 generations at the University of Camerino, Italy. The strain is originally derived from the Sardinian preferring strain (sP) generated at University of Cagliari, Italy (54). In addition to their alcohol-preferring phenotype, msP rats display anxiety- and depressive-like behavior. Interestingly, these traits can be attenuated by repeated intragastric alcohol administration or treatment with the tricyclic antidepressant desipramine (64). These data suggest that alcohol has an antidepressant-like effect in msP rats and that the anxiety- and depressive-like behavior might contribute to the alcohol preferring phenotype. Previously, the msP strain has been extensively pharmacologically characterized (for a review see Ciccocioppo et al. (54), but more recent studies have focused more on identifying genetic variation responsible for the phenotype of these rats. For example, paper IV in this thesis pointed to a role for CRHR1. The study showed that msP rats express higher levels of CRHR1 transcript in several relevant brain regions. The up-regulated CRHR1 expression was associated with a haplotype variant in the promoter region of the CRHR1 gene. Pharmacological data further supported the notion that CRHR1 is an important mediator of alcohol preference and anxiety-like behavior in msP rats (65).

1.3.3 Validity of AA and msP models

Although human alcoholism may not be possible to fully model in experimental animals, several models that reflect clinically relevant aspects of the human condition have been generated (for an interesting discussion see Koob et al (66). The validity of an animal model can be assessed using three criteria; 1) predictive validity, 2) construct validity and 3) face validity. Predictive validity refers to the criterion that the model should respond to drugs that are clinically effective for the human condition to be modeled, without being affected by drugs that are not. A good animal model also fulfils the criteria of construct validity, meaning that the model is dependent on similar fundamental biological mechanisms as those underlying the human condition. Finally, the model is said to display face validity if the behavioral characteristics and manifestations of symptoms that are associated with the human disease are also present in the animal model.

AA and msP rats have both been shown display predictive validity for the two drugs approved for the treatment of alcoholism, naltrexone and acamprosate (49;54;67). In addition, several experimental drugs with preliminary data supporting efficacy in human alcoholism have been shown to be effective in msP rats. These include 5HT3 receptor antagonists and $GABA_B$ antagonists (54;68). The predictive validity of these models is, however, by no ways complete. Selective serotonin reuptake inhibitors (SSRIs) have been repeatedly shown to suppress alcohol consumption in several animal models of alcoholism including msP rats but this effect has failed to translate into successful clinical trials (69-72). Since the repertoire of approved clinically effective drugs is currently restricted to naltrexone and acamprosate it is hard to estimate the true value of AA and msP lines for predicting clinical efficacy. The value of the AA and msP lines is likely to be revealed further as more drugs become available.

The issue of construct validity is often hardest to assess in regard to animal models since the underlying events responsible for the human condition are rarely completely known. However, the notion that selection for high alcohol preference in these lines also leads to enrichment of specific alleles is in itself an element of construct validity, since it shows that, as in humans, the propensity to consume large amounts of alcohol can be inherited. Theoretically, a good genetic model would be expected to carry genetic

variants similar to those that have been linked to alcoholism in humans. Two examples that show AA and msP lines to at least partially satisfy this criterion, are the selection for genetic factors of the opioid system and CRH systems, respectively (61;65).

The most important behavioral manifestation of related to dependence in these lines is obviously that both AA and msP rats consume pharmacologically relevant amounts of alcohol. AA rats, in addition, exhibit a high degree of impulsivity, a behavioral trait which is also present in early onset alcoholics and who have a distinct heritability and pharmacogenomic profile (6;73). In contrast, in the msP line, excessive alcohol consumption is accompanied by high trait anxiety (64;65). An interesting feature of msP rats is that they ingest alcohol in a binge-like manner, i.e. most of the drinking is done in bouts followed by a period of relative abstinence. This correlates very well with a study by Treutlein et al showing an association between a genetic variant of CRHR1 and binge-drinking in humans, considering that CRHR1 is thought to be a major contributing factor to the msP phenotype (54;74).

1.3.4 AA and msP rats – two different models of alcoholism

Given the considerable behavioral differences between AA and msP rats, it might be argued that these lines represent two inherently different animal models of alcoholism. Considering that alcohol preference is most likely a polygenic trait in which multiple sets of candidate alleles may result in the same phenotype, it is conceivable that the selection process has accumulated two different sets of alleles in AA and msP rats resulting in the same overall phenotype, such that both lines share high alcohol preference (6). This could also explain the differences in co-segregated traits between the two lines. This notion may be also relevant for the human condition. Alcoholism is not a unitary disorder as reflected by the DSM-IV criteria. It is possible that the two different lines model different subpopulations of alcoholic patients. The AA strain would here represent an “impulsive” phenotype, which is characterized by decreased anxiety and increased impulsivity. In humans, behavioral inhibition is crucial for the ability to pursue long-term rewards at the expense of immediate gratification. Impaired behavior control has been shown to increase the risk for antisocial personality disorder, conduct disorder and addictive disorders (73;75;76). The msP rats, on the other hand

could be regarded as an “anxious” phenotype in which alcohol preference is driven by anxiety- and depression like states, corresponding to the subpopulation of alcoholic patients with late onset, in whom mood and anxiety disorders are common (54). While this notion emphasizes the importance of evaluating these models independently when screening for candidate genes, it may also have implications for drug screening in these lines (see. 4.5.2).

1.4 THEORIES ON DEPENDENCE

Historically, addictive disorders have commonly been regarded as being caused by lack of character or discipline. As a more scientific view of addiction evolved initial theories suggested the compulsion for drug taking was driven by either the euphorogenic properties (i.e., positive reinforcement) or the desire to alleviate withdrawal (i.e., negative reinforcement) (3;77-79). Recently, due to the immense progress in the fields of genetics, molecular neurobiology and imaging, a deeper and more complex understanding of the biological and genetic aspects of addiction has emerged. A number of new theories have been conceptualized to incorporate these new findings. Three dominating theories are presented here to give a background and put the research presented later in a broader perspective (80-82). They have been selected because they represent different lines of thought and because of their importance for the research field. A common theme among these theories is that they are trying to provide a unifying framework that explains the key features of dependence; motivation, craving, relapse and control. It should be noted that they have originated in research on other addictions, and the extent to which they apply to alcoholism varies, or, in some cases, remain unclear.

1.4.1 Positive reinforcement by addictive drugs, mesolimbic dopamine and the theory of incentive sensitization

A universal feature of most common drugs of abuse is their ability to activate the mesolimbic dopamine (DA) system, which was early postulated to constitute a common final pathway mediating drug reinforcement (79;83;84). Numerous attempts to refine this initial formulation have followed. These have pointed to the role of mesolimbic DA not so much as a direct mediator of drug reward, but rather as a signal for associative learning,

or a signal that is predictive of reward (85). The theory of incentive sensitization by Robinson & Berridge may represent the most important among the refinements of the DA theory of reward (82;86). A central concept of this theory is that repeated drug 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 ("incentive motivation") is enhanced – increased drug appetite or "wanting". This is to be distinguished from the ability of the drug to produce its pleasurable effects, or "liking", which usually declines as dependence progresses. This selectively increased motivation for drug seeking is referred to by Robinson & Berridge as "incentive sensitization". Sensitization, or reverse tolerance refers to an increase in drug effects after repeated drug administration. Although sophisticated behavioral experiments may be able to distinguish between "wanting" and "liking", most of the incentive sensitization theory rests on data that are only indirectly related to motivation. Under many – but not all – conditions psychomotor stimulation, i.e. exploratory locomotor activity in response to a drug dose, largely reflects the activity of mesolimbic DA. Using this as an indirect measure, sensitization has been observed for most drugs of abuse in experimental animals. After repeated amphetamine injections in animals, an increase in motor behavior, locomotion and explorative behavior can be seen. Proponents of the incentive sensitization theory have argued that this can be viewed as a proxy marker for increasing salience of the drug. In addition to that sensitization is induced by most drugs of abuse; the extraordinary persistence of drug-induced locomotor sensitization is another feature making it attractive as a mechanism underlying dependence (87). The primary candidates for genes underlying sensitization are obviously genes involved in dopaminergic neurotransmission (88). While there is preclinical evidence supporting a role for incentive sensitization in dependence it remains controversial whether this phenomenon occurs in patients with addictive disorders. Although considerable research efforts for quite some time now have been devoted to modulation of the mesolimbic DA transmission involved in drug reward, these have not led to successful development of clinical treatments.

1.4.2 The theory of hedonic allostasis

The recruitment of negative affect during development of dependence is a fundamental concept for the theory of hedonic allostasis as proposed by Koob et al (81;89;90). Here drug dependence is viewed as a progression from an impulsive state, where drug intake is casual, into a compulsive state where it is chronic and relapsing in nature. This process is illustrated in the three stages of the "cycle of dependence"; preoccupation-anticipation, binge-intoxication and withdrawal-negative affect. In the initial stages of dependence, the cycle is mainly driven by elements associated with impulsivity and the positive reinforcing effects of drugs. As the cycle progresses, negative affect is gradually recruited and dependence becomes more compulsive. At this stage the cycle is mainly driven by negative reinforcement. This process is referred as an "allostatic shift". In contrast to the well established physiological term homeostasis i.e., maintaining stability around a set point, allostasis refers to maintaining stability at the cost of shifting the set point outside of the normal homeostatic range when physiological systems are chronically challenged. The theory of hedonic allostasis suggests that chronic drug insults disturb homeostatic mechanisms regulating mood and affect. After each successive cycle a new allostatic set point is set and more negative affect is recruited, further driving the cycle of dependence. In search of underlying mechanisms, research has been focused on genes and regions known to be involved in fear- and anxiety related behaviors. Of particular interest is the role of corticotrophin releasing hormone (CRH) and its receptors in the amygdala complex. Several studies have showed that induction of dependence in rats by repeated cycles of alcohol exposure and withdrawal results in an increase of alcohol preference and stress sensitivity. These behavioral changes are associated with increased expression levels of several CRH-related genes in the amygdala. Furthermore, in vivo antagonism of the CRHR1 receptor attenuate behavioral manifestations of alcohol dependence (91). Imaging techniques have provided additional evidence for mood regulation in dependence; it has been shown that the intensity of self-reported craving in cocaine abusers is significantly correlated to amygdala activity (4). The theory of hedonic allostasis is attractive from a pharmacotherapeutic perspective since attenuation of negative affect most likely provides an intrinsic incentive for compliance with treatment. It should be noted that the negative affect

recruited by repeated drug use appears distinct from typical anxiety and depressive disorders, as demonstrated by the fact that drugs effective in these conditions (benzodiazepines and SSRIs) are not effective in preventing relapse (70;71;92). Instead, converging animal and human data indicate that the allostatic state is characterized by a shift in affective processing, so that anxiety level and mood may be normal in the resting state, but responses to stressors are exaggerated while responses to normally pleasurable stimuli are attenuated or absent (93-95).

1.4.3 Dependence as pathology of motivation and choice

In a recent review, Kalivas & Volkow proposed a theoretical framework to explain the central features of dependence (80). In their view, addiction is pathology of motivation and choice. A key feature of addictive disorders is the increased motivation to obtain drugs and decreased behavioral control to resist them. The amygdala, nucleus accumbens and the prefrontal cortex have all been shown to be important brain regions for the activation of motivated adaptive behaviors. While the amygdala has been identified as an essential region for fear-motivated behaviors, the nucleus accumbens has been shown to play an important role in reward-mediated behaviors (3;96). On the contrary, the prefrontal cortex seems to play a minor role in ascribing positive or negative valance to individual stimuli but possess a more integrative function in determining valance and behavioral response (97;98). Together these regions define what can be referred to as a motivational circuit, important for guiding behavior. Addictive disorders are viewed here as a dysregulation of the motivational circuit following repeated drug use, with loss of behavioral control as the end-result. There are several pieces of evidence suggesting a role for this circuit in addictive disorders, especially for glutamatergic prefrontal cortical efferents. Firstly, using imaging techniques it was shown that self-reported craving is highly correlated to activity of the prefrontal cortex in cocaine abusers (99-101). Secondly, inactivation of the prefrontal cortex in animal models prevents cue-, drug- and stress-induced reinstatement of drug-seeking (102-104). Thirdly, antagonism of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the nucleus accumbens prevents drug- and cue-induced reinstatement of drug-seeking (105;106). In search for neurodaptations involved in the dysregulation of the motivational circuit, research has focused on genes involved in modulating glutamate release and affecting

postsynaptic response. Inhibitory metabotropic glutamate autoreceptors mGluR2/3 constitutes an example of the former and scaffolding proteins like Homer and PSD-95 are examples of post-synaptic proteins (107;108). However, even if cortical glutamatergic pathways are involved in the development of dependence they may prove hard to target by pharmacological intervention. Glutamate is the most abundant excitatory neurotransmitter in the brain and modulating it is likely to interfere with basal brain functions, possibly limiting the applicability of this approach. Per contra, there are preliminary studies showing some promise for modulation of glutamate in treatment for cocaine dependence. In a pilot study, Mardikian et al demonstrated that treatment with N-acetyl-cysteine, a widely used drug for treatment of chronic pulmonary conditions, resulted in complete termination or reduced drug use (109). The underlying mechanism for this effect may be restoration of basal glutamate levels in the nucleus accumbens.

1.4.4 Alcoholism – a special case

The theories described above have been developed primarily with psychostimulants in mind. There are several reasons for this. Psychostimulants produce more striking behavioral effects, and the biological target mechanisms through which they act are well characterized. These features make it easier to study the impact on addictive drugs using psychostimulants rather than alcohol. There are however several pieces of evidence that suggest alcohol to differ from psychostimulant drugs.

Amphetamine and other psychostimulant are higly reinforcing. Animals will readily lever press hundreds or thousands of times for a single dose of amphetamine or cocaine, and granted unlimited access, both animals and humans will often neglect satisfying their basic needs such as eating and sleeping, to the point of dying in many cases (79). Alcohol, on the other hand, is a weak acute reinforcer, and animals will rarely lever press more than a few tens of times for a dose of this drug. This presumably reflects the difference in the effects of the two drugs on mesolimbic dopamine release, a system crucial for the reinforcing properties of drugs. Amphetamine induces a much higher peak dopamine release compared to alcohol (84). Furthermore, alcohol acts on multiple molecular targets, and in contrast to psychostimulants, affects both stimulatory and sedative systems in several

brain regions. Cocaine and amphetamine inhibit dopamine reuptake mechanisms or increase vesicular release, respectively (110;111). The molecular substrates for alcohol are presumably greater in number and less well defined. Initially, alcohol was thought to exert its effects through non-specific lipid membranes interactions (112). A series of studies, however, showed that alcohol physically interacts with numerous proteins, including, the NMDA and GABAA receptors (113;114). In addition to these systems, alcohol interacts indirectly with a multitude of pathways.

Because of the relatively modest reinforcing properties of alcohol, in comparison to psychostimulants, it could be argued that the theory of incentive sensitization is less applicable to alcoholism compared to cocaine addiction, for example. In fact, recent imaging studies show reduced rather than increased dopamine release in the nucleus accumbens of alcoholics following stimulation by the stimulant drug methylphenidate (115)

The hedonic allostasis theory is maybe the theory that has been most developed with alcoholism in mind. Prolonged exposure to alcohol in rodents has been shown to increase alcohol preference and sensitivity to stress. These behavioral changes are accompanied by up-regulation of the CRH system in the amygdala. Interestingly a similar phenotype can be observed in genetically selected msP rats, which display increased alcohol-preference and anxiety-like behavior. In concordance these studies, msP rats also exhibit increased expression of CRHR1 which most likely is driven by genetic variation at this locus. Finally, both excessive alcohol consumption and reinstatement of alcohol-seeking behavior can be blocked in both these models by CRHR1 antagonists. These data suggest that the theory of hedonic allostasis is a very relevant concept for alcoholism (91).

A strong support for a role of glutamatergic signaling in alcoholism has been provided by acamprosate, which most likely exerts its therapeutic effects by dampening glutamatergic signaling (116). Pharmacological modulation of alcohol-related behaviors has focused on NMDA and AMPA receptors. NMDA receptor channel blockers have been showed to reduce alcohol consumption and alcohol-seeking in rodents (117;118). There is also preliminary evidence suggesting that NMDA antagonists reduce cue-induced craving in alcoholics (119). Furthermore, blockade or deletion of specific subunits of the AMPA receptor complex significantly reduce cue-induced

reinstatement of alcohol-seeking in experimental animals (120;121). In line with these results, topiramate, an anti-convulsant drug that blocks AMPA receptors has recently been showed to reduce heavy drinking in alcoholic patients (122). In a pilot study, Heinz et al postulated that a neural network comprised of the medial prefrontal cortex and nucleus accumbens circuit might be important for alcoholism. They showed that cue-induced activation of the striatum and medial prefrontal cortex is associated with subsequent relapse in alcoholics (123). In summary, the results above show that modulation of glutamate signaling may be a viable approach for pharmacological treatment of alcoholism.

1.4.5 Points of convergence and therapeutic implications

The theories presented above represent different lines of research. Even if they focus on different aspects of addictive disorders they are not by necessity mutually exclusive. On the contrary, it can be argued that they cover overlapping aspects of dependence. Dysregulation of mechanisms involved in rewarding aspects of alcohol are likely to be important in the initial phase when dependence is established. Recruitment of negative affect and loss of behavioral control, however, are more important factors in maintaining dependence in latter stages of the disorder. A unifying theme is that they emphasize the importance of neuroadaptive changes as a result of repeated drug use in driving the dependent state. However, it is also clear that the concept of susceptibility to dependence mediated by genetic variation fits in with any of these theories. Importantly, the three theories make different predictions about which aspect of addictive disorders that would be most suitable to target by pharmacotherapy. The incentive sensitization theory predicts reward modulation as the main target whereas the other theories predict stress relief and behavioral control, respectively, to be more important. Each of these theories has their advantages and disadvantages and ultimately, the validity of either of them will be decided by their ability to generate research that can provide new drug targets and medications that are efficient in treating alcoholism.

1.5 HIGH THROUGHPUT SCREENING FOR CANDIDATE GENES

1.5.1 Hypothesis driven research vs. high throughput screening

The theories described above are examples of hypothesis driven research which all emphasize different aspects of dependence and more importantly have different predictions for what genetic and molecular substrates are expected to be crucial for the its development. The rapid development of high throughput tools (i.e. expression microarrays) has offered an alternative way of identifying genetic factors underlying addiction. This approach involves using microarrays to screen for differentially expressed genes in animal models with differences in alcohol preference. The major advantages of such an approach is the sheer number of genes that can be queried in one single experiment and the reduction of "discovery bias" inherent to the method. In contrast to hypothesis driven research that focuses on genes with a particular function or region-specific expression relevant for the phenotype, the high throughput nature of microarrays allows for analysis of genes with no previous known association. In the best case, this can result in discovery of completely novel candidate genes and the development of new hypotheses as exemplified by the studies below (1.5.3).

1.5.2 Quantitative trait loci analysis

Quantitative trait locus (QTL) analysis is a method that is used to map genomic regions that are important for inheritance of polygenic traits. By comparing the correlation between specific markers and the quantitative trait value for each individual in the entire population it is possible to acquire an overall measurement of how close a certain genomic region is associated with the trait. QTL analysis can be used to map continuous physical traits such as weight and height but have also been applied to complex behaviors such as alcohol preference, and are then referred to as behavioral QTLs (bQTLs) (124;125). QTL analysis can also be used in combination with high throughput screening approaches to increase the likelihood that differentially expressed genes are truly related to the relevant phenotype. Finally, this method can be used to identify genes that are regulated at their own loci (i.e. cis-regulated) (126). For this purpose, gene expression is viewed as the

continuous trait and the resulting QTL is referred to as expression QTL (eQTL).

1.5.3 Screening for genes involved in complex behaviors

High throughput screening approaches have been applied to identify genes involved in alcohol-related behaviors. Tabakoff et al screened for genetic determinants underlying the phenotype of acute functional tolerance (AFT) to alcohol in mice (127). AFT is defined as tolerance that develops during a single exposure to alcohol. The initial step of measuring AFT in mice involves training them to balance on a wooden dowel rod. The mouse is then administered a dose of ethanol and when the mouse loses its balance a blood sample is taken to estimate the blood ethanol concentration (BAC0). A second blood sample is then acquired when the mouse regains balance (BAC1). At this point, the mouse is injected a second time and a third blood sample is acquired when it once more regains its balance (BAC2).The AFT is then measured as the difference between BAC2 and BAC1. Furthermore, in humans, low sensitivity to the sedative properties of alcohol, i.e. high AFT, has long been regarded as a risk factor for developing alcoholism (128). The strategy used in this study involved obtaining expression profiles from brains of mice selectively bred for high and low AFT and comparing these profiles to bQTLs for AFT. The results suggested that genes involved in N-Methyl-D-Aspartate (NMDA) receptor signaling are important mediators of AFT (127). In a continuation of this study, a similar platform was used to identify genes for alcohol preference and AFT in selected lines, with the addition of eQTL analysis. Even though filtering of gene expression data through bQTLs reduces the number of differentially expressed genes that are not related to the studied phenotype, it might occlude genes which are not by themselves located under a bQTL but whose main regulatory elements are (i.e. trans-regulated genes). By overlying behavioral QTLs (for alcohol preference or AFT) with eQTLs it is more likely that these genes are included in the analysis (126). Finally, Mulligan et al performed a microarray meta-analysis of expression profiles from three selected mice lines and four inbred strains with innate difference in alcohol preference. The analysis revealed consistent patterns of differentially expressed genes between the alcohol-preferring and non-preferring lines. Further bioinformatic analysis suggested that several molecular pathways are

involved in determining alcohol preference including most notably, the MAPK pathway (129).

1.5.4 Screening for candidate genes in AA and msP rats

The strategy used here to identify candidate genes for alcohol preference in AA and msP rats is summarized in Figure 2. It consists of two phases. The initial phase involves screening for differentially expressed genes and selecting candidate genes for further study. The second phase entails confirmation and functional validation of the candidate gene in question. Microarray platforms, and in one case in situ hybridization (paper IV) was used for screening for differentially expressed genes. A selection of candidate genes was then done, based on several criteria. These included fold-change, statistical significance, involvement in relevant biological process and previous literature suggesting a role for the specific gene in alcohol preference. Currently there are no QTL data on alcohol preference available from either AA or msP rats, but if the relevant gene resided under a QTL from other alcohol preferring lines, that was taken into regard. Rigorous statistical analysis and careful candidate gene selection are requirements for successful identification of candidate genes. As the process focuses more and more on individual genes, labor and level of commitment is increased. An inherent problem with high throughput approaches and microarrays in particular is the high false discovery rate. To counter this problem, the selected candidate genes were confirmed using alternative methods, for example, Real-Time PCR, Western blot and receptor binding. Sequence analysis was then performed to identify underlying genetic variation contributing to the expression differences. Finally, functional validation of the confirmed candidate genes was performed. This is the most arduous step in the process and can involve everything from measuring receptor activity to behavioral analysis of knockout mice. This is an outline of the approach used to identify the candidate genes described in this thesis; it should be pointed out that other kinds of analysis and methods can be used successfully to reach the same objective.

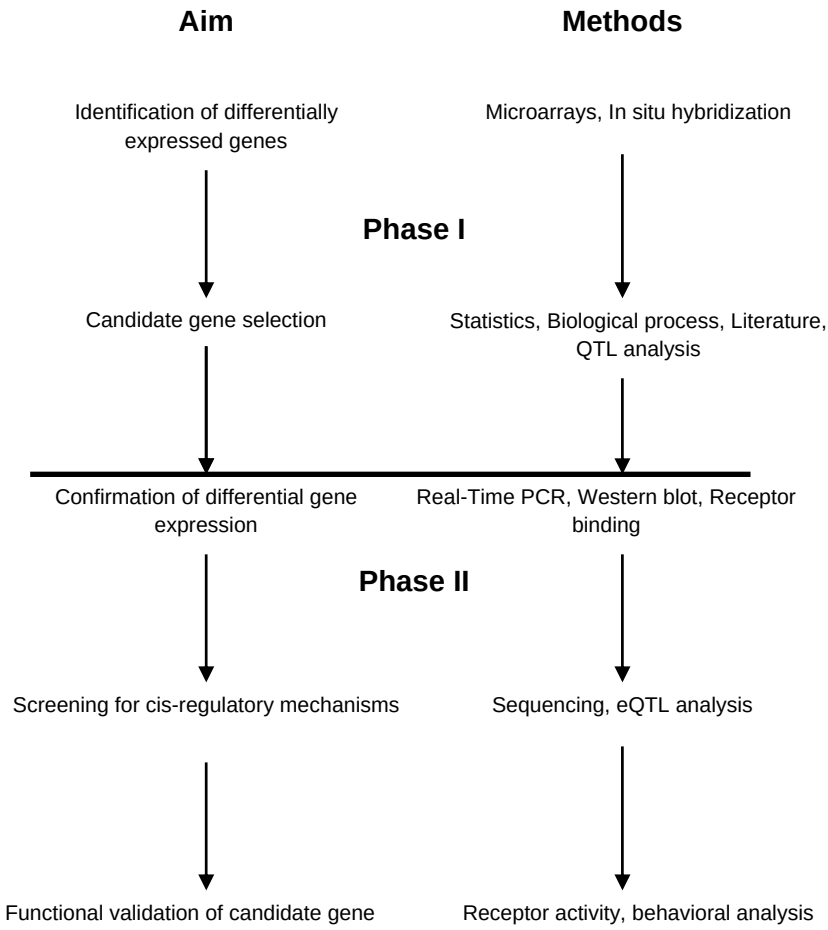

Figure 2. Flow chart showing the strategy used in this thesis to identify candidate genes for alcohol preference in AA and msP rats. The strategy is divided into two phases, an initial screening phase followed by a secondary phase involving confirmation and functional validation of the relevant candidate gene.

2 AIMS

2.1 OVERALL AIMS

The overall aim of this thesis was threefold. Firstly, to identify candidate genes underlying the alcohol preferring phenotype in the AA and msP lines, secondly to identify cis-regulating elements driving differential expression and thirdly, to establish a mechanism by which these genes contribute to the phenotype.

2.2 SPECIFIC AIMS

- To screen and confirm genes that are differentially expressed in key brain areas for addiction (i.e., hippocampus, nucleus accumbens, amygdala and medial prefrontal cortex) of AA rats compared to ANA rats. (paper I)

- To investigate the role of beta-arrestin 2 (ARRB2) in alcohol preference using AA rats and ARRB2 knockout mice. (paper II)

- To identify genes differentially expressed in the medial prefrontal cortex of AA rats compared to ANA rats, with special emphasis on genes involved in glutathione metabolism, and to assess genetic variation affecting expression levels (paper III)

- To study genetic factors contributing to the alcohol preferring and anxiety-like phenotype of msP rats. (paper IV)

- To determine molecular and genetic factors underlying the differential response to the NMDA receptor antagonist neramexane between alcohol-preferring AA and msP rats. (preliminary data)

3 METHODS

3.1 ANIMALS

3.1.1 Genetically selected rat lines

Drug-naïve, male alcohol-preferring AA and ANA non-preferring rats (National Public Health Institute, Helsinki, Finland) were used in paper I, II and III. Drug-naïve, male Wistar rats were used as a reference strain in paper IV. Drug-naïve, male msP rats (University of Camerino, Camerino, Italy) were used in paper IV and in preliminary results. For further details see the individual papers. All animals were sacrificed through decapitation during the light phase. The brains were directly frozen in -40°C isopentane and then stored in -70°C until further use.

3.1.2 Mice

ARRB2 knockout mice were used in paper II to assess the effect of ARRB2 on alcohol-related behaviors. The generation of mice lacking ARRB2 by homologous recombination has been described previously by Bohn et al (130). Mutant and wild-type (wt) mice were obtained as a kind gift from Professor Robert Lefkowitz, Duke University and bred at NIAAA. They were housed together in standard Plexiglas cages with food and water ad libitum. The colony room was maintained on a 12 hr light /dark cycle with lights on at 6 A.M. Animals were between 12 months age at the time of testing. Only male mice were used in experiments. Animal care and handling procedures were in accordance with National Institutes of Health Guidelines.

3.2 EXPRESSION ANALYSIS

3.2.1 Microarray analysis

High throughput oligonucleotide microarray (Affymetrix, Santa Clara, CA, USA) screening was applied in paper I and III and preliminary results to identify differentially expressed genes. In paper I, total RNA from each brain region was extracted from nine animals from AA, ANA or Wistar strains respectively and each divided in three pools per strain. Target preparation and hybridization was done according to the manufacturers protocol (Affymetrix, Santa Clara, CA, USA). Targets were hybridized to Affymetrix rat neurobiology U34 arrays containing probe sets for approximately 1200 genes. After staining and washing in an automated fluidic station, the arrays

were scanned in a Hewlett-Packard gene array scanner using the affymetrix MAS 5.0 software to generate CEL-files of probe intensity values. dChip 1.1 (www.dchip.org) software was used to read probe signals and to estimate probe set expression intensity values for each transcript based on the Affymetrix annotation of the probe sets. In paper III, target preparation for individual samples (n=8/strain) from medial prefrontal cortex of AA and ANA rats was done as described for paper I, except that RAE230A arrays were used. In comparison to the U34 arrays, the RAE230A arrays use a reduced number of probes for each transcript, resulting in a higher number of genes that can be queried per chip (approximately 15000). Another advantage of the RAE230A chip over the U34 chip is the dispersion of probes for each gene over the entire surface of the chip, thereby reducing the possibility of hybridization bias. CEL files obtained from MAS 5.0 software were inspected for regional hybridization bias and quality control parameters, as previously described (131). Gene chips that passed quality control (CEL-files) were then imported to the Gensifter software package (gs2.genesifter.net) which allows generation of expression estimates using the Robust Multichip Averages (RMA) algorithm and statistical analysis using Welch's two-sample t-test, assuming unequal variances. Correction for multiple testing was done by adjusting the p value for family-wise error rate according to Holms's sequentially rejective multiple test procedure. Cohen's d' statistic was used for meta-analysis of separate studies according to the following formula: $d = 2\,t / \sqrt{df}$, where t is the t value from each experiment and df is the degrees of freedom. The direction of change was post hoc coded into *d*; positive values indicate up-regulation in preferring lines (132). Expression profiles were obtained for AA and msP medial prefrontal cortex as described for paper III (preliminary results).

3.2.2 Real-Time PCR

Real-Time PCR was used to confirm differential candidate gene expression in paper I and III. Reverse transcription (RT+) was performed according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). To control for genomic DNA contamination parallel reactions without enzyme were performed (RT-). One hundred ng of RNA was used for all the RT reactions; the RT reactions were subsequently diluted five times and stored at -20°C. Using 5 µl of the diluted sample, a Real-Time PCR reaction was performed in 50 µl containing Taqman universal master mix (Applied

Biosystems, Foster City, CA) and a gene specific primer/probe set. The Applied Biosystems 7700 or 7900 HT sequence detector was used to perform the PCR. Primer / probe concentrations were optimized according to manufacturer's protocol. For sequence information see the individual papers.

3.3 PROTEIN ANALYSIS

3.3.1 Western blot

Western blot was performed in paper II and III to confirm differences in protein levels. Tissue samples from the hippocampus of AA and ANA rats were boiled in pre-warmed SDS extraction buffer (0.45 M Tris-HCl, pH 8.5, 2.5% glycerol, 4% SDS, 0.5 mM DTT, 25 μg/ml MG-132 (Affinity Biosciences, Golden, CO, USA) and 6mg/ml COMPLETE (Roche, Indianapolis, IN, USA) protease inhibitors for 3 min and then sonicated (Paper II). Alternatively, samples were extracted in lysis buffer consisting of 137 mM NaCl, 20 mM Tris-HCl ph 8.0, 1% Nonidet P-40, 10% glycerol, 1% protease inhibitor cocktail (Sigma, St. Louis, MO, USA) (Paper III). Protein extracts were aliquoted and stored at -80°C until further analysis. Protein concentration was determined by the DC protein assay (Biorad Biosciences, Hercules, CA, USA). Samples containing equal amounts of total protein were separated on a 10% tris-glycine gel and blotted onto nitrocellulose membranes using the Xcell II system (Invitrogen, Carlsbad, CA, USA). Membranes were then immunoblotted with a primary mouse ARRB2 antibody (Santa Cruz Biochemicals, Santa Cruz, CA, USA) (paper II) or primary GSTA4 antibody (U.S. Biological, Swampcott, MA, USA) (paper III) and an anti-mouse secondary antibody from rabbit (Biorad Hercules, CA, USA). Detection and quantitation was performed using the FUJI LAS-3000 system and the Multigauge Software (FUJIFILM, Tokyo, Japan). The ARRB2 signal was normalized against total protein content as measured by Memcode staining (Pierce, Rockford, IL, USA) (133). Gsta4 levels were normalized to beta-actin levels (Cell Signaling Technology Inc., Beverly, MA, USA). Protein levels were compared by one-way ANOVA

3.4 GENETIC ANALYSIS

3.4.1 Sequencing analysis

Sequencing analysis was performed using genomic DNA from 15-20 animals each of AA and ANA animals (Paper II and III), msP and Wistar

strains (paper IV), msP and AA lines (Preliminary results). DNA was isolated from whole blood using the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer. Primer pairs were designed to amplify approximately 2kb of the promoter and the 5'UTR and the 3'UTR of the relevant gene, except for ARRB2, where the whole gene was sequenced (paper II). PCR was subsequently performed on a Tetrad 2 thermal cycler (MJ Research, Waltham, MA, USA) using Clonetech Advantage PCR kit (BD Biosciences). The PCR products were purified by QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Sequencing was done by employing the primers used in the initial PCR reaction, and ABI PRISM Big Dye Terminator v3.0 sequencing kit according to the manufacturer's manual. The analyses were performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

3.4.2 Haplotype reconstruction and bioinformatic analysis

Haplotype reconstruction was performed using the Haploview program (Paper II-IV, preliminary results) (134). The WebQTL tool (www.genenetwork.org) (135) was used to define genomic regions associated with the expression of distinct genes (i.e. eQTLs) (paper II). Linkage with ARRB2 expression is represented as LOD-scores (logarithm of odds) and plotted against chromosomal position. Seven different datasets from recombinant inbred mouse lines (BxD RI) between the C57BL/6J and DBA/2J parental lines were used: 1) UCHSC BXD whole brain from the University of Colorado at Denver, 2) OHSU/VA B6D2F2 brain and 3) OHSU/VA B6D2F2 striatum from the Oregon Health Sciences University, 4) HQF BXD striatum from the University of Tennessee, 5) The Hippocampus Consortium, 6) VCU BXD prefrontal Cortex from The Virginia Commonwealth University and 7) GE-NIAAA cerebellum.

3.5 BEHAVIORAL ANALYSIS OF ARRB2 MUTANT MICE

3.5.1 Voluntary alcohol consumption

Voluntary alcohol consumption was assessed in a two bottle-free choice continuous access paradigm (paper II). Mice were allowed ad libitum access to two bottles containing either water or alcohol for four weeks. Bottles and mice were weighed every second day to follow consumption and the side for the two solutions were changed daily to avoid development of side

preference. Alcohol concentration was increased gradually from day 2-4 (2% alcohol), to day 4-6 (4% alcohol) and to days 7-28 (6% alcohol). To control for putative differences in taste preference, the same mice were allowed ad libitum access to either sucrose (5%) or quinine (0.02 mM) for 14 days and taste preference ratios were acquired. Mean intake during the 6% period was analyzed using one-way ANOVA.

3.5.2 Alcohol induced locomotion

Locomotion was assessed after a single intraperitoneal injection of alcohol (i.p, 0.75g/kg). Infrared locomotor cages were used (27.9 cm x 27.9 cm test environment with three 16 beam I/R arrays; Med-Associates, St. Albans, VT, USA) and activity was recorded for 30 min in 10-min intervals. Results were analyzed using two-way ANOVA for treatment and strain and Tukey's HSD post-hoc test (paper II).

3.5.3 Alcohol clearance

Blood levels of alcohol were measured after a single alcohol injection (i.p., 3.5 g/kg,). Samples were obtained from the tail at 15, 60, 120 and 240 min after injection. The samples were subsequently analyzed using Analox analyzer (Analox Instruments, Luneburg, MA, USA). Data were analyzed using two way ANOVA with genotype as between subjects and time as within subjects (repeated measures) factors (paper II).

3.5.4 Rotarod

Four month-old mice were placed on a rotarod (Ugo Basile, Varese, Italy) turning at a fixed rate of 10 rpm. The mice learned to remain on the rod during three 60-s training periods. Saline or increasing doses of alcohol (0.5 mg/kg dose, range from 0.5-3 mg) were injected i.p. After 5 min, each mouse was tested three times in succession for its ability to remain on the rod. The cut-off time was 60 s. The ED_{50} was calculated via nonlinear regression (sigmoidal dose- response curve) for each subject using GraphPad Prism v4 (GraphPad Sofware Inc., San Diego, CA USA) and the different genotypes were compared by one-way ANOVA (paper II).

4 RESULTS AND DISCUSSION

4.1 GLOBAL EXPRESSION PROFILING IN AA AND ANA RATS (PAPER I)

Identifying differentially expressed genes in genetic models of alcoholism may uncover previously unidentified susceptibility genes and point to new targets for drug development. For this purpose, microarray expression profiles from AA, ANA and non-selected Wistar rats were obtained. The Wistar strain was included as a reference strain. The analysis was focused on four key brain areas known to be involved in addiction, i.e., cingulate cortex, nucleus accumbens, amygdala and hippocampus. Comparing gene expression profiles between the lines, only modest differences could be detected. Elevated neuropeptide Y (NPY) transcript levels were detected and confirmed using Real-Time PCR in the hippocampus of ANA rats compared to AA and Wistar rats. This result is in concordance with previous studies using in situ hybridization (136). However this difference is not likely to contribute to the alcohol preferring phenotype of AA rats, since expression in AA rats did not differ from Wistar rats. The two other main findings of this study was the differential expression of a cluster of genes involved in MAPK signaling and the GPCR regulator ARRB2, in the nucleus accumbens, and in all four regions, respectively. Interestingly, MAPK signaling has been suggested as a pathway involved in alcohol preference, in an extensive study of the expression profiles in alcohol preferring mouse strains (129).

4.2 VALIDATION OF ARRB2 AS A CANDIDATE GENE FOR ALCOHOLISM (PAPER II)

In a study by Arlinde et al (paper I) (137) decreased ARRB2 levels was observed expressed in several brain regions of AA rats compared to ANA rats. Here, ARRB2 was further investigated in order to extend the initial findings and characterize the functional impact of ARRB2 on alcohol-related behaviors. Expression analysis using in situ hybridization revealed elevated ARRB2 expression in the nucleus accumbens and in several subregions of hippocampus of AA rats compared to ANA rats. A possible explanation for the discrepancy between these results and those from paper I was provided

by subsequent sequence analysis. It is quite likely that the observed differences in signal reflect genetic variation affecting probe hybridization rather than true differences in expression levels. A SNP was located in a region of the ARRB2 gene to which several probes were targeted (Exon 13). Similar observations have been made by others comparing gene expression profiles from animals with different genetic background (138). Increased ARRB2 protein levels and the discovery of a haplotype specific to the AA rats further support that the increased ARRB2 expression levels found by in situ hybridization reflect the true condition (see below).

The ARRB2 expression differences were paralleled by similar differences in protein levels, as demonstrated by Western blot analysis of hippocampal samples from AA and ANA rats. These findings were accompanied by a 7-marker haplotype variant in complete linkage dysequilibrium, which segregated fully between the lines. The haplotype variant spanned the whole ARRB2 gene, from approximately 1500 base pairs upstream of the translation start site to the 3' UTR of the gene (Figure 3). Additional evidence for cis-regulation of the ARRB2 gene was provided by bioinformatic analysis. Data analysis of expression profiling from recombinant inbred mouse lines of the BXD RI panel and genome-wide allelic marker association was used to identify genomic regions that are important for regulation of ARRB2 (www.genetwork.org) (135). A single distinct and highly significant eQTL for ARRB2 was observed at its own locus in hippocampal, striatal and prefrontal cortex tissue but not in cerebellum. These findings strongly suggest that variation at the ARRB2 locus has region-specific impact and is important for ARRB2 expression in the hippocampus and striatum, the regions that show the largest ARRB2 expression differences between AA and ANA lines.

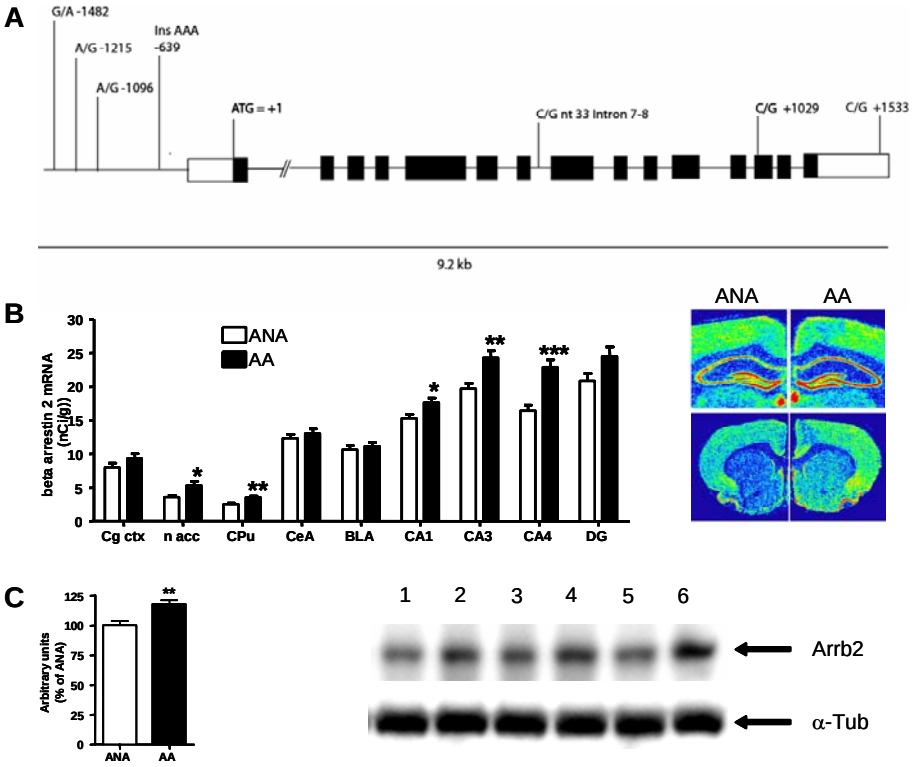

Figure 3. Genetic variation at the Arrb2 locus is associated with increased gene expression. A) AA rats are homozygous for a novel haplotype block (analysis by Haploview), comprised of one insertion and six SNPs. The ANA line carries the wild-type sequence according to ENSEMBL database (ENSRNOG00000019308). No other genetic variation was detected. The nucleotide position is calculated from the A in the start codon of the mRNA sequence (NM_012911.1) which is designated as +1. Positions in the promoter are calculated in the 5' direction starting from the nucleotide preceding the start codon, which is designated as -1. Exons are shown as boxes; the coding region is colored in black and untranslated regions are colored white. SNP positions are indicated by lines with the wild-type allele is given first. Introns are not shown at full length. We sequenced the loci of 15 animals per line. B) In situ hybridization shows elevated Arrb2 mRNA levels in AA as compared to ANA rats. Expression values were compared region-wise by one-way ANOVA followed by Holm's sequentially rejective multiple test procedure. *P < 0.05; **P < 0.01; ***P < 0.001; n = 8 animals/line. To the right: representative autoradiogram of coronal brain sections showing increased Arrb2 expression in hippocampus (upper panel) and dorsal and ventral striatum (lower panel). Abbreviations: Cg ctx, cingulate cortex; CPu, caudate putamen; n acc, nucleus accumbens; CeA, central amygdaloid nucleus; BLA, basolateral amygdaloid nucleus, CA1 to CA4, Cornus Ammon areas; DG, dentate gyrus. C) Western blot analysis revealed elevated Arrb2 protein levels in the hippocampus of AA rats compared to ANA rats. Protein levels of the housekeeping gene alpha-tubulin were unaltered between the two strains. Densitometric measures of both proteins were normalized to the mean of the ANA group and analyzed using one-way ANOVA **P < 0.01; n = 6 animals/line, Representative Western blots are shown to the right. Lanes 1, 3, 5 are samples from ANA rats, lanes 2, 4, 6 from AA rats.

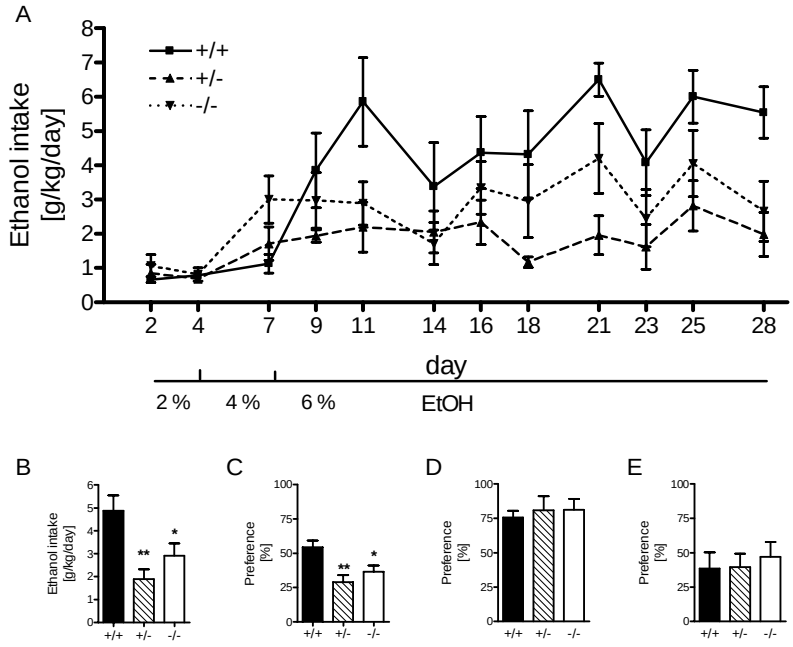

Figure 4. Decreased voluntary alcohol consumption in Arrb2 mutant mice. A) Continuous access, two-bottle, free-choice alcohol consumption was monitored over four weeks. Alcohol concentration was increased gradually from 2% to 6% (v/v). B, C) Arrb2+/- and Arrb2-/- mice consume significantly less alcohol and have a lower preference ratio as compared to wild-type (wt) mice. Mean alcohol intake for the 6% alcohol solution was analyzed by one-way ANOVA: $F[2,22] = 7.1$, $P < 0.01$; Fisher's post hoc test vs. Arrb2+/+: *$P < 0.05$; **$P < 0.01$. Alcohol preference: $F[2,22] = 7.0$, $P < 0.01$; Fisher's post hoc test vs. Arrb2+/+: *$P < 0.05$; **$P < 0.01$. There was no statistical difference between $Arrb2^{+/-}$ and $Arrb2^{-/-}$ mice. A reciprocal increase in consumption from the non-alcohol bottle, but not in total fluid intake was observed. D, E) Taste preference to sucrose (5%) and quinine (0.02 mM), respectively. Mice were allowed access to sucrose and quinine in a two-bottle, free choice, continuous access paradigm vs. tap water for seven days, and taste preference ratios were acquired. Results were analyzed using one-way ANOVA. There were no significant differences between wild-type and Arrb2 mutant mice.

ARRB2 knockout mice were obtained to assess whether the differential expression of ARRB2 in AA rats is causally involved in alcohol-related behaviors. Both homozygous and heterozygous mutant mice displayed reduced voluntary alcohol consumption compared to wt mice, as assessed by the two-bottle free choice paradigm (Figure 4). Further tests were done to rule out that the altered alcohol consumption is due to changed taste preference or alcohol metabolism. These control experiments established that neither of these factors is likely to contribute to the observed phenotype.

Drug-induced psychomotor locomotion stimulation is widely regarded as a proxy measurement of rewarding dug effects. With this in mind, ARRB2 knockout mice were tested for alcohol-induced locomotion. In contrast to wt mice, ARRB2 mutant mice did not exhibit increased locomotion behavior following alcohol injection, suggesting a role for ARRB2 in mediating alcohol's rewarding effects. In agreement with this view, ARRB2 knockout mice also showed diminished c-fos activation, an indicator of neuronal activity, in the shell of nucleus accumbens following acute alcohol administration.

ARRB2 is a crucial regulator of GPCR signaling. It binds the receptor following ligand stimulation and phosphorylation, facilitating receptor desensitization, internalization and G-protein independent signaling (139). The results presented herein suggest that ARRB2 may be important for the rewarding effects of alcohol and that this effect is driven by genetic variation at the ARRB2 locus. There is additional evidence pointing to role for ARRB2 in alcohol preference. A robust up-regulation of ARRB2 was observed in mouse lines selected for high alcohol preference in comparison to their low-preferring counterparts (129). However, it should also be pointed out that ARRB2 does not seem to be increased in alcohol-preferring iP rats, nor does it reside within any of the QTLs for alcohol preference in rodents that have been described so far.

The exact mechanism for how ARRB2 could affect alcohol preference is currently unknown, but there are studies pointing towards opioid and dopaminergic systems. ARRB2 interacts both with the mu-opioid receptor and the dopamine D2 receptor. For example, mice lacking ARRB2 show increased and sustained analgesic response to morphine, a prototypical opiate acting mainly through the mu-opiate receptor (130;140). It has also recently been reported that locomotor stimulation by psychostimulants is partly mediated by an ARRB2 dependent formation of a kinase/phosphatase scaffolding signaling complex at the D2 receptor (141). Both these systems are likely to be affected by changes in ARRB2 expression. The disinhibition of GABAergic interneurons in the VTA by stimulation of mu-opioid receptors, and the resulting dopamine release in the nucleus accumbens is an integral process in the acute rewarding effects of alcohol (25;142;143). If ARRB2 modulated alcohol consumption through this mechanism, the mutant

mice would be expected to consume increased levels of alcohol, since they would be experiencing more reward. This notion, and the studies showing that conditioned place preference and extracellular dopamine release in response to morphine is actually increased in ARRB2 mutant mice, suggests the positive correlation between ARRB2 levels and alcohol preference observed here cannot easily be attributed to opioid mechanisms (144). Instead, the findings that ARRB2 expression is increased in the striatum of AA rats and that c-fos induction by alcohol is blunted in ARRB2 mutant mice suggests that dopamine signaling may be more important for mediating the effects of ARRB2 on alcohol preference.

4.3 GSTA4: A POSSIBLE ROLE FOR ALCOHOL PREFERENCE AND LONGEVITY (PAPER III)

Two independent studies have shown decreased expression of GSTA4 in alcohol-preferring P rats and the inbred iP line derived from it, compared to their respective non-preferring counterparts (145;146). Subsequent sequence analysis also revealed four single nucleotide polymorphisms in the GSTA4 gene, suggesting a possible relation between this locus and the alcohol preferring phenotype observed in these rats. In light of these results we queried our database of expression profiles from the medial prefrontal cortex of AA and ANA rats for differential expression of glutathione metabolizing genes. A number of genes were found to differ between the two lines. Among these, GSTA4 was the gene that was the most significant and robustly altered. Differential expression of this gene was independently confirmed using Real-Time PCR. This method was also used to extend the analysis to other key regions for addiction, i.e. nucleus accumbens, amygdala and hippocampus. In contrast to the P and iP line findings, an approximately 2-fold GSTA4 expression increase was detected in all four brain regions examined (Figure 5). This expression difference also translated into protein levels as shown by Western blot analysis of hippocampal tissues from AA and ANA rats.

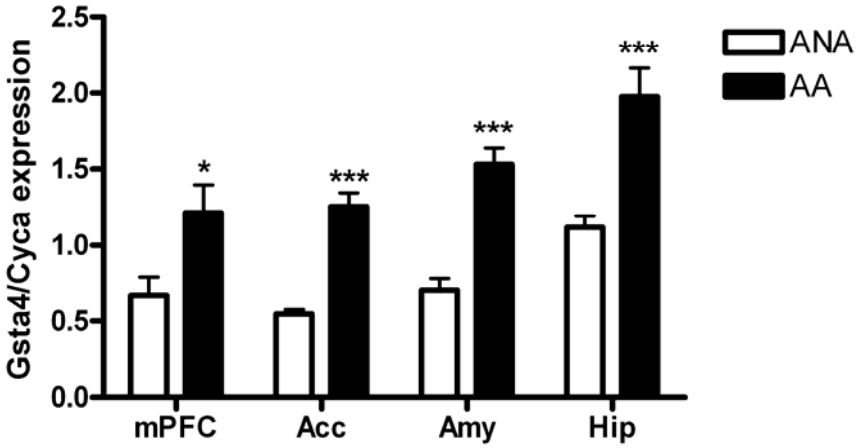

Figure 5. Expression analysis of GSTA4 in four key brain areas for addiction. Real-Time PCR analysis revealed an approximately two-fold increase in GSTA4 expression in the medial prefrontal cortex, nucleus accumbens, amygdala and hippocampus of AA rats compared to ANA rats. Cyclophilin A gene expression was used as endogenous reference and values are shown as the ratio of GSTA to Cyclophilin A expression in the respective brain area. Abbreviations: mPFC, medial prefrontal cortex; Acc, nucleus accumbens; Amy, amygdala and Hip, hippocampus. Statistical analysis was done using one-way ANOVA for each region *P < 0.05; ***P < 0.001, n=8/strain

In search of genetic variability potentially underlying the differential GSTA4 expression in AA and ANA rats sequence analysis was performed. In addition to the wt allele and the variants described for the iP line, analysis of the AA and ANA lines revealed the existence of a GSTA4 haplotype not previously described, here designated as var3. The haplotype consisted of six SNPs in complete linkage dysequilibrium and spanned from approximately 400 base pairs upstream of the translation start site to the 3' untranslated region (UTR) of the gene. The var3 exhibited a differential distribution between the two lines. Whereas ANA rats were all homozygous for the var3 variant, AA rats displayed roughly equal allelic frequency of the var3 and the wt allele. Together, these results suggest a correlation between GSTA4 haplotype and expression levels. To assess the putative impact of GSTA4 genotype, gene expression in the amygdala was plotted against genotype, independent of strain. GSTA4 expression displayed a gene dose relationship dependent on genotype, with var/var genotype conferring the lowest expression levels and wt/wt the highest (Figure 6). A substantial proportion of the variance (59%) was explained by genotype.

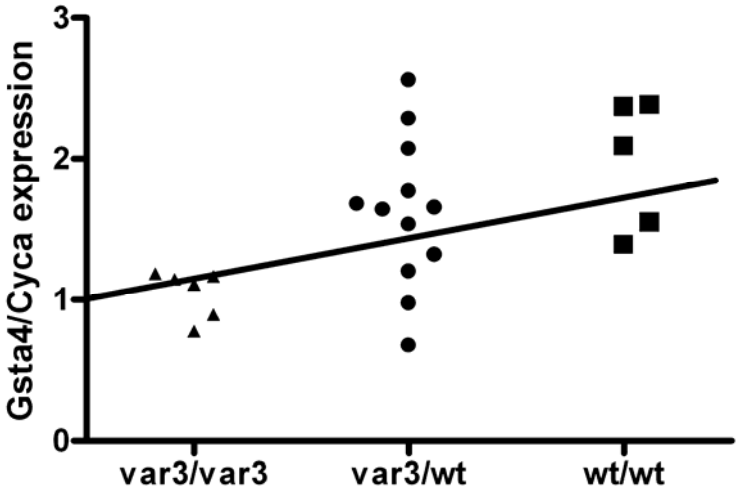

Fig 6 Correlation between GSTA4 genotype and expression levels. Genotype was assigned to all animals and then correlated to expression levels in the amygdala. The analysis yielded a highly significant correlation. For linear regression analysis the genotype was transformed into the gene dose of wt allele. The regression line is described by y=0.69x + 1.13 Pearsons r = 0.77 the proportion of the variance explained by the genotype is $r^2 = 0.59$.

The glutathione transferases (GSTs) are a large family of proteins whose main function is the disposal of exogenous toxic compounds and adaptive, antioxidant response to reactive oxygen species (147-149). There is, however, also research suggesting a role for GSTs in alcoholism. Oxidative stress is one the probable mechanisms involved in alcohol-induced neural damage (150-152). Furthermore, the metabolism of dopamine, a neurotransmitter important for alcohol reward is dependent on GST activity (153). Nevertheless, in light of the expression data from the alcohol-preferring iP rats, which show the opposite pattern compared to AA rats (i.e. decreased expression), it is hard to conceive a role for GSTA4 in determining alcohol preference. There are, however, several other possible explanations for this finding. One possibility is that GSTA4 is located close to another locus that is selected for alcohol preference. Possibly supporting this idea is that GSTA4 has been shown to be in the vicinity of a suggestive QTL found in the iP rats (124). Alternatively, genetic variation at the GSTA4 locus might be a result of a phenomenon known as genetic fixation. Theoretically, the selection procedure enriches for alleles involved in alcohol

preference, thus reducing genetic heterogeneity. If the starting pool of GSTA4 variants is small it is possible that two different variants are enriched in AA and ANA lines even if GSTA4 is not selected for alcohol preference. Finally, it cannot be excluded that GSTA4 is, in fact, selected for its effects on alcohol preference and that it exerts its influence on this phenotype in an unknown way.

It is presently unclear what the functional consequences of altered brain GSTA4 expression are. It might contribute to other phenotypes that have co-segregated with alcohol preference. Increased life-span and anxiety-like behavior are examples of two such traits (56;57). Interestingly, ANA rats exhibit a significantly shorter life-span compared to AA rats and also to common laboratory rats. It is possible that a down-regulation of GSTA4 expression driven by the haplotype variant var3 is responsible for the decrease in life-span. This hypothesis is in agreement with studies of GSTA4 knockout mice which are more susceptible to oxidative stress and show degenerative changes earlier in life (154). Another theory is that GSTA4 is involved in regulating anxiety-like behaviors. The AA line display decreased measures of anxiety compared to ANA rats (56). In contrast to alcohol preference, this phenotype would correlate well with GSTA4 expression, since AA and P rats display opposite patterns of expression, and anxiety-like behavior. In further support of this view, it was recently reported that genes involved in controlling glutathione levels in the brain, and that uses glutathione as a cofactor, regulate anxiety in mice (155).

4.4 GENETIC VARIATION AT THE CRHR1 LOCUS INCREASES THE VULNERABILITY FOR EXCESSIVE ALCOHOL SELF-ADMINISTRATION AND REINSTATEMENT OF ALCOHOL-SEEKING (PAPER IV)

Excessive voluntary alcohol intake is a key clinical feature of alcoholism, and is the phenotype selected for in the selectively bred rat lines used in this thesis. Another clinically prominent feature of alcoholism is the increased vulnerability for relapse i.e., return to alcohol seeking after a prolonged period of abstinence. Three categories of environmental stimuli have been shown to facilitate relapse into drug seeking in both humans and animal models: 1) consumption of a small “priming” dose of ethanol, 2)

presentation of ethanol associated cues and 3) stress. It is more than likely that these stimuli interact with pre-existing genetic susceptibility factors in a G × E dependent manner to trigger relapse.

In order to identify genetic susceptibility factors for excessive voluntary alcohol intake and stress-induced reinstatement of alcohol-seeking, we carried out a focused differential gene expression screen in genetically selected alcohol-preferring msP rats. During the selection of msP rats, an anxiety-like phenotype has co-segregated with ethanol preference (see 1.3.2). To assess if the increased sensitivity to stress contributed to the excessive ethanol intake and elevated propensity to return to alcohol seeking following stressful stimuli, msP rats were tested in the stress-induced reinstatement paradigm. Compared to non-selected Wistar rats, msP rats require less stressful stimuli (i.e. lower shock intensity) to return to alcohol-seeking after extinction of self-administration. Gene expression profiling of stress-related genes was carried out in order to identify genetic substrates underlying the phenotype observed in the msP rats. In situ hybridization analysis revealed several genes that were differentially expressed in relevant brain areas between msP and non-selected Wistar rats. CRHR1 was one of the genes that were most robustly and consistently changed and was therefore further analyzed. msP rats exhibit increased levels of CRHR1 transcript in several brain regions including hippocampus, nucleus accumbens and amygdala. These changes were paralleled by even greater increases in the density of CRHR1 binding sites in cingulate cortex and amygdala, indicating that the expression differences also translate into differences in protein levels. The functional role of elevated CRHR1 expression and function for both excessive voluntary alcohol self-administration and stress-induced reinstatement was demonstrated using the brain penetrant CRHR1 antagonist antalarmin. Untreated msP rats showed significantly higher self-administration than msP rats. Doses of antalarmin that left self-administration unaffected in non-selected Wistar rats reduced it in the msP line, to a level equal to that found in the Wistar animals. Thus, CRHR1 antagonism fully rescued the self-administration phenotype of the msP line. Secondly, doses of antalarmin that were ineffective in blocking stress-induced reinstatement in Wistar rats fully blocked it in msP rats. These findings demonstrate that phenotypic differences between msP and Wistar rats for these two behaviors can fully be accounted for by the

elevated expression and function of CRHR1 found as an innate susceptibility factor in the msP line. These findings parallel and are consistent with an extensive accumulation of data indicating that up-regulation of CRHR1 expression and function induced by a history of alcohol dependence results in what is essentially a phenocopy of the msP line (for review, see Heilig & Koob (91).

In search of genetic factors underlying the elevated expression and function of the CRHR1 gene, this locus was then screened for genetic variation to identify cis-regulating elements. Sequence analysis identified a dinucleotide haplotype, approximately 2000 base pairs upstream of the first start codon. The Wistar rats were all homozygous for a haplotype variant identical to that described for rattus norvegicus (G-G, wt) whereas msP exhibited variation at the CRHR1 locus with a approximately mendelian distribution of wt alleles and the variant haplotype (A-A, var). A chi-square analysis of the allele distribution yielded a highly significant association between allele frequencies, suggesting that the two alleles segregated during selection for alcohol preference in msP rats.

The CRH peptide and its associated receptor, CRHR1, have been extensively studied in the context of the hypothalamic-pituitary-adrenal (HPA) axis activation (156;157). In addition to this function, the CRH system has been implicated in regulating anxiety-like behavior. In support of this view, genetic ablation of the CRHR1 receptor or CRH itself results in impaired stress response and reduced anxiety-like behavior in mice (157;158). Furthermore, pharmacological inhibition of the CRHR1 receptor attenuates measures of experimental anxiety in several animal paradigms (159-161). Interestingly, the efficacy of the antagonists in decreasing anxiety levels seems to be dependent on base-line anxiety levels. For example, CRHR1 antagonism is more effective in animals with high innate anxiety levels or in animal exposed to stress. This is probably related to fact that the CRH system is quiescent during normal conditions (162). The results presented above suggest that genetic variation at the CRHR1 locus might be a key factor in determining the anxious phenotype of msP rats. In addition, variation at this locus interacts with stress, and reduces the threshold for stress-induced reinstatement of alcohol-seeking. Thus, innate differences of CRHR1 levels are likely to constitute a susceptibility factor for the

development of addiction that exerts its effect in a G × E dependent manner. These and other preclinical data correlate well with two recent reports implicating genetic variants of the human CRHR1 with alcohol-related behaviors. Blomeyer et al reported a significant interaction between a haplotype tagging SNP in the CRHR1 gene and stressful life events, resulting in more frequent and higher maximum alcohol drinking (37). Treutlein et al reported a genetic association of CRHR1 with binge drinking and alcohol intake patterns (74). The emerging data surrounding CRHR1 strongly suggests a role for this gene in the development of addiction and suggests that it might constitute a viable target for pharmacotherapy.

4.5 A DIFFERENT KIND OF G × E INTERACTION: GENETIC VARIATION AT THE NR3A LOCUS (PRELIMINARY DATA)

Glutamatergic neurotransmission has long been in focus as a treatment target for alcoholism. In particular, several studies point to an important role for NMDA receptors in the development of addiction (163). Chronic alcohol consumption results in increased NMDA receptor mRNA levels and differential expression of specific subunits (164). Furthermore, NMDA receptor antagonists have been shown to attenuate several behavioral manifestations of addiction, including voluntary alcohol consumption and reinstatement of alcohol-seeking behavior (118;165). Neramexane is one of the non-competitive NMDA antagonists that have been deemed especially promising for treatment of alcoholism. Many findings are in line with this proposal, most notably, neramexane has been shown to reduce voluntary alcohol intake and prevent acquisition and expression of conditioned place preference in rodents (117;166). In addition, it was recently shown that neramexane suppresses voluntary alcohol consumption in genetically selected AA rats (Vengeliene et al, unpublished). This effect was, however, not observed in another animal model of alcoholism, the alcohol-preferring msP line, suggesting that genetic factors might underlie the differential response. In search of such factors, microarray expression profiles from the medial prefrontal cortex of AA and msP rats were compared and the NMDA receptor subtype 3 A (NR3A) gene was found to be significantly up-regulated in msP rats compared to AA rats. This finding was further confirmed using Real-Time PCR. NR3A is a dominant negative subunit of

the NMDA receptor complex. In vitro studies have shown that increased levels of NR3A result in a decreased conductance through the NMDA receptor. Furthermore, NMDA receptors which are composed of NR3A subunits display significantly reduced sensitivity to receptor antagonism (127;128). These two findings might offer an explanation for the differential response to neramexane observed between AA and msP rats. It is conceivable that the increased NR3A expression in msP rats results in an overall down-regulation of NMDA signaling, rendering these rats insensitive to further suppression by neramexane.

Here, we re-sequenced the NR3A gene in AA and msP rats, to identify genetic variation driving the differential expression. A previously unknown trinucleotide haplotype variant was found in msP rats, while the AA rats were all homozygous for a variant identical to that described for Wistar rats (Figure 7). The msP rats exhibited an approximately equal distribution between the wt and the variant allele (0.58 and 0.42, respectively). These results suggest that innate genetic differences at the NR3A locus between AA and msP rats may contribute to differential gene expression and predict treatment response to neramexane. This finding is an example of a type of G × E interaction that is likely to become more important for clinical treatments in the future (i.e. gene × drug interactions).

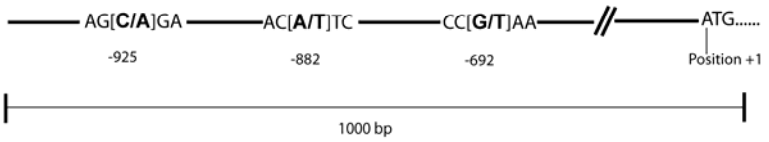

Figure 7. Genetic variation at the NR3A locus. Sequence analysis revealed three SNPs in a region approximately 700 and 1000 base pairs upstream of the start codon in the NR3A promoter in msP rats. The SNPs were analyzed using the haploview program and found to be in allelic identity. The variant allele was unique for msP rats; AA rats, in contrast exhibited a genotype identical to that found in public databases (ENSEMBL ID ENSRNOG00000005723). SNPs are shown at their respective location upstream of the start codon, with the wt allele first followed by the msP-specific variant.

4.5.1 Personalized medicine

The concept of personalized medicine represents the idea that if genetic information about a person is available, it is possible to customize medical treatments that will be especially beneficial to that person. The underlying notion is that by knowing the genotype of a specific gene in an individual it is possible to predict and tailor treatment responses. This notion emerged in the wake of the human genome sequencing project and has since then attracted substantial attention. Currently, there are very few studies providing examples of such interactions in regards to psychiatric diseases. The A118G polymorphism of the human mu-opiate receptor is, however, one example that is relevant to alcoholism. This polymorphism is situated in the coding region of the receptor (Exon 1) and results in an amino acid substitution, possibly increasing the affinity of the receptor (18). A series of studies suggests that this mutation could play an important role for treatment of addiction. In two initial reports it was demonstrated that carrying the G allele increases cortisol response to the mu-opiate antagonist naltrexone in healthy volunteers (32;167). These findings, in addition to previous studies showing that naltrexone selectively attenuates alcohol-induced euphoria in individuals with a family history of alcoholism (168), suggested that treatment response to naltrexone for alcoholism could be dependent on the A118G variant. In a study addressing this specific question, Oslin et al showed that carrying the G variant indeed increases the therapeutic response to naltrexone in treatment of alcoholism (29). This finding has been followed up by other studies replicating the effect and thus the A118G variant is now one of the few validated candidates for personalized medicine in psychiatry (30;169;170). The studies presented here point to the possibility that additional genotype selective treatment targets might emerge.

4.5.2 A possibility for translational pharmacogenetic studies

Genetic variance and increased expression of the NR3A gene in msP rats seems to confer resistance to suppression of alcohol consumption by the NMDA receptor antagonist neramexane. In AA rats, on the other hand, which display a similar genotype as non-selected Wistar rats, suppression of consumption is readily seen follow drug administration. Similarly, the innate up-regulation of CRHR1 expression and function in msP rats make this line a model selectively sensitive to antagonists for this target. These and other

observations suggest that AA and msP rats model different aspects of alcohol addiction (see 1.3.4) and that they might be differently sensitive to drugs for treatment of alcoholism. A similar situation has been described for the A118G mutation in the human mu-opiate receptor, indicating that animal models might reflect a phenomenon that is related to the human condition (i.e. selective efficacy dependent of genotype).

This raises the interesting prospect that genetic models of alcoholism might be used to identify genes underlying the differential treatment response. Such a strategy would include obtaining a pharmacological profile for drugs relevant for clinical treatment of alcoholism in a panel of genetically selected alcohol-preferring rat lines. Lines that display a divergent response to a specific drug would then be screened for gene expression differences and genetic variation in a similar fashion as described for NR3A.

Although it might be viewed as desirable to design a pharmacotherapeutic approach to which most patients are responsive to, this has proved exceedingly hard. As an example, naltrexone, one of the most widely used drug for treatment of alcoholism, is only effective in a subset of patients. It is likely that a range of pharmacological interventions will have to be designed to reach increased treatment response in the full spectrum of patients. The strategy described above might provide a way to identify genetic variation important for treatment response in alcoholic patients.

5 SUMMARY

Global expression profiling by microarrays identified members of the MAPK pathway and the GPCR regulator, ARRB2, as differentially expressed in several brain regions of AA rats compared to ANA rats.

Further expression analysis of ARRB2, revealed increased transcript levels in the hippocampus and striatum of AA rats compared to ANA rats. Differential expression was accompanied by different levels of ARRB2 protein levels in the hippocampus. The elevated expression was associated with a 7-marker haplotype in complete linkage dysequlibrium, which segregated fully between the lines, and was unique to the preferring line. In addition, a single, distinct, and highly significant eQTL was detected at the ARRB2 locus in hippocampus and striatum, supporting a cis-regulatory mechanism of gene regulation. Validation of the role of ARRB2 in alcohol preference was provided by knockout mice. ARRB2 mutant mice display significantly lower voluntary alcohol consumption and alcohol-induced locomotion. Together, these results suggest that ARRB2 modulates acute responses to alcohol and is an important mediator of alcohol reward.

GSTA4 was identified as differentially expressed in a microarray-based study of the medial prefrontal cortex of AA and ANA rats. Sequence analysis revealed genetic variation at the GSTA4 locus. Increased gene expression could be confirmed for several brain regions and was highly dependent on GSTA4 genotype. The results suggested that ANA rats seem to be impaired in metabolic pathways that metabolize reactive oxygen species. It is unlikely that genetic variation at the GSTA4 locus contributes to alcohol preference per se but it may influence co-segregated traits such as longevity and anxiety-like behavior.

Alcohol-preferring msP rats display increased sensitivity to stress and a lowered threshold for stress-induced reinstatement of alcohol-seeking compared to non- selected Wistar rats. Expression analysis revealed a robust up-regulation of CRHR1 transcript and protein in several brain regions of msP rats, most notably, the hippocampus and the amygdala. Antagonist studies showed that doses of a CRHR1 antagonist that were ineffective in Wistar rats, rescued both self-administration and reinstatement phenotypes of msP rats. Sequence analysis identified a dinculeotide haplotype variant of

the CRHR1 gene, only present in msP rats. These data indicate that CRHR1 is a key factor in determining the anxiety-like and alcohol-preferring phenotype of msP rats.

Previous results have shown that alcohol-preferring AA and msP rats exhibit a differential response to the NMDA receptor neramexane with respect to alcohol consumption. Microarray analysis indicated that this effect might be due to expression differences of the NR3A gene between the lines. Sequence analysis was performed to identify genetic variation underlying the differential response. While AA rats displayed a similar haplotype variant to that described for wt rats, msP rats showed variation at the NR3A locus. The results suggest that genetic models of alcoholism may be used in translational pharmacogenetic studies to identify loci that affect drug response in humans.

6 CONCLUDING REMARKS

Twin and adoption studies have shown that the risk for developing alcoholism and other addictive disorders has moderate heritability. In spite of significant efforts to identify genetic susceptibility factors for alcoholism, very few candidate genes have thus far been verified. The benefits of identifying such genes would be several. It would grant increased knowledge about fundamental biological mechanisms underlying addictive disorders and may ultimately provide novel targets for pharmacotherapy. Furthermore, the notion that alcoholism is a disorder with a substantial hereditary component is a view that is still controversial and not accepted by a substantial part of the society today. Hopefully, increased knowledge about underlying biological factors will contribute to lessen the stigmatization which still surrounds the disorder.

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