

GENETIC VARIATION AND BRAIN GENE EXPRESSION IN RODENT MODELS OF ALCOHOLISM: IMPLICATIONS FOR MEDICATION DEVELOPMENT

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Much research on experimental animals that is aimed to decipher genetic factors involved in alcoholism has been devoted to either models of innate alcohol-related phenotypes or responses after acute alcohol challenge. Such focus has, however, limitations when it comes to the pathogenetic mechanism underlying alcohol addiction, because the progression into the disorder takes years and genetic

as well as environmental factors may exert different influences along this trajectory. Animal models of the neuroadaptations involved in the development of dependence exist, but have been difficult to implement for genetic and genomics analysis. Consequently, currently available data have been difficult to reconcile with the human condition and could be misleading in predicting targets for medication development. This review will illustrate strengths and pitfalls of genomic approaches in rodent models of alcoholism and emphasize the need for convergent lines of evidence to improve the predictive value of such studies. Examples of a convergent research approach include validation studies for *Agt*, *Arb2*, *Crlr1*, *Grin3a*, and *Npy*.

I. Alcohol Addiction, Genetic Susceptibility, and Neuroadaptation

Humans like to consume alcohol for various reasons, mostly because it changes emotional states, but also for its taste or its ability to alter palatability of food. Individuals differ in their response to alcohol, which may range from strong euphoria to outright aversive reactions in some people, but the majority of individuals experience and appreciate the mild anxiolytic or sedative effects of alcohol. These responses depend largely on individual constitutive factors. After some trial-and-error period, most people develop a stable pattern of consumption that may be modulated by various life events, but is generally characterized by control over the drinking situation. Unfortunately, some people cannot maintain controlled drinking. Over years they gradually develop an alcohol use disorder, in the most severe cases alcohol addiction. The disorder is characterized by compulsive, uncontrolled alcohol intake and chronic relapses that can occur even after years of abstinence. Alcohol consumption in this stage is mainly motivated by negative drives, such as anxiety, low mood or increased sensitivity to stress, and is thus fundamentally different from occasional, but controlled drinking that is generally positively motivated (Koob and Volkow, 2010). The risk to develop alcoholism is to a large proportion (50–60%) heritable, but shared by dozens or even hundreds of genes that interact with each other and with the environment, a pattern typical for most psychiatric disorders (Goldman *et al.*, 2005).

In contrast to most addictive substances, such as central stimulants or opioids, which have well-established effects on specific targets, alcohol affects a wide range of neurochemical systems (Spanagel, 2009). Alcohol has no high affinity receptor or binding site and the weak reactivity of the molecule results in low potency to produce its pharmacological effects. Nevertheless, a few specific binding sites have been identified for alcohol including the NMDA, GABA_A, 5-HT₃, and

nACh receptors as well as L-type calcium channels and GIRKs (for recent reviews see [Harris *et al.*, 2008](#); [Spanagel, 2009](#)). Physiological relevant doses of alcohol, ranging from 5 to 100 mM blood and brain concentrations, alter the function of these targets and produce the acute neural effects that lead to the feeling of intoxication ranging depending on the dose, from disinhibition to sedation and even hypnosis. On the neural level, the primary effects of alcohol are followed by a wave of indirect effects involving a variety of neurotransmitter and neuropeptide systems—mainly monoamines, endogenous opioids, and endocannabinoids—which are thought to underlie the positively reinforcing effects. These are likely to be most relevant in early stages of alcohol use when alcohol associated cues are predictive of positively reinforcing alcohol effects, leading to what can be called “reward craving.” A key factor in determining an individual’s response to alcohol, including the feeling of positive effects or of being intoxicated, is genetic predisposition. Various candidate genes for these reactions have been put forward from human genetic studies ([Ray and Hutchison, 2004](#); [Schuckit *et al.*, 2009](#)). However, it is still a matter of debate to what extent these traits and associated candidate genes contribute to an increased risk for developing alcoholism.

One example of how genetic factors may mediate individual responses to alcohol is a single-nucleotide polymorphism within the first exon of μ -opioid receptor OPRM1. The variant A118G affects binding of endogenous ligands and the G-allele is consistently associated with increased experience of euphorogenic effects of alcohol ([Bond *et al.*, 1998](#); [Kakko *et al.*, 2008](#); [King *et al.*, 1997](#); [Ray and Hutchison, 2004](#); [Wand *et al.*, 2002](#)). A functionally equivalent variant in *rhesus macaques* is associated with increased psychomotor stimulation by alcohol, increased alcohol preference, and increased frequency of alcohol consumption to the level is associated with in the rhOPRM1 gene ([Barr *et al.*, 2008](#)). Although the convergence of phenotypic responses is compelling, translation on the level of sequence variation may not be possible, in particular when it comes to assigning complex functional or behavioral effects to a single-point mutation that could be in linkage disequilibrium with an unknown number of other variants. One possibility for such a direct interrogation of distinct human genetic variants is to generate mice that carry human DNA sequences, sometimes referred to as humanized mice. In the case of OPRM1 A118G this was accomplished by replacing the endogenous mouse exon 1 with either allele of huOPRM1 A118G ([Ramchandani *et al.*, 2010](#)). The resulting mouse lines thus differ only in a single nucleotide. We demonstrated that G-allele carrying mice have a strongly enhanced alcohol-evoked release of dopamine in the nucleus accumbens compared to mice homozygote for huOPRM1 A-allele, thereby linking this individual polymorphism to a distinct neurochemical mechanism. These findings were paralleled by PET imaging of healthy human subjects demonstrating increased alcohol-induced displacement of the D2 ligand ^{11}C -raclopride in the

ventral striatum of human G allele carriers compared to AA homozygotes (Ramchandani *et al.*, 2010). Together, these studies provide converging evidence across species that genetic variation at the OPRM1 locus modulates the acute responses to alcohol. The mouse model will be suitable to investigate whether excessive activation of brain reward systems by alcohol in OPRM1 118G carriers confers susceptibility for alcohol use disorders and to what extent this initial response to alcohol is changed once dependence has developed. In humans, being an OPRM1 118G carrier does not seem to predict increased risk for alcohol use disorders (Arias *et al.*, 2006). However, this conclusion is drawn from a meta-analysis of available data for A118G, and it is likely that such an approach pools genetically and pathophysiologically highly heterogeneous populations within the current diagnostic categories of alcoholism.

A critical point that deserves to be emphasized is that the responses to alcohol, both in regards to its pharmacological effects and to what comprises the motivational forces driving alcohol-related behaviors, change with repeated use. If excessive alcohol consumption progresses, brain stress systems involving various neuropeptides (e.g., CRH, NK1, or NPY) are becoming engaged and increasingly sensitized, which in turn results in further escalation of alcohol use and high propensity to relapse, which is now driven by negative reinforcement or “relief” craving (Heilig *et al.*, 2009). Genetic factors may produce their effects at any stage of the addiction trajectory: initially by mediating direct drug effects, providing high or low motivation for drug taking, then through different adjustments to increasing or rapidly changing alcohol levels, and finally by building up powerful incentives to maintain an obviously aberrant, negative behavior. Most individuals that are exposed to alcohol, even those subjected to potentially harmful environmental factors, do not develop addiction. This suggests that the brain is equipped with adaptive systems providing resiliency toward such insults. However, in some instances adaptive responses or the breakdown of adaptive capacity in one or several brain systems may lead to the development of addiction (Koob, 2003).

A further important factor in the acute and chronic responses to alcohol is pharmacokinetics, because individuals differ widely in alcohol metabolism. The primary metabolite of alcohol is acetaldehyde, which when it accumulates can trigger a number of unpleasant vegetative reactions known as “flushing response.” This reaction may act as a deterrent to further alcohol use. As an exception to the rule there is no major contribution of individual genes in complex psychiatric disorders, certain variants within alcohol metabolizing genes, that is, the alcohol/aldehyde dehydrogenase gene complex exert indeed strong protective effects on the risk of developing alcoholism (Edenberg, 2007), but do so likely at the expense of decreased defense against reactive metabolites and increased cancer liability (Brooks *et al.*, 2009; Sinclair, 2006).

II. Animal Models and the Trajectory of Alcohol Addiction

The purpose of an animal model of psychiatric disorders cannot be to perfectly copy the human condition, but to inform about (neuro)biological mechanisms underlying the disease process and to provide a tool for hypothesis testing, for example, if a molecular target might be useful for medication development. There is still some debate whether an animal model should cover as much of the symptomatology of a given psychiatric disorder as possible, for example, fulfill all diagnostic criteria of the Diagnostic and Statistical Manual for Mental Disorders (American Psychiatric Association, 1994) for alcoholism, or whether it is more useful to focus on a distinct core symptom that is recognized as a significant obstacle in treatment outcome. From a clinical perspective, the latter strategy appears to be more relevant. First, alcoholics, at least in early stages of their disease, present a highly heterogeneous, sometime diagnostically challenging clinical picture, which makes it difficult to identify common mechanisms. Second, the major problem in treating alcoholism is relapse and the compulsive mechanisms that may lead to it. Thus, the occurrence of relapse behavior or the intensity of craving may have little to do with tolerance to alcohol's pharmacological effects or the severity of the acute withdrawal reaction, two main diagnostic criteria for alcoholism. In fact, some of the most valid rodent models for medication development in alcoholism, that are, the alcohol deprivation effect or reinstatement of alcohol seeking, do not encompass any signs of tolerance or physical withdrawal (Egli, 2005; Sanchis-Segura and Spanagel, 2006).

Our understanding of the neurobiology of alcoholism comes largely from animal studies and is validated by at least two major developments: first, by the fact that all existing medications for this disorder are directly derived from animal experiments (Egli, 2005), and second the general concordance with an increasing amount of human brain imaging studies (Volkow *et al.*, 2003). Laboratory rodents can adequately model distinct characteristics of an addictive behavioral syndrome related to excessive drug intake, seeking and craving. These models have shown good predictive validity for identifying targets for novel pharmacological treatments for alcohol dependence (Egli, 2005). Consequently, these models are expected to be useful for genetic and genomic studies aimed to identify genes involved in the development of alcohol addiction.

Three classes of animal models can be recognized that have been considered for the study of genes and transcriptome responses in alcoholism. The first class addresses acute or short-term effects of alcohol and has been of key importance for identifying the pharmacological targets of alcohol and in studying its immediate behavioral consequences. The second class of models addresses genetically encoded vulnerability for the development of alcoholism and has been mainly

used to study traits related to initial alcohol responses. Within this category fall some inbred mice lines with exceptional high innate alcohol preference as well as rodent lines that have been developed by selective breeding for high or low alcohol consumption. The third category is based on alcohol-induced neuroadaptations, relating to the fact that addiction inevitably requires exposure to the drug. Animals either have access to alcohol over long periods of time or are involuntarily exposed to sufficiently high alcohol levels to induce dependence, which may reflect various states in the progression of the disorder.

As illustrated in Fig. 1, the three types of models can be placed along the addiction trajectory, and accordingly could be considered as relatively separate entities that may or may not be connected by shared neurobiological mechanisms. In other words, genetic factors that play an important role in mediating

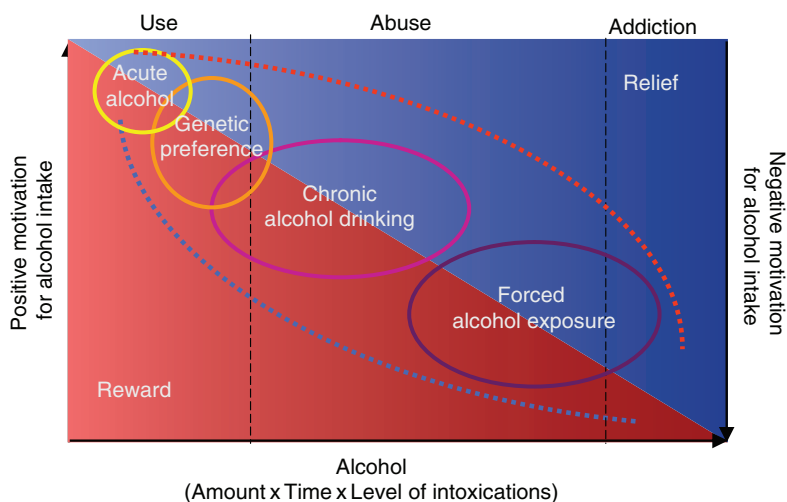


FIG. 1. Conceptual framework placing the common classes of animal models along the trajectory of addiction. Alcohol dependence progresses over time from initial, largely positively reinforcing, pleasurable alcohol effects (reward), to be maintained by relief from a negative emotional state (negative reinforcement). Genetic and environmental factors interact with each other and determine an individual's response to alcohol. Importantly, the influences of these factors vary along the trajectory of accumulated alcohol effects. Thus, an individual may experience alcohol consumption for a long time as positive, but finally show a rapid break down of the reward system (red dotted line). Alternatively, one's constitution may entail sensitized stress systems which could rapidly take-over the incentive for repeated alcohol use and entrap this person by powerful negative reinforcement already early in the addiction process (blue dotted line). Animal models can only address distinct aspects of the addiction trajectory. Widely used genetic models and acute alcohol challenges cover the early, primarily positively reinforced phases of alcohol actions, but miss the neuroadaptive processes characterizing the progression into addiction. Chronic alcohol consumption and forced intoxication procedures have been shown to involve various degrees and aspects of negative reinforcement, but are generally difficult to utilize for candidate gene discovery (See color plate 2).

early alcohol responses, such as the opiate–dopamine cascade in positive reinforcement, may be completely disengaged when behavioral responses are uncoupled from normal motivational control at the final stages of addiction. Similarly, stress response genes, such as the corticotropin-releasing factor receptor subtype 1 (CRHR1), may have little weight for some of the initial positive emotional actions of alcohol. The distinction of the various models seems to be obvious, but to neglect these facts is problematic for studying the mechanisms underlying alcohol addiction and particularly for identifying therapeutic candidate targets. A wealth of candidate genes has been obtained using acute and genetic models but the relevance of many of these genes remains to be established. For long-term alcohol exposure models, extensive genomic and transcriptome studies are lacking, but the emerging data suggest that these experiments can result in the identification of candidate genes that show greater consilience with human studies.

In the following we will briefly review some of the genetics and genomics findings from the various types of animal models. We will present our view on the strength and weaknesses of these models and their applications. Our main position is that clinically relevant genetic and genomic factors for the etiology of alcohol addiction can only be identified by attending to the drug-induced neuroplasticity and the formation of robust addiction-related behaviors, that is, by using animal models of the disease state. Trait models contribute importantly to the understanding of alcohol's various mechanisms of action, but they may be of limited use for finding mechanism involved in alcoholism, because none of the initial alcohol response traits is strongly associated to the human disorder. The best chance for finding consilience with human data and so to increase our knowledge about the human condition, results from animal studies searching for multiple lines of evidence that converge on a candidate mechanism.

III. Acute Pharmacological Responses to Alcohol

A. TRANSCRIPTOME STUDIES OF ACUTELY ALCOHOL-RESPONSIVE GENES

Genome-wide expression analysis of the acute responses to alcohol has been performed by several groups (Kerns *et al.*, 2005; Rulten *et al.*, 2006; Treadwell and Singh, 2004; Uddin *et al.*, 2005). Large numbers of genes affected by the alcohol challenge in a brain region-wise manner have been reported, but as to be expected from alcohol's complex pharmacology, no prominent mechanism could be unraveled. The most robust response to alcohol show gene clusters related to plasticity, myelination, neurogenesis, metal ion homeostasis, and cellular stress

response. In the process, web-based tools were developed for browsing the alcohol-responsive transcriptome (e.g., at <http://bioinfo.vipbg.vcu.edu/ERGR> (Guo *et al.*, 2009)).

Without doubt, genome-wide expression experiments give important clues about mechanistic aspects of the acute alcohol response. The findings converge with *in vitro* and tissue culture methods for promoter analysis and point to a distinct, but large set of genes that could be regulated by alcohol through shared promoter elements, including the cAMP-response (CRE), activator protein-1 (AP-1), specificity protein-1 (SP-1), and the alcohol response (ARE) binding elements (recently reviewed by Pignataro *et al.*, 2009). Importantly, the mechanisms involved in the acute responses to low and high doses of alcohol are conserved across a wide range of species, including worms, flies, fish, and rodents (Davies *et al.*, 2003; Moore *et al.*, 1998; Peng *et al.*, 2009). The functional implication of these acutely responding genes for alcohol's various effects has only been proven for relatively few candidates and requires generally multiple lines of evidence dissecting the actions of involved genes. A recent study tested the importance of decreased *Kras* expression by alcohol in mutant mice showing either up- or down-regulation of *Kras* (Repunte-Canonigo *et al.*, 2010). Genetic interference with the expression of this small G-protein was associated with altered transcriptomic response to alcohol challenge involving distinct signal transduction pathways such as PI3K, NF κ B, and Jak/Stat signaling. These findings are functionally validated by the demonstration that reduced *Kras* expression prevented the development of addiction-related behavior, that is, increased voluntary alcohol consumption after chronic intermittent alcohol vapor intoxication (see Section V.B.). Notably, *Kras* is also strongly upregulated in the brain of genetically high alcohol-preferring (HAP, see Section IV.A.) mice (Mulligan *et al.*, 2006). Given the pleiotropic actions of alcohol, an involvement of the RAS pathway is to be expected, but the convergence on distinct members of the RAS family and their interacting proteins may point to specific roles of these molecules for distinct alcohol responses. It will be interesting to see whether such observations will hold up as candidate genes for human alcohol consumption or dependence.

Furthermore, the induction of transcription factors such as *Fos* or *Egr1* by alcohol has been highly useful for delineating the brain sites that respond acutely to alcohol (reviewed in Vilpoux *et al.*, 2009). However, comparing maps of alcohol-sensitive regions from naïve- and alcohol-experienced rats clearly shows that this response is changing strongly with the development of dependence (Hansson *et al.*, 2008). Such information is lacking for nearly all acutely alcohol-responsive genes, but addressing these issues will importantly contribute to our understanding of how transcriptional control mechanisms participate in the disease process.

B. MODELS DERIVED BY REVERSE GENETIC DESIGN

Transgenic and knockout animal models have been utilized to a large extent for studying the role of individual candidate genes in the acute or early responses to alcohol. A recent review covering nearly 150 mouse mutants found that the majority of these lines show at least some alteration in alcohol consumption and other related phenotypes (Crabbe *et al.*, 2006). The results from these mutant lines emphasize the complexity of alcohol-related traits. Because the response to alcohol is intricately linked to a wide range of neurobiological systems, it will be difficult to prove alcohol-specific mechanisms in individual global gene knockouts. Conditional genetic interference will allow more specific perturbation, but the problem remains that alcohol responses are likely mediated by combined actions of many rather than a few genes. Furthermore, existing genetically engineered lines are so far rarely studied for neuroadaptations that are brought about by chronic alcohol use and that may be involved in the development of addiction. The importance of such studies is exemplified by knockout mice for the *Cnr1* gene. These mutants are not impaired in their acute responses to alcohol, but show dependence-related phenotypes after long-term exposure to the drug (Chu *et al.*, 2007; Sillaber *et al.*, 2002). In addition, the deletion of a single gene may have widespread consequences for the transcriptomic response to alcohol. For example, mice deficient for the gamma subunit of protein kinase C, *Prkcg*, do not develop tolerance to alcohol, and more than 100 genes react different to chronic, high-dose alcohol treatment between mutant and wild-type mice (Bowers *et al.*, 2006). The study identified a *Prkcg*-dependent gene, *Kcnk1*, which codes for a potassium channel and was related to resistance to tolerance.

In summary, experiments involving acute or short lasting alcohol challenges show wide spread genomic responses pointing to mechanisms of action. These responses change with repeated or chronic exposure to alcohol, but so far these dynamics have been little studied in the various experimental paradigms, and this information is particularly missing for the many transgenic lines showing altered initial alcohol responses. Another underdeveloped area is the validation of findings for the human condition. Investigations on human postmortem brains are not very helpful in this regard, because these studies cannot be designed in a manner that proves causality. A potential approach could be to study transcriptome responses of peripheral blood cells in humans after well-controlled alcohol challenge. Comparison of such data to corresponding blood and brain expression profiles from experimental animals could give important clues about translatable genomic responses to alcohol and may ultimately result in accessible biomarkers for diagnostic and monitoring of alcohol use disorders.

IV. Models of Genetic Susceptibility: Selected Rat Lines and Inbred Mouse Strains

A. GENETIC MODELS DERIVED BY SELECTIVE BREEDING

A common strategy to establish the heritability of a distinct phenotype or disorder is the so called forward genetic approach. The term includes phenotype-driven selective breeding and selection of animal lines with innate phenotypic differences. Among the earliest attempts to develop a disease model was selective breeding for the amount of alcohol an animal is willing to consume under the assumption that a high preference for alcohol may be a contributing factor to developing alcohol addiction. The first of these experiments was successfully reported by Mardones of the University of Chile more than 60 years ago (Mardones *et al.*, 1953). Animals were selectively bred for their daily consumption of a 10% alcohol solution. Within a few generations there was virtually no overlap in consumption between animals of the high- and low-preferring lines. Ultimately these researchers found that variation within alcohol metabolizing enzymes, in particular variants of the aldehyde dehydrogenase *Aldh2*, predict the major proportion of variance in alcohol consumption (Quintanilla *et al.*, 2006). The validity of this experimental approach is clearly demonstrated by the well-established fact that in humans certain variants within the alcohol/aldehyde dehydrogenase complex have a clear protective effect on the risk for developing alcoholism (Edenberg, 2007).

Due to its simplicity, many laboratories around the world have tried to replicate Mardones' experiment and succeeded in establishing at least eight rat and one mouse line with high and low alcohol preference (recently summarized in Crabbe *et al.*, 2010). Genetically preferring rats will consume about 5–10 g/kg/day and mice up to 20 g/kg/day. The underlying idea is that the selection pressure applied gradually leads to enrichment of alleles promoting or preventing alcohol drinking in the high- and low-preferring lines, respectively. Genetic factors controlling the behavior should thus be more easy to identify. By and large, it has been proven difficult to link observed differences conclusively to alcohol-drinking behavior. Because behavioral traits are multifactorial controlled and genes have pleiotropic actions, the different alcohol-preferring lines show considerable phenotypic diversity. Some of the behavioral or neurochemical traits may in fact be co-segregated with alcohol preference. In line with our general concepts of human alcoholism, co-segregated traits may fall into the anxiety domain as observed in the P and sP rat lines (Colombo *et al.*, 1995; Hansson *et al.*, 2006; Stewart *et al.*, 1993), or alternatively could reflect increase impulsivity like in AA rats (Möller *et al.*, 1997). The major source of line differences, however, is random fixation of alleles that are neutral to the selective pressure. These are expected to segregate randomly, but in populations with a small number of breeders such alleles will either be fixed or lost with time. Hence,

each of these lines and their respective controls represent a unique combination of selected and random traits. One way to partially control for the effects of random fixation is to generate multiple replicate lines for a selection experiment, and approach that has also been utilized in the breeding of some alcohol-preferring lines (i.e., HAD/LAD rat and HAP/LAP mouse lines).

1. *Transcriptome Analysis Illustrates Caveats of Selected Lines*

Comparing genome-wide expression patterns from the pairs of lines is a common approach to identify candidate genes whose differential expression is the result of allelic segregation. Various brain regions from alcohol-naïve animals have been subjected to this type of analysis resulting in identification of large numbers of differential expressed genes between the respective high and low alcohol-preferring lines (Arlinde *et al.*, 2004; Björk *et al.*, 2006; Ciccocioppo *et al.*, 2006; Edenberg *et al.*, 2005; Kimpel *et al.*, 2007; Mulligan *et al.*, 2006; Sommer *et al.*, 2001, 2006; Worst *et al.*, 2005). A somewhat surprising result of these studies was the notion of extremely low overlap in differential gene expression between the various models. A recent direct comparison of brain expression profiles from three alcohol-preferring rat lines gives a possible explanation for this observation by demonstrating that about one-third of the 6000 interrogated gene probes were significantly different expressed between P, HAD, and AA rats (Fig. 2; Matthaues *et al.*, 2009).

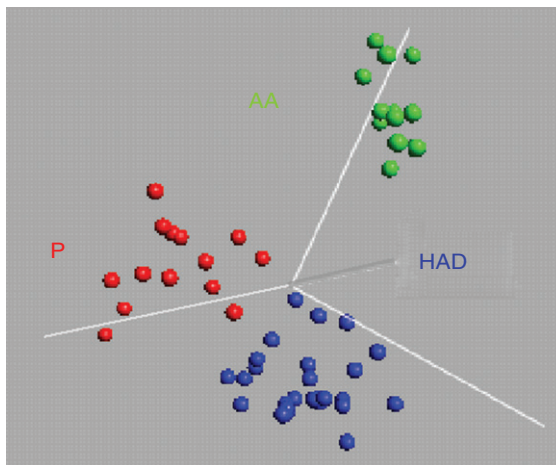


FIG. 2. Profound differences in brain gene expression between three selected lines for high alcohol preference. Principal component analysis was used to analyze microarray expression profiles from caudate-putamen or amygdala of AA, P, and HAD rats (Matthaues *et al.*, 2009). Each blob represents the summarized expression data from an individual microarray. Data are normalized for the effect of brain region. Analysis was done using Qlucore Omics Explorer (<http://www.qlucore.com>).

Because of the factors described above, the causal relationship between enrichment of alleles and increased alcohol consumption is often unclear, constituting a major hurdle in identifying candidate loci and genes for a specific trait as exemplified by studies on *Gsta4* (glutathione S-transferase alpha 4) in P/NP and AA/ANA rats. *Gsta4* was identified as differentially expressed in a high-throughput expression screen of several brain regions of P and NP rats (Liang *et al.*, 2004). Prompted by the observation that *Gsta4* is also located in proximity of a quantitative trait locus for alcohol preference in mice (Belknap and Atkins, 2001) the authors further investigated this gene and confirmed that alcohol-preferring P rats compared to non-preferring NP rats display a robust decrease in both *Gsta4* mRNA and protein levels. In contrast, alcohol-preferring AA rats show nearly twofold higher *Gsta4* brain expression compared to their low preferring genetic counterpart line (Björk *et al.*, 2006). P and ANA lines carry a different variant haplotype of the gene correlated with lower expression, suggesting that the differences are most likely mediated by cis-regulatory factors. The opposing correlations between alcohol preference and *Gsta4* expression observed in the two pairs of selected lines suggests that this gene is not likely involved in alcohol preference but was likely subject to random genetic fixation or may interact with the different genetic backgrounds of the founder lines by yet unknown mechanism (involving epistasis) pointing to the complexity and heterogeneous nature of alcohol preference.

Behavioral assessments of the three alcohol-preferring lines revealed also a potential pitfall of the genetic models, that is, a narrowed behavioral reaction norm. This term describes the amount of change in a quantitative phenotypic trait displayed in response to a certain amount of environmental challenge or pressure (Levine *et al.*, 1979). A line generated through the pressure of selective breeding may be “locked into” a particular phenotype, and thus will be less suitable for studies addressing changes in behavioral phenotype and gene expression patterns in relation to, for example, pharmacological or environmental challenge. Thus, for the P, HAD and AA lines it appears that that selection for high alcohol preference does not predict responsiveness in terms of alcohol-related behaviors. Only P rats, but not AA or HAD animals, responded with increased alcohol consumption to challenges such as alcohol deprivation or environmental stress (Vengeliene *et al.*, 2003).

2. Gene Expression, Pharmacoresponse, and Medication Development

Albeit selected lines have limitations when it comes to identifying genetic components intimately associated with alcohol-drinking behavior, they have become valuable tools for medication development (Egli, 2005). In spite of that alcohol addiction is a highly prevalent psychiatric disorder, only two medications,

the glutamatergic modulator acamprosate and the opioid antagonist naltrexone, are internationally approved for the treatment. These drugs have provided proof-of-concept in large clinical studies, but also point to a need for identifying additional pharmacological treatments (Bouza *et al.*, 2004). Established differences in genomic and neurochemical profiles between the various lines may not be a disadvantage, but rather can be an asset for medication development. Thus, some lines are responsive to certain classes of drugs, but may be inadequate for testing other compounds as illustrated in the following example.

Alcohol, as mentioned earlier, physically interacts with and antagonizes the NMDA receptor. Accordingly, NMDA antagonists have been proposed for the treatment of alcoholism (Spanagel, 2009). Based on preclinical findings, neramexane, a noncompetitive NMDA receptor antagonist, has been deemed especially promising. However, clinical studies with alcohol-dependent patients have so far yielded negative results, probably due to insufficient dosage (Spanagel and Kiefer, 2008). Neramexane suppresses voluntary alcohol consumption in AA rats but not in another alcohol-preferring line, the msP line (Spanagel, 2009). In search of genetic factors underlying such a difference in pharmacoresponsiveness, microarray-derived brain expression profiles were compared between these lines and the gene for the NMDA receptor subtype 3 A, *Grin3a*, was found to be significantly upregulated in cortex of msP rats compared to AA rats. The increased expression is likely due to genetic variation at the *Grin3a* locus in the msP rats (KB, unpublished data). NMDA receptors composed of NR1–NR3A subunits exhibit a reduced sensitivity to channel blockers compared with NR1–NR2A receptors (Chatterton *et al.*, 2002). Thus, it is possible that the increased NR3A expression in msP rats results in an overall downregulation of NMDA signaling, rendering these rats insensitive to further suppression by neramexane. Markedly elevated expression levels of NR3A have also been found in the brains of psychiatric patients (Mueller and Meador-Woodruff, 2004), supporting the notion that NMDA receptor blockers can provide a therapeutic venue in alcohol-dependent patients only when sufficient doses of these drugs are applied.

The concept of personalized medicine, that is, achieving increased treatment response by the use of genotype directed treatment has received considerable attention recently. Studies like the one described above raises the interesting prospect that selected lines 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. Follow up on these findings would include going back to the clinic and conduct targeted genetic screens in patients where the selection of candidate genes is based on the rodent data.

3. Candidate Gene Selection from Selected Lines Using Convergent Lines of Evidence

Even though identification of candidate genes for alcohol preference is difficult and multiple lines of evidence are needed to link any of these findings with good confidence to the phenotype, such support has indeed been compiled for some genes, here exemplified by studies on β -arrestin 2 (*Arrb2*), neuropeptide Y (*Npy*) and angiotensinogen (*Agt*).

Arrb2 was identified as a differentially expressed gene in a global expression screening of several brain regions of AA and ANA rats (Arlinde *et al.*, 2004). Further investigation of this gene was incited by studies showing that mouse lines selected for high alcohol preference display robustly upregulated *Arrb2* levels compared to their non-preferring counterparts (Mulligan *et al.*, 2006). The major function of the β -arrestins is to facilitate desensitization and internalization of G-protein coupled receptors following agonist stimulation (Björk *et al.*, 2010; Lefkowitz and Shenoy, 2005). *Arrb2* is a critical modulator of μ -opioid receptor signaling (Bohn *et al.*, 1999, 2003). Interestingly, alcohol-preferring AA rats exhibit increased *Arrb2* mRNA transcript and protein levels in several brain regions compared to ANA rats, and these differences appear to translate to protein levels (Björk *et al.*, 2008). To assess the functional importance of *Arrb2* for alcohol-related behaviors, mice lacking this gene were tested in a number of paradigms. Mutant mice display both reduced voluntary alcohol consumption and alcohol-evoked psychomotor stimulation, suggesting a role for *Arrb2* in mediating the rewarding effects of alcohol. There are currently no data regarding a role of ARRB2 in human alcoholism, but an involvement of β -arrestins in nicotine dependence is strongly suggested by human genetic studies (Sun *et al.*, 2008).

An extensive body of evidence from genetically modified animals exists supporting a role for *NPY* in alcohol preference (reviewed in (Thorsell *et al.*, 2006) and relapse behavior (Cippitelli *et al.*, 2010; Gilpin *et al.*, 2003)). Two of the selected rat lines, P and AA, show decreased *NPY* mRNA levels in several brain areas compared to their respective genetic counterparts (Arlinde *et al.*, 2004; Caberlotto *et al.*, 2001; Ehlers *et al.*, 1998). Expression of the peptide in the amygdala appears of special importance, not only for the trait anxiety shown by P rats, but also for alcohol consumption and relapse-like behaviors as demonstrated by specific interference with gene expression in this region using recombinant viral vectors (Thorsell *et al.*, 2007). As for *Arrb2*, the expression differences are likely due to *cis*-regulatory factors and genetic variants possibly leading to reduced expression have been identified (Spence *et al.*, 2005). These studies are of relevance for the human condition because the importance of variation in the *NPY* promoter for brain *NPY* expression and its association with stress responses and alcohol consumption was also demonstrated in primates, including *Rhesus macaques* and humans (Lindell *et al.*, 2010; Sommer *et al.*, 2010). Together,

these findings suggest that variation at NPY locus interacts with the degree of stress loading raising the possibility that human NPY variation could potentially increase risk of alcohol dependence more so among individuals with especially traumatic life experiences and early or high cumulative levels of stress exposure. In support of this argument, the only reports of a link between NPY variation and alcohol dependence have studied individuals with late-onset alcoholism or samples highly represented by war veterans (Mottagui-Tabar *et al.*, 2005; Zhu *et al.*, 2003). These studies underline the importance of gene–environment interaction for the development of alcoholism and further studies in this direction are warranted.

A wider ranging approach for candidate gene identification was recently proposed by merging brain gene expression data with human genetic linkage data, as well as human postmortem tissue data and information on biological function (Rodd *et al.*, 2006). This convergent functional genomics approach combined the analysis of three animal paradigms based on the P and NP rats at base line condition and their response to treatments with alcohol. Microarray gene expression data from five key brain regions (frontal cortex, amygdala, caudate–putamen, nucleus accumbens, and hippocampus) were analyzed. The integration of multiple independent lines of evidence, each by itself lacking sufficient discriminatory power, led to the identification of high probability candidate genes, pathways, and mechanisms for alcoholism, among those highly interesting targets for potential pharmacological intervention with existing non-addictive agents including ACE inhibitors for the angiotensin pathway, and mood stabilizers (lithium for PKC epsilon, lamotrigene and zonisamide for alpha-synuclein), or olanzapine for GABA-A1 receptor. The approach was validated using the angiotensin-converting enzyme (ACE) inhibitor lisinopril that markedly decreased alcohol intake in P rats (Rodd *et al.*, 2006). In line with the P rat, also other selected lines with high alcohol preference, that is, AA rats and HAP mice, show increased brain expression of the angiotensin precursor gene *Agt* (Saba *et al.*, 2006; Sommer *et al.*, 2006). A role for *Agt* as a positive modulator of alcohol consumption is further strengthened by work done in mutant mice with various types of genetic modifications in the angiotensin-signaling pathway (Maul *et al.*, 2001, 2005). Importantly, alcohol addicted rats show a long-lasting increase of brain *Agt* expression and respond to ACE inhibition with a more pronounced decrease in alcohol consumption compared to nonaddicted rats (Sommer *et al.*, 2007). Interaction of brain angiotensin and CRH systems has been suggested as an alcohol intake promoting mechanism (Sommer and Saavedra, 2008). These interactions may take place on a pre-wired background in the genetic models or may emerge as a dysregulation between various neurochemical systems during development of alcohol dependence.

B. APPLYING INBRED MICE STRAINS FOR THE INVESTIGATION OF ALCOHOL-RELATED PHENOTYPES

The use of mice in the search for alcohol-related genes has been different from rat. This is due to the availability of a large number of different inbred strains, which widely differ in their phenotypes and cover a rich reservoir of the natural genetic variance of this species. Inbred lines result from brother–sister mating for at least 20 generations and are genetically homogeneous, in other words, individuals of the same line are genetically identical. Provided the genotype is stable, these lines allow cumulative data collection over many generations. Great innate differences in alcohol preference between inbred strains of mice have been long known (McClearn and Rodgers, 1959). For example, the C57BL/6 consumes about 10 times more of a 10% alcohol solution compared to the DBA/2 line, and these differences in alcohol preference have remained stable over nearly 50 years (Wahlsten *et al.*, 2006). Consequently, much less emphasis has been given to selective breeding for alcohol preference/consumption.

Inbred mouse lines, in particular of the C57BL/6 and DBA/2 lines, have been intensely used to generate intercross populations (B6D2) for gene mapping using quantitative trait locus analysis. This approach correlates continuous measures of a distinct trait, for example, alcohol preference or withdrawal severity, with the information from allele specific markers spaced out across the genome. It is thereby possible to link the trait to one or several regions of the genome. With the availability of high-resolution genomic maps of the mouse genome and advanced methods to generate mapping populations, this approach becomes increasingly sensitive. Large scale re-sequencing of the mouse genome has produced high density maps of the genetic variation between more than 50 commonly used inbred mouse strains and identified over eight million genetic differences between them (<http://mouse.perlegen.com/mouse/index.html>), and thus allowed the construction of haplotype blocks of the mouse genome (Frazer *et al.*, 2007). Today, more than hundred QTLs for alcohol-related traits are recorded, again showing the complexity of the genetic control underlying these phenotypes. A database summarizing information on alcohol-related QTLs and related fine-mapping by congenic strains (mice that possess a short interval of DNA surrounding the QTL from a donor strain transferred onto the distinct genetic background of a recipient strain) is available at www.ohsu.edu/parc/data/qtl/by_phen.shtml

The major challenge of QTL mapping remains the identification of QTGs, the genes responsible for the trait. The difficulties arise from the fact that QTLs represent a relatively large genomic region that could harbor hundreds of genes. Thus far only one gene, *Mpdz*, implicated in protein–protein interactions and a known interaction partner of 5-HT₂ and GABA_B receptors, has been

conclusively linked to be the source of the effect of a QTL for alcohol withdrawal seizure intensity. The path to this discovery from the initial description of the QTL to mounting enough sufficient evidence for proving the case of the candidate took a substantial amount of time (Buck *et al.*, 1997; Shirley *et al.*, 2004). A second strong candidate QTG is *Scn4b* (voltage-gated sodium channel, subunit beta-4), which has been extracted out of a large meta-analysis of differential expression between alcohol-preferring mouse lines and fine mapping of a QTL using congenic lines for Chr9 (Mulligan *et al.*, 2006). It remains to be proven that *Scn4b* is the sole source of the effect of this QTL.

C. GENETICAL GENOMICS: COMBINING QTL MAPPING AND GENOME WIDE-WIDE EXPRESSION PROFILING AS A SHORT CUT TO CANDIDATE GENE IDENTIFICATION?

Differentially expressed genes can be mapped to alcohol QTLs and those who pass this filter can be searched for polymorphisms. Using this strategy the carnitine transporter, *Slc22a4*, was found be linked to alcohol sensitivity (loss of righting response) and to contain several promoter polymorphisms that disrupt putative transcription factor binding sites (Maclaren *et al.*, 2006). Interestingly, the human ortholog maps to a genomic region associated with human alcohol sensitivity in linkage studies.

Moreover, gene expression can be seen as a quantitative trait by itself and consequently be mapped to a distinct position in the genome, thereby delineating loci that are involved in the control of expression of individual genes (this has been termed expression QTL or eQTL for distinction from QTLs obtained for a behavior, in this context referred to as bQTL). By overlaying a bQTL with eQTLs for genes differentially expressed in the mapping population one can considerably narrow down the number of genes that arises from expression profiling studies and that might be in control of the behavior. This integration of transcriptional profiling and linkage analysis has been termed “genetical genomics” (Jansen and Nap, 2001). Extensive data on brain expression difference between the C57BL/6 and DBA/2 mice and their derivative intercross or recombinant lines have been collected by several consortia and revealed potential regulatory networks involved in various alcohol-related phenotypes. These data are comprehensively and freely available to the community on the WebQTL site, <http://www.genenetwork.org> (Chesler *et al.*, 2005).

For alcohol-related phenotypes, this approach has been repeatedly used and refined to identify candidate genes in mice and rats (Hu *et al.*, 2008; Saba *et al.*, 2006; Tabakoff *et al.*, 2008, 2009). Data from this elegant set of studies are

available for further mining at <http://phenogen.uchsc.edu/PhenoGen/index.jsp> (Hoffman *et al.*, 2010). One interesting conclusion from these experiments is that the genetic networks controlling alcohol action at low doses, that is, alcohol preference, seem to be completely different from the ones involved in tolerance to high doses of alcohol (Saba *et al.*, 2006). Furthermore, it appears that some of the candidate genes for alcohol preference in mice implicate the olfactory system (Tabakoff *et al.*, 2008). Support for this hypothesis comes from experiments demonstrating that stimulation of the rat tongue with a drop of aqueous alcohol *in vivo* alters expression profiles in the trigeminal ganglion, which in turn could trigger some of the gustatory or bodily responses to alcohol (Matsumoto *et al.*, 2006). It remains hitherto unclear if these findings are a species-specific characteristic of the rodent model or if they can be generalized to human alcohol consumption.

Recent advances in rat genome research allow extending the genetical genomics approach toward this species. The HxB/BxH panel of recombinant inbred rat strains is extensively characterized for cardiovascular and metabolic traits but has received little attention so far in the field of behavioral and psychiatric genetics. Alcohol-related traits are well distributed among the 25 or so recombinant inbred lines so that bQTLs for alcohol consumption could be determined in this population, the most robust one located on Chr1. The overlap of brain eQTLs with bQTLs for alcohol drinking produced a set of 12 candidate genes involved in GABA signaling, activation of dopaminergic neurons as well as energy metabolism and caloric intake (Tabakoff *et al.*, 2009). This study also asked whether these results are relevant for human alcohol consumption by testing association of about 850 gene-related SNPs with daily intake levels in two human populations. Significant association was found for some variants related to GABA genes. The study, thus, presents initial evidence for convergence of human and animal genetic data.

The low number of candidate genes derived from the above experiments points to an important problem of the genetical genomics approach: detection of highly specific candidate genes comes at the expense of sensitivity. The magnitude of this problem becomes evident when one compares the number of genes that are contained under a single QTL and that are actually differentially expressed. Such an experiment was reported recently for the highly significant alcohol preference QTL on Chr4 in P rats (Liang *et al.*, 2010). This QTL was isolated from its genomic background by producing reciprocal chromosome 4 congenic strains. In other words, the genomic region containing the QTL from the P line was transferred onto the NP background and vice versa. Any true difference in gene expression that is caused by locus specific genetic variation should carry along with this switch. Indeed, more than 70 cis-regulated, differentially expressed genes could be assigned to this QTL by this method, and these are proposed as strong candidates for affecting alcohol consumption. On the

other hand, there is no hint of an alcohol preference QTL on Chr4 in the HxB/BxH panel of rat lines, and also with the AA model no overlap in differential expression can be found, except for a few genes such as *Snca* (α -synuclein), *Dgki* (diacylglycerolkinase iota), and *Npy* (Arlinde *et al.*, 2004; Sommer *et al.*, 2001, 2006; WHS, unpublished data). Thus, while the QTL on Chr4 seems to be highly important for drinking behavior in P rats, the same genes may not play a major role for controlling this behavior in the other rat models. This obviously poses a problem for generalizing findings from rodent genetic models to human alcohol consumption or alcohol use disorders and highlights the significant genetic heterogeneity underlying alcohol preference.

D. COMMON THEMES FROM TRANSCRIPTOME STUDIES IN GENETIC MODELS OF ALCOHOLISM

Despite differences in experimental design and the difficulties in linking a specific candidate gene to the high-drinking phenotype, a recurring theme in examining potential transcriptome differences between selected lines under alcohol-naïve conditions are two major functional groups, neuroplasticity and metabolism. Nearly all studies point to few, brain region-specific signal transduction pathways (e.g., cAMP/CREB/PKA/MAPK, Jak/Stat, NF- κ B, AKT) or plasticity markers (neuropeptides, synaptic proteins) and a wide range of differences in metabolic pathways. Differential expression of signaling molecules under alcohol-naïve conditions seems to predict some alcohol-responsive genes. Thus, most of the responsive genes after an acute alcohol challenge of C57BL/6 mice show constitutively different levels between alcohol-naïve C57BL/6 and DBA/2 animals (Rulten *et al.*, 2006). Key pathways identified in this study are also differentially expressed between AA and ANA rats, and are functionally affected by an acute alcohol challenge as demonstrated by different phosphorylation of AKT, GSK3 β , and ERK1/2 in these rat lines (Neznanova *et al.*, 2009; Sommer *et al.*, 2006). On the other hand, high alcohol preference does not predict general responsiveness to the drug. In fact, alcohol-avoiding DBA/2 mice show a stronger transcriptome response in the prefrontal cortex as well as stronger locomotor activation upon acute alcohol challenge relative to high-drinking C57BL/6 mice (Crabbe *et al.*, 1982; Kerns *et al.*, 2005).

Observed alterations in metabolic gene expression are only partially attributable to variances in the ADH/ALDH complex. Alcohol avoidance seems to emerge very rapidly during selective breeding, which in fact would support a major gene effect on this behavior. On the other hand, expression of *Aldh2* is higher in DBA/2 compared to C57BL/6 mice (Bhave *et al.*, 2006), which is not

consistent with the general notion of a protective effect of this enzyme against increased alcohol consumption (Edenberg, 2007). An alternative possibility to be considered is that metabolic adaptation to higher alcohol levels may be an essential co-selected trait when selective pressure toward high alcohol preference is applied. Support for this hypothesis comes from two independent lines of evidence. Human brain imaging data demonstrate that low doses of alcohol decrease brain glucose metabolism in human subjects to a much larger degree than expected from observed cognitive and behavioral symptoms (Volkow *et al.*, 2006). Upon 0.5 g/kg alcohol, which leaves subjects alert and only moderately intoxicated, whole-brain glucose metabolism could be reduced by 30%, which is close to levels encountered by doses of anesthetics that induce unconsciousness. To substitute this energy deficit, brain metabolism has to be able to shift toward alternative substrates, such as acetate which can enter the brain through active transport mechanisms (Cruz *et al.*, 2005; Pawlosky *et al.*, 2010; Waniewski and Martin, 1998). Furthermore, organisms that consume large amounts of fermented food seem to be enzymatically well equipped to ensure that the ingested alcohol does not interfere with their survival. An extreme case was recently reported where the penta-tailed treeshrew (*Ptilocercus lowii*) in its natural habitat frequently consumes alcohol doses that would intoxicate humans without showing signs of intoxication (Wiens *et al.*, 2008). Taken together, it appears that coping with high alcohol levels requires metabolic flexibility that likely reaches beyond tolerance mediated by rapid metabolism through the ADH/ALDH complex, but may be obtained through a wide range of tuning mechanism to secure sufficient ATP production for proper brain functioning.

E. CONSILIENCE BETWEEN CANDIDATE GENES FOUND IN GENETIC ANIMAL MODELS AND FOR HUMAN ALCOHOLISM

With the growing number of candidate genes for which sufficient evidence for their involvement in acute or early alcohol responses is mounted, the question arises to what extent these findings are relevant for the human condition. In other words, are the genes that mediate innate alcohol-related behaviors in mice the same as those that comprise risk factors for human alcoholism?

Some examples for such overlap can indeed be found. MPDZ, identified as a QTG for withdrawal seizure intensity in mice (Shirley *et al.*, 2004), was associated with alcohol-related phenotypes in two human populations, though not with alcohol withdrawal seizures (Karpyak *et al.*, 2010; Tabakoff *et al.*, 2009). Likewise, in an attempt to find overlapping confirmed QTLs for specific alcohol-related behaviors in mice, that is, alcohol preference and alcohol withdrawal severity,

and any alcohol-related phenotype in humans, including DSM-IV defined alcoholism, it was realized this is not an easy task (Ehlers *et al.*, 2010). In the end, two cases of overlap were found. A human QTL on distal chromosome 1 for alcohol dependence overlaps with the mouse QTL for alcohol withdrawal on distal chromosome 4 that was conclusively linked to *Mpdz*. In the second case, a human QTL for alcohol dependence severity and withdrawal on distal chromosome 15 maps on the region of mouse Chr9, where a broad QTL for alcohol preference was found. Thus, the paired phenotypes are only loosely matched. The authors conclude that exact phenotypic matching might not be necessary or optimal for finding genes that affect alcohol-related behaviors. From the human disease perspective, this is frustrating news because it implies that either existing data are not sufficiently comparable or the applied models do not adequately reflect the clinical phenotype. As often, truth lies in both arguments and an extensive discussion of this subject including what might be necessary to reach better consilience between clinical and animal research fields on alcoholism can be found in a recent special issue of *Addiction Biology* (Crabbe, 2010).

On the animal side, there has been a strong bias toward easy assessable phenotypes, that is, alcohol preference, tolerance or withdrawal severity, but judging by the positions expressed in the consilience papers we still lack a good understanding of how these animal responses can be translated into human alcohol behaviors. To illustrate the point, we will briefly go over some issues raised in this debate regarding these phenotypes. Preference drinking in rodents relates to alcohol's reinforcing effects, but this test lacks any measure of the motivational component of this behavior (Leeman *et al.*, 2010; Stephens *et al.*, 2010). It is possible to measure reinforcement and reward sensitivity quite precisely in animal models, but for these tests genetic or transcriptome data are hitherto lacking. As detailed above it is still a matter of debate to what extent increased alcohol preference in humans may contribute to the risk of developing alcoholism (Arias *et al.*, 2006). Also a low level of response to alcohol intoxication has some degree of heritability in humans and seems predicative for developing alcohol addiction (Schuckit *et al.*, 2009). In animals this genetic relationship is often assessed by the loss of righting reflex (LORR), in which hypnotic doses of alcohol are injected and time is measured until the animal regains the ability to right itself. Plenty of genetic and expression data have been obtained for this test, but its validity for human low level of response to alcohol is not demonstrated (Crabbe *et al.*, 2010). Finally, seeking relief from acute withdrawal symptoms by renewed alcohol intake is one diagnostic criterion for alcoholism (DSM-IV). A hallmark feature of alcohol withdrawal is hyperexcitability, which can be relatively easily measured in animals as withdrawal-induced seizures. Although the genetic determinants of withdrawal seizure susceptibility are well characterized, it is uncertain how this susceptibility connects to the development of an addictive process (Heilig *et al.*, 2010).

In summary, most of the innate responses in commonly employed animal models miss the aspect of alcohol and behaviorally induced neuroadaptations that underlie the motivational shift occurring during the progression into addiction. Therefore, many of these efforts might fail to detect disease relevant genetic mechanisms. The high alcohol consuming phenotype has been viewed as an important methodological advantage for pharmacological studies, and it has been repeatedly argued that these models possess good predictive validity based on the fact that they have played an important role in the development of available medications for alcoholism. Such an argument carries the risk of being circular and given the low efficacy of current treatments, the suitability of a particular animal model for testing a particular drug should be continuously reevaluated. Further development of genetic models should reach beyond stable intake patterns, which likely reflect controlled drinking. Changing environmental conditions may provide a challenge for animals with high alcohol preference that may drive them to override their internal control mechanisms preventing intoxication. It has been shown that scheduling alcohol access to short periods at the beginning of their active (circadian dark) phase can make rodents to engage in more harmful drinking patterns (“too fast – too much”). Selection for this type of drinking was successfully demonstrated recently and these mice consistently reach blood alcohol levels >1 g/L (Crabbe *et al.*, 2009; Rhodes *et al.*, 2007). It will be interesting to see what genes control those safety signals and the propensity of an animal to ignore them. Apart from high alcohol intake, other traits predictive of an increased addiction risk should be subjected to behavioral genetics analysis. Such traits may include impulsive choices, impaired extinction of conditioned alcohol responses or increased sensitivity for reinstatement of alcohol-seeking behavior. Future research will show to what extent new models will contribute to increased consillience of human and animal data and to a better understanding of alcohol addiction.

V. Modeling Addiction-Related Neuroadaptations by Long-Term Alcohol Access or Forced Intoxication

The studies reviewed above have mainly focused on the acquisition of alcohol drinking or the maintenance of relatively constant and controlled drinking behaviors. This work has led to the identification of genetic determinants of alcohol's positive reinforcement processes. The emergence of uncontrolled, compulsive alcohol consumption and seeking behaviors typically seen in addicted individuals is likely driven by negative motivational forces, probably triggered as an opponent process by acute, protracted, or conditioned withdrawal. The allostatic dysregulation that is hypothesized to underlie this development requires

long-lasting neuroadaptations of the reinforcement systems (Koob and Le Moal, 2001).

Until recently, the study of long-term neuroadaptations in alcohol addiction has been limited, in part because of lack of suitable animal models. A major obstacle of experimental alcohol research is that even in high-preferring lines voluntary alcohol drinking rarely reaches blood or brain alcohol concentrations (BACs) required for dependence induction (> 1 to 2 g/L). Pharmacological effects to modulate this level of intake may be of limited use for modeling treatment of human alcoholism (Egli, 2005; Heilig and Egli, 2005). In order to drive the type of stable plasticity associated with or underlying dependence, robust changes in multiple transmitter systems are likely to be required. However, glutamate signaling is thought to be of particular importance and can likely only be provided by high enough alcohol concentrations that lead to consecutive withdrawal reactions.

Large amounts of alcohol are indeed consumed voluntarily also by nonselected laboratory rodents under certain experimental conditions. For example, by scheduling access to alcohol drinking to the beginning of the circadian dark phase, rats can overcome the internal control signals that limit their intake and thus engage in excessive drinking (Rhodes *et al.*, 2005). In a very different approach, rats will increase their alcohol consumption in time when given access over many months and may actually double or triple their daily intake (Holter *et al.*, 1998; Wolfgramm *et al.*, 2000). Also, alternate access to 20% alcohol and water every other day has successfully been used to increase voluntary alcohol consumption in a matter of weeks (Steensland *et al.*, 2007; Wise, 1975). The excessive intake observed under all these procedures can be selectively reduced by acamprosate or naltrexone, providing validation for their use in medication development. However, their genetic or transcriptome foundation has yet to be established.

Two well-established models for studying aspects of relapse behavior are the reinstatement of alcohol seeking and the alcohol deprivation effect (Bachteler *et al.*, 2005; Katner *et al.*, 1999; Sanchis-Segura and Spanagel, 2006; Shaham *et al.*, 2003). In the former, animals are trained to respond for alcohol in self-administer chambers. Then, no reinforcement is given during the self-administration sessions until operant responding is extinguished. Following extinction, a priming dose of alcohol, stress or conditioned stimuli can reinstate alcohol-seeking behavior. Although the obtained BACs during alcohol self-administration are below 1 g/L, the procedure clearly induces long-term neuroadaptation within addiction-related brain circuits. However, reinstatement of alcohol seeking can be elicited both in alcohol dependent and in nondependent animals, and thus, the specificity of the underlying processes for alcohol addiction is unclear. Nevertheless, the blockade of CRHR1 receptors prevents stress-induced reinstatement of alcohol seeking only in dependent animals, demonstrating that the

mechanisms leading to this behavior in dependent and nondependent animals are different (Gehlert *et al.*, 2007). Various pharmacological targets interfere with reinstatement behavior, but so far experiments to analyze the genetic factors or transcriptomic responses are lacking.

The term alcohol deprivation effect (ADE) refers to a phenomenon that is observable across many species including mice, rats, monkeys, and humans (Spanagel, 2009). Long-term access to alcohol, when interspersed by short periods where access is prevented (deprivation) will result in temporarily excessive alcohol consumption. In normal, unselected male rats, daily intake may increase from normal 1–2 g/kg alcohol to above 6 g/kg after the deprivation. Importantly, repeating the pattern of access and deprivation progressively elicits a compulsive-like alcohol seeking. The uncontrolled and compulsive component of this drinking behavior is demonstrated by taste adulteration of the alcohol solution with quinine, a highly bitter substance that usually produces strong taste aversion in rats (Spanagel and Holter, 2000; Vengeliene *et al.*, 2009). The ADE is widely used in preclinical medication development and the results for many compounds have been systematically reviewed recently by Spanagel (2009).

A. NEUROADAPTATIONS INVOLVED IN COMPULSIVE-LIKE BEHAVIORS AFTER LONG-TERM ALCOHOL ACCESS

Long-term alcohol access with repeated deprivation periods has substantial effects on the striatal and amygdala transcriptome (Matthaus *et al.*, 2009). From the overall expression changes several networks could be identified related to heme synthesis, immunoregulation, and cytoskeleton. Whether these alterations reflect compensatory mechanisms to the long-term alcohol intake or are involved in the addiction-related behavioral responses remains to be established. Another finding from this experiment was upregulation of striatal dopamine D3 receptors and the demonstration of their functional involvement in the behavioral control of the ADE by specific antagonists (Vengeliene *et al.*, 2006). These data are highly relevant for human alcoholism because a functional polymorphism in the human DRD3 gene (Ser9Gly) affects P300 event-related potentials, an EEG based endophenotype consistently associated with alcoholism (Mulert *et al.*, 2006). Of even greater importance seems to be the fact that the chronic alcohol intake altered expression in a set of genes that was predicative for finding susceptibility genes for human alcoholism in a genome-wide association study (Treutlein *et al.*, 2009). Out of fifteen SNPs, that could be confirmed in the replication study, three were put forward by the animal experiment. These three SNPs were in CDH13 (cadherin 13), ADH1C (alcohol dehydrogenase 1C), and GATA4 (GATA

binding protein 4). Thus, combining animal and human data in such a convergent approach can indeed result in increased consilience for our knowledge about human alcoholism.

A new study in the long-term access/repeated deprivation model focused on alterations in glutamate signaling in the striatum associated with development of compulsive-like drinking behavior (Vengeliene *et al.*, 2010). Expression of many glutamatergic genes in the dorsal striatum was altered by the long-term alcohol access. Importantly, about a third could be reverted to baseline by treatment with a glycine receptor antagonist that showed long-lasting anti-relapse properties in this model. Further data mining points to an important role of *Adcyap1* (adenylate cyclase activating polypeptide 1, also known as PACAP) in mediating this effect. These findings fit well with the proposed role of striatal circuits in habit formation and that their dysfunction may underlie compulsive drug-seeking behavior (Vanderschuren and Everitt, 2005).

Another study testing the effects of 1-year access to alcohol (Sake) was conducted in Fisher rats. However, because in this study rats did not have a choice between alcohol and nonalcohol solutions, it is unlikely that similar neuroadaptive response should have taken place as in the ADE model. Gene expression profiling was combined with proteomics analysis. No overlap with the above reviewed studies was reported, but a strong downregulation of NADH dehydrogenase (ubiquinone) Fe-S protein 1, *Ndufs1*, was found pointing again to altered mitochondrial function in the brain of alcohol-drinking rats.

B. FORCED ALCOHOL EXPOSURE, SENSITIZATION OF STRESS SYSTEMS, AND THE POST-DEPENDENT STATE

The major disadvantage of the long-term alcohol access model may be its high logistic requirements, which are difficult to afford for most laboratories. Alternatively, forced alcohol exposure can produce over relatively short periods of time a behavioral profile consistent with alcohol addiction. Forced intoxication can be administered via different routes, by offering alcohol-containing liquid diet as the sole source of food, by intragastric alcohol gavages, by breathing alcohol vapor or by systemic injection (Majchrowicz, 1975; Rogers *et al.*, 1979). For a long time, chronic alcohol administration via these routes was known to produce robust withdrawal reactions (Becker, 2000), but only transient motivation to self-administer alcohol (Roberts *et al.*, 2000). Thus, in contrast to the clinical situation, increased alcohol preference as well as most of the induced neuroplasticity was fading away along with the recovery from the acute withdrawal reaction. The missing piece in these experimental setups was

consideration of the temporal pattern of alcohol exposure. Even in heavy drinkers, the brain is not bathed constantly in high alcohol concentrations, but has to cope with ever changing alcohol levels, from rapidly rising back to zero, thereby going repeatedly through cycles of intoxication and various degrees of withdrawal. Once the element of intermittent exposure patterns was introduced into the routes of chronic administration, researchers began to see behavioral consequences that persist long beyond completion of physical withdrawal (Lopez and Becker, 2005; O'Dell *et al.*, 2004; Rimondini *et al.*, 2002). Progressively increasing activation of glutamatergic transmission over repeated withdrawals is likely to provide the signal for this plasticity (De Witte *et al.*, 2003).

One of the most reliable and versatile tools for precise control of brain alcohol exposure is vapor exposure. Using this approach it was shown that prolonged brain alcohol exposure to levels commonly occurring in human alcoholics (~ 150 – 250 mg/dL or 35–55 mM) leads to behavioral consequences that seem to be relevant for alcoholism (Rimondini *et al.*, 2002). Prolonged duration (Rimondini *et al.*, 2003) and an intermittent pattern of exposure (O'Dell *et al.*, 2004; Rimondini *et al.*, 2002), two features that mimic the exposure profile in clinical alcoholism, appear critical for induction of the key behavioral consequences that persist long beyond completion of physical withdrawal: (1) escalation of subsequent voluntary alcohol intake, measured using both two-bottle free-choice drinking (Griffin III *et al.*, 2009; Lopez and Becker, 2005; Rimondini *et al.*, 2002) and operant responding for alcohol (Roberts *et al.*, 2000); (2) sensitization of behavioral stress responses (Breese *et al.*, 2005b; Overstreet *et al.*, 2002; Sommer *et al.*, 2008; Valdez *et al.*, 2002, 2003, 2004). The resulting behavioral syndrome has been termed the “post-dependent state” (Heilig and Koob, 2007; Heilig *et al.*, 2009). This term is used to describe the sum of the behavioral and neuroadaptive consequences that are induced as an animal becomes dependent on alcohol, and that remain for extended periods of time thereafter even in its absence. Examples of such long-lasting neuroadaptations after intermittent alcohol vapor exposure from our own work are summarized in Fig. 3.

Other methods that lead to repeated cycles of intoxication and withdrawal exist, such as for example through forced liquid diet or intragastric alcohol gavages. These have been recently reevaluated for inducing a post-dependent state (Braconi *et al.*, 2010; Gilpin *et al.*, 2009). Although perhaps less potent and less easy to control, the procedures can induce similar sets of behavioral consequences (for review see Breese *et al.*, 2005a).

Although vapor exposure by itself and the rapidly changing alcohol levels are a demanding challenge to the animals, the procedure is safe and we found no overt health problems in post-dependent rats that had been abstinent for 3 weeks. Besides numerous unchanged blood parameters, also basal corticosterone levels and alcohol metabolism were normal. (Rimondini *et al.*, 2002, 2008). Alcohol vapor exposure has been criticized to be a highly non-physiological method of

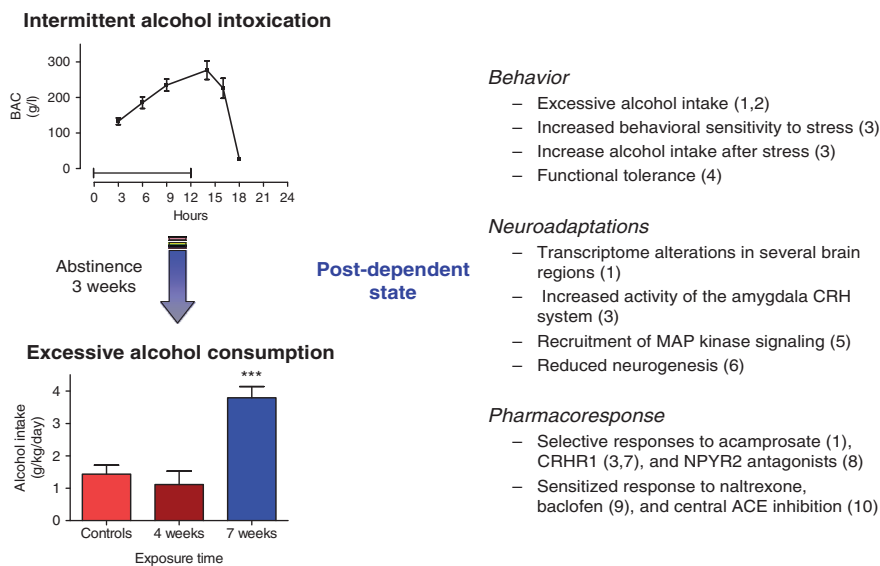
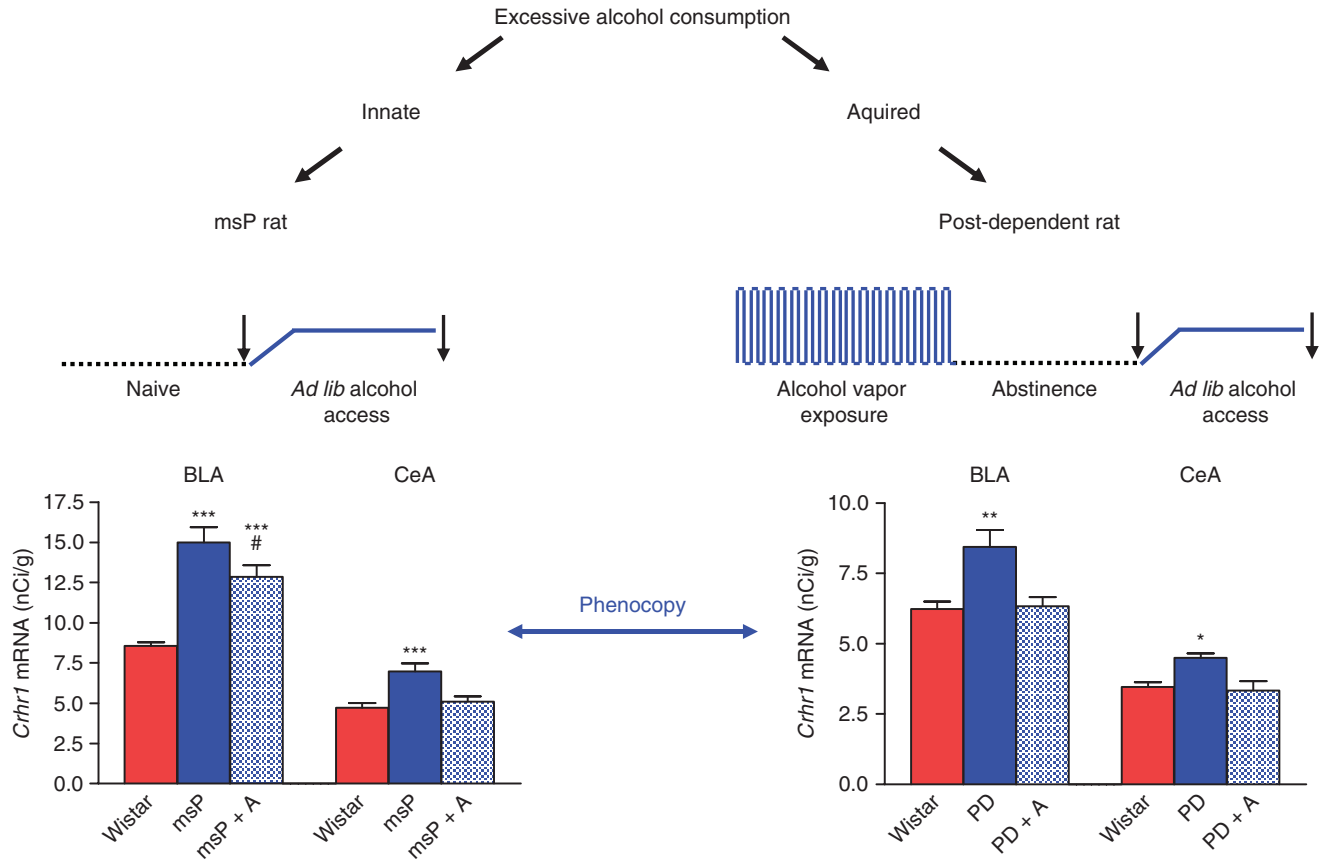


Fig. 3. Intermittent alcohol intoxication leads to the development of a post-dependent state. Left: The top graph shows blood alcohol concentrations in rat obtained during one daily cycle of alcohol vapor exposure and withdrawal. The bottom graph shows voluntary alcohol consumption in a two-bottle free-choice, continuous access paradigm (6% alcohol in water vs. water only) after 4 and 7 weeks of daily intermittent exposure cycles. Seven weeks exposed animals develop a persistent phenotype of excessive voluntary alcohol consumption and increased behavioral sensitivity to stress (post-dependent state). Right: Summary of our observations in post-dependent rats. Numbers in parenthesis refer to the following publications: (1) Rimondini *et al.*, 2002; (2) Rimondini *et al.*, 2003; (3) Sommer *et al.*, 2008; (4) Rimondini *et al.*, 2008; (5) Hansson *et al.*, 2008; (6) Hansson *et al.*, 2010; (7) Gehlert *et al.*, 2007; (8) Rimondini *et al.*, 2005; (9) unpublished results; (10) Sommer *et al.*, 2007. *** $p < .001$.

alcohol delivery. In fact, alcohol inhalers (AWOLTM) have been introduced for human consumption, which clearly demonstrates that, given the choice, humans voluntarily use alcohol vaporization as a method of intoxication. Scientific reports on the health consequences of this consumption pattern in humans are still lacking. Exposed animals show no apparent pathology in respiratory function. Olfactory and gustatory systems have not been investigated so far; however, since vapor exposed animals do not differ in preference for sweet and bitter taste from controls, basic function of these systems seems to be intact. To this point, the only known long-term pathology in post-dependent rats is secondary osteoporosis (Torricelli *et al.*, 2007), a condition that is commonly seen also in alcoholic patients (Kanis *et al.*, 2005).

A key neuroadaptation in post-dependent animals and widely replicated finding is the recruitment of the CRH system within the amygdala brain region



(Heilig and Koob, 2007), which is critically involved in mediating dependence-induced increased behavioral stress responses, elevated alcohol self-administration and alcohol-seeking behavior during both acute and protracted abstinence (Funk *et al.*, 2006; Gehlert *et al.*, 2007; Sommer *et al.*, 2008; Valdez *et al.*, 2003).

Interestingly, robust *Crhrl* upregulation was also demonstrated in the genetically selected, alcohol-preferring Marchigian Sardinian Preferring (msP) rat and could be linked to increased stress reactivity, excessive alcohol self-administration, and increased propensity for relapse-like behavior in this line (Hansson *et al.*, 2006). An intriguing observation is that both post-dependent and msP rats given *ad lib* access to alcohol will reverse increased amygdala *Crhrl* transcripts down to control levels, suggesting that alcohol intake may act as a functional CRHR1 antagonists in these animals. Thus, msP animals seem to share similar features with post-dependent animals and may therefore represent a corresponding behavioral *phenocopy* of post-dependent rats (Fig. 4). It has to be pointed out that msP rats only share distinct neurochemical features with post-dependent animals. In fact, *Crhrl* expression in msP rat is upregulated across many brain regions (Hansson *et al.*, 2006), while in post-dependent animals increased expression of this gene is restricted to the amygdala (Sommer *et al.*, 2008). In other words, investigations in msP cannot replace studies in the more laborious neuroadaptation model but seem to provide an excellent opportunity for functional validation of specific targets. Such convergent use of genetic and neuroadaptation models appears as a useful path to increased consilience with human data. Indeed, genetic variation at the *Crhrl* locus as a susceptibility factor for excessive alcohol drinking might have parallels in higher species, including rhesus macaques (Barr *et al.*, 2009) and humans (Chen *et al.*, 2010; Nelson *et al.*, 2010; Treutlein *et al.*, 2006).

Fig. 4. Convergent evidence from genetic and neuroadaptation rat models for a role of amygdala *Crhrl* expression in excessive alcohol consumption. Increased *Crhrl* mRNA levels are clearly seen within the basolateral (BLA) and central (CeA) amygdala brain regions in both post-dependent and msP rats by means of in situ hybridization. Upon *ad lib* access to alcohol a downregulation of *Crhrl* transcripts toward control levels occur within these amygdala regions in animals of both models, suggesting that alcohol is voluntarily consumed to counteract the overactivity of the CRH system in this region. Right: Post-dependent rats (Wistar) were generated by intermittent alcohol vapor exposure for 7 weeks (rolling line) and sacrificed (black arrows) either after 3 weeks of abstinence (PD) or after additional 3 weeks of *ad lib* access to alcohol (PD+A) in a continuous access, two-bottle free-choice paradigm (6% alcohol vs. water). During this period PD+A rats consumed 3.5–4 g/kg/day alcohol. Alcohol-naïve Wistar rats were used as controls. Left: Genetically preferring msP rats were sacrificed either alcohol naïve (msP) or after *ad lib* access to alcohol (msP+A, 10% alcohol vs. water) for 15 day during which they consumed 7.1–7.7 g/kg/day alcohol. Nonselected, alcohol-naïve Wistar rats were used as controls. Bar graphs showing quantitative *Crhrl* expression levels (mean \pm SEM, $n = 7$ to 8) from in situ hybridization on amygdala sections. Statistical analysis was performed by region-wise one-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Wistar controls; # $p < 0.05$ msP+E vs. msP). Data have been partly reported previously (Hansson *et al.*, 2006, 2007b; Sommer *et al.*, 2008).

C. TRANSCRIPTOME STUDIES OF THE POST-DEPENDENT STATE

While the post-dependent state model appears highly versatile and is increasingly utilized in pharmacological experiments, only a few studies have assessed transcriptome-wide responses associated with the neuroadaptations involved in the addiction-related behaviors. One major problem in the design of such studies is to choose the relevant time points. Experimental data obtained during alcohol intoxication, in early withdrawal or after prolonged abstinence will likely point to very different groups of genes. Yet, among the sparse reports using the intermittent alcohol vapor intoxication there is surprising overlap.

To capture long-term neuroadaptations we chose a three week abstinence period. At this point effects of acute withdrawal have passed and most of the expression changes should have stabilized. Also, because the behavioral symptoms seen in the post-dependent state are subtle, no grave alterations in gene expression are to be expected either potentially being below the sensitivity threshold of the microarray platform. Nevertheless, our initial study found dysregulation of about 30 genes in the medial prefrontal cortex or amygdala persisting 3 weeks into abstinence (Rimondini *et al.*, 2002). Although the evidence from this microarray experiment was modest, several findings were pointing to pharmacological targets and have been functionally validated in subsequent studies, for example, the endocannabinoid system, MAP kinases pathways, and glutamate transporter (Hansson *et al.*, 2007a, 2008; Karlsson, 2009). An extended analysis points to the prefrontal projection neurons as the major place of long-term plasticity with profoundly decreased expression of several cAMP/PKA/CREB regulated transcription factors (e.g., *Egr1*, *Fos*, *Junb*, *Nr4a1*) and functional important effector genes including *Bdnf*, *Homer1*, and *Scn4b* (WHS, unpublished data). Moreover, the transcription factors show a blunted response to alcohol challenge (Hansson *et al.*, 2008). Interestingly, some of these genes seem to be downregulated in the medial prefrontal cortex of intermittently alcohol vapor exposed animals already during intoxication (*Egr1*, *Nr4a1*, *Homer1*) and in early withdrawal (*Egr1*, *Bdnf*) (Melendez *et al.*, 2006; Repunte-Canonigo *et al.*, 2007). Together, these data are highly consistent and point to the possibility that the cAMP/PKA/CREB pathway in the medial prefrontal cortex has lost, at least in part, the ability to generate an adaptive response in the dependent state. This would have important functional implications likely to be contributing to the addiction-related behaviors.

The same network of transcription factors, that is, *Fos*, *Jun*, *Junb* and *Nr4a1* and *Nr4a3*, was found to be upregulated in the nucleus accumbens of P rats that had either continuous or intermittent access to alcohol for 2 months and were sacrificed 15 hours after the last alcohol access period (Bell *et al.*, 2009). These rats may have reached blood alcohol levels up to 0.8 g/L, which is likely not sufficient to induce neuroadaptations of the type found in the studies reviewed above using

intermittent alcohol vapor exposure. The results from these different models are obviously difficult to compare, but the finding of correlated expression in this regulatory network could point to opposing activity states in the two regions after long-term exposure to alcohol. Increased activity in the nucleus accumbens is produced by the pharmacological effects of alcohol, but higher doses may also involve neurotoxic actions, for which the mPFC seems to be particularly vulnerable to, and hence result in decreased activity of this region. On the behavioral level, activation of accumbal and inhibition of prefrontal activity may both lead to increased alcohol intake, but while the former seems to reflect adaptation within the homeostatic borders of normal or controlled function, the latter appears to be dysregulation leading to loss of control.

Although post-dependent animals express addiction-related phenotypes such as excessive voluntary alcohol consumption and increased anxiety responses, the model is not informative about predisposing factors for these behavioral responses. Instead post-dependent animals can inform how repeated alcohol intoxication and withdrawal impact on systems that mediate these phenotypic responses. Thus, alterations in gene expression are not necessarily found within the genes mediating the initial response to alcohol, but reflect a pathological process that has recruited additional pathways which in turn set the stage for aberrant response on the systems level which become evident as addictive behaviors. Thus, in post-dependent animals we find a mix of pharmacological effects of alcohol including tolerance and withdrawal reactions as well as neurodegenerative and compensatory responses to alcohol. These vary in a brain region specific manner and together comprise the pathological phenotype. In contrast, long-term access models such as repeated ADE exposure are likely to be driven by the reinforcing effects of alcohol and resulting in a rewiring of brain circuits and formation of habitual responses. For example, sensitized stress responses are found in post-dependent, but not in repeated ADE exposed animals. Thus, both types of neuroadaptation models result in different behavioral syndromes associated with distinct transcriptomic profiles, but both reflect important aspects of human alcoholism.

D. ATTEMPTS TO COMBINE GENETIC AND NEUROADAPTATION MODELS FOR STUDYING GENE-ENVIRONMENT INTERACTIONS

After identifying genetic factors underlying alcohol responses and neuroadaptive mechanisms involved in the development of addictive behaviors, it appears to be a logical continuation of these lines of research to ask, how these two main categories of causal factors interact and to what extent these models are suitable

to study such interactions? There seem to be severe limitations to such an approach resulting from the complexity of each type of model. For example, we mentioned that some of the genetic lines have a severely narrowed behavioral reaction norm and thus only a limited ability to react to challenge, as exemplified by the observation that AA and HAD rats do not express an ADE (Vengeliene *et al.*, 2003). In addition, we found that both AA and ANA rats show resistance to the intermittent alcohol vapor exposure procedure; specifically they do not alter their pattern of alcohol consumption (Hoffman *et al.*, 2003; Sommer *et al.*, 2005). In our experience, this is unusual because using the intermittent vapor exposure paradigm we robustly induce post-dependent excessive alcohol drinking in a variety of rat lines, including Wistar, Sprague-Dawley, Fisher, and a genetically modified rat line (Sommer *et al.*, 2007). If the findings in AA and ANA rats do indeed represent innate resistance to developing a post-dependent state, it would be highly interesting to identify involved genetic factors.

Other genetic lines might be more useful to study the genetic determinants underlying the development or maintenance of a post-dependent state. Withdrawal seizure prone and withdrawal seizure resistant (WSP/WSR) mouse lines were selectively bred for high or low alcohol withdrawal severity following chronic ethanol vapor exposure (Kosobud and Crabbe, 1986). A recent study examined 15 common inbred mouse lines after 3 days of intermittent alcohol vapor intoxication for withdrawal severity (Metten *et al.*, 2010). In line with previous results, the severity of handling induced convulsions during alcohol withdrawal is strongly determined by genotype, but the genetic factors seem to act independent of the intoxication paradigm, that is, the same genes are likely to be involved in withdrawal severity regardless whether intoxication was chronic, intermittent, or acute. Given the logistic efforts necessary for maintaining the intermittent exposure paradigm over several weeks, there is justified hesitation to embark on selective breeding for susceptibility or resilience to developing a post-dependent state.

In summary, neuroadaptations that occur after a prolonged history of alcohol access or exposure seem to persist long into abstinence, some of those probably for the lifetime of the individual, and reflect important aspects of human alcoholism. Several interesting candidate mechanisms have been put forward by these models, most notably a widespread dysregulation of glutamatergic genes in the repeated ADE model and the sensitization of brain stress and fear systems in the post-dependent state.

VI. Conclusions

The last decade has seen a widespread use of high-throughput methods for transcriptome analysis to study brain function, and a wealth of genome-wide expression data from various models has been accumulated by alcohol

researchers. We have considered commonly used rodent models in alcoholism research from the perspective of their potential place on the disease trajectory. Such a view emphasizes mechanistic differences in the observed responses to alcohol as they relate to alcoholism. Taking into account the dynamics of the addiction process, which are mainly driven by time-dependent brain exposure to alcohol, led us to critically reconsider the potential knowledge gain that could be obtained from the study of initial responses or relatively brief exposure to alcohol in terms of the disorder and its treatment. In fact, the large number of genetic and genomic investigations based on experimental paradigms mostly reflective of acute alcohol effects or its controlled use has resulted in surprisingly few candidate genes with potential implications for addiction treatment. Instead, the research focus should be shifted toward alcohol-induced long-term neuroadaptations, where initial transcriptome studies have already put forward interesting and verifiable candidate mechanisms. The emerging transcriptome studies from these models point to alterations in distinct vulnerable brain regions and circuits, such as stress response and fear circuits, rather than widespread dysregulation. Thus, future genomics analysis may focus on affected networks and subpopulations on the basis of detailed neuroanatomical, pharmacological, and in vivo neuroimaging mappings. Most importantly, evidence needs to be collected from multiple experimental lines including different types of animal models as well as data from human observations in order to reach increased consilience about what are the genetic and transcriptional mechanism underlying the clinical condition of alcohol addiction and how to apply this knowledge to better satisfy the largely unmet medical needs.

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