Identification of Quantitative Trait Loci in Alcoholism

Thesis

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Ich dachte darüber nach, in wie hohem Maße die Naturwissenschaft es sich zum Plan gemacht hat, absolut alles erklären zu wollen. Worin sich natürlich die Gefahr versteckt, allem gegenüber, das sich nicht erklären lässt, vollständig blind zu werden.

Maya
oder Das Wunder des Lebens
J. Gaarder

Abbreviations

5-HT	5-hydroxytryptamine-3, serotonin
A	Adenine / Adenosine
ADH	Alcohol dehydrogenase
ALDH2	Acetaldehyde dehydrogenase 2
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
bp	Base pair
C	Cytosine / Cytidine
cAMP	Cyclic adenosine monophosphate
Chr	Chromosome
cM	Centimorgan
cm	Centimeter
CPP	Conditioned place preference
dB	Decibel
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleoside triphosphate
EDTA	Ethylenediamine tetraacetic acid
EtOH	Ethanol
F	Degree of freedom
F1	First filial generation
F2	Second filial generation
FAEE	Fatty acid ethyl ester
FAM	6-carboxy-fluorescine
g	Gram
G	Guanine / Guanosin
GABA	*γ*-aminobutyric acid
h	Hour
HEK cells	Human embryonic kidney cells
HEX	Hexachloro-6-carboxy-fluorescine
HPLC	High performance liquid chromatography
i.p.	Intraperitoneal
IMP	Multiple imputation
kb	Kilobase pair
kDa	Kilodalton
kg	Kilogram
LOD	Logarithm of the odds

LOG	Common logarithm
m	Meter
M.musculus	Mus musculus (house mouse)
mA	Milliampere
MEOS	Microsomal ethanol oxidation system
$MgCl_2$	Magnesium chloride
min	Minutes
mRNA	Messenger RNA
msec	Milliseconds
n	Sample size
n.s.	Not significant
nAChR	Nicotinic acetylcholine receptor
NAD (P)	Nicotinamide adenine dinucleotide (phosphate)
ng	Nanogram
NMDA	*N*-methyl-D-aspartic acid
OD	Optical density
P450	Cytochrome P450IIE1-CYP2E1
PA	Polyacrylamide
PCR	Polymerase chain reaction
pd	Postnatal day
Pde6b	Phosphodiesterase 6B
PK	Protein kinase
QTL	Quantitative trait locus / loci
RNA	Ribonucleic acid
ROX	6-carboxy-X-rhodamine
sec	Seconds
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
T	Thymine / Thymidine
TAE	Tris-acetate-EDTA
TBE	Tris-Borate-EDTA
TET	Tetrachloro-6-carboxy-fluorescine
Tris	Tris (hydroxymethyl) aminomethane
U	(Enzyme) *Unit*
VTA	Ventral tegmental area
µl	Microlitre

INDEX

1 INTRODUCTION

“ Trying to describe the process of becoming an alcoholic is like trying to describe air. It’s too big and mysterious and pervasive to describe. […] It’s a slow, gradual, insidious, elusive becoming. *“*
(KNAPP 1996)

In Germany, more than 9 million people consume alcohol in a manner posing a risk to health, 1.3 million are considered to be alcohol dependent. Just about 10 per cent undergo therapy – often much too late after 10 to 15 years of dependence. 20 per cent of the 12- to 25-year-old adolescents consume alcohol in regular terms. The economic costs of alcohol-related diseases are estimated to amount to 20 billion Euro per year. Each year, 42.000 people die either directly (via alcohol abuse) or indirectly (e.g. via accidents caused by alcohol) of the consequences of alcohol consumption (German Federal Ministry of Health). Contemplating all these facts, alcohol misuse is among the biggest social problems worldwide and the aetiology of this complex disease is thus of great interest in scientific and medical research.

There are considerable differences in the definition of alcoholism and related terminology among the medical community, treatment programs, and the general public. The *Journal of the American Medical Association* (JAMA) defines alcoholism as a primary, chronic disease characterised by impaired control over drinking, preoccupation with the drug alcohol, use of alcohol despite adverse consequences, and distortions in thinking (MORSE and FLAVIN 1992). It is debated whether dependence as defined by JAMA is physical (characterised by withdrawal), psychological (based on reinforcement), or both. The *Diagnostic and Statistical Manual of Mental Disorder*, 4th edition (DSM-IV) defines alcohol abuse as repeated use despite recurrent adverse consequences. It further describes alcoholism as a chronic relapsing disorder, which is characterised by a preoccupation with obtaining alcohol, loss of control over its consumption, development of tolerance, dependence, and impairment in social and occupational functioning. According to the DSM-IV definition, alcoholism is equivalent to *Substance Dependence on Alcohol*.

Alcohol represents an organic compound in which a hydroxyl group (-OH) is bound to a carbon atom of an alkyl or substituted alkyl group. Among the different alcoholic compounds, the one suitable for drinking is ethanol (EtOH) (Figure 1). Ethanol is a product of the alcoholic fermentation process. Via this common biological reaction, glucose and water are converted

in the presence of ferment (usually yeast) to produce ethanol and carbon dioxide. Since any source of glucose is sufficient to produce alcohol via the fermentation process, it forms the basis of numerous alcoholic beverages worldwide (KOOB and LE MOAL 2006).

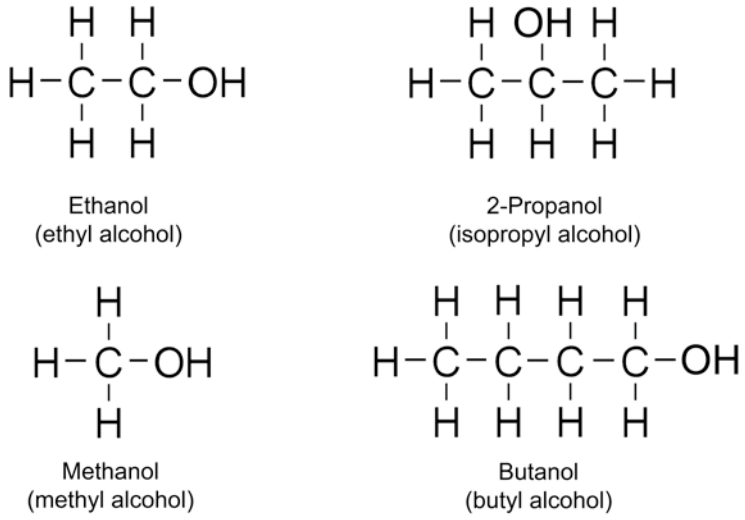


Figure 1. Chemical structure of ethanol and other alcohols. Ethanol is also known as EtOH, using the common organic chemistry notation of representing the ethyl group (C_2H_5) with Et.

Many neurobiological and environmental factors are known to influence ethanol-drinking behaviour, whereas the nature and extent to which these variables govern the propensity to drink alcohol varies among individuals (BECKER and LOPEZ 2004). In addition, inherited factors also contribute substantially to an individual's susceptibility to alcohol abuse and alcoholism. Twin registry and adoption studies have shown that the heritability of alcoholism may be as high as 50-60 per cent (HIGUCHI *et al.* 2006). Thus, the genetic constitution and environmental experience interact to alter both direct alcohol actions and molecular mechanisms that indirectly affect ethanol-related behaviours (LOVINGER and CRABBE 2005).

1.1 Metabolism of alcohol

Alcohol is detoxified and removed from the blood through oxidation, preventing the alcohol from accumulating and destroying cells and organs. Most of the consumed alcohol is metabolized in the liver, but the small quantity that remains unmetabolized permits the measurement of alcohol concentration in breath and urine. Via the absorption in the stomach and the intestines alcohol is passed into the blood. Until all the consumed alcohol has been metabolized, it is distributed throughout the body, affecting the brain and other tissues (BOSRON *et al.* 1993). In the liver, the enzyme alcohol dehydrogenase (ADH) mediates the conversion of alcohol to acetaldehyde, which is then rapidly converted to acetate, water and

carbon dioxide by the aldehyde dehydrogenase (Figure 2a). It was shown that genetic variation in this pathway contributes to individual differences in alcohol elimination in human populations. In Asian populations for example, inactivation of the acetaldehyde dehydrogenase 2 (ALDH2) is believed to cause the alcohol-induced flush reaction after drinking alcohol (WALL *et al.* 1993).

In the liver, alcohol is also metabolized by the microsomal ethanol oxidation system (MEOS) catalyzed by cytochrome P450IIE1-CYP2E1 (P450; Figure 2c). This pathway was shown to be responsible for metabolic tolerance to alcohol, as its activity is increased after chronic alcohol drinking (LIEBER 2005). Moreover, metabolism by P450 results in a significant release of free radicals, which in turn diminish reduced glutathione and other defence systems against oxidative stress that play a major pathogenic role in alcoholic liver disease. In a third reaction pathway, alcohol is metabolized in a non-oxidative way by the fatty acid ethyl ester (FAEE) synthase, which leads to the formation of fatty acid esters. The highest concentrations of FAEEs are found in organs that are vulnerable to the toxic effects of alcohol, including the liver (BEST and LAPOSATA 2003) (Figure 2d). Regardless to the amount that has been consumed, the liver only metabolizes a certain amount of alcohol per hour. The rate of alcohol metabolism depends, in part, on the amount of metabolizing enzymes in the liver, which varies among individuals and again has genetic determinants (BOSRON *et al.* 1993).

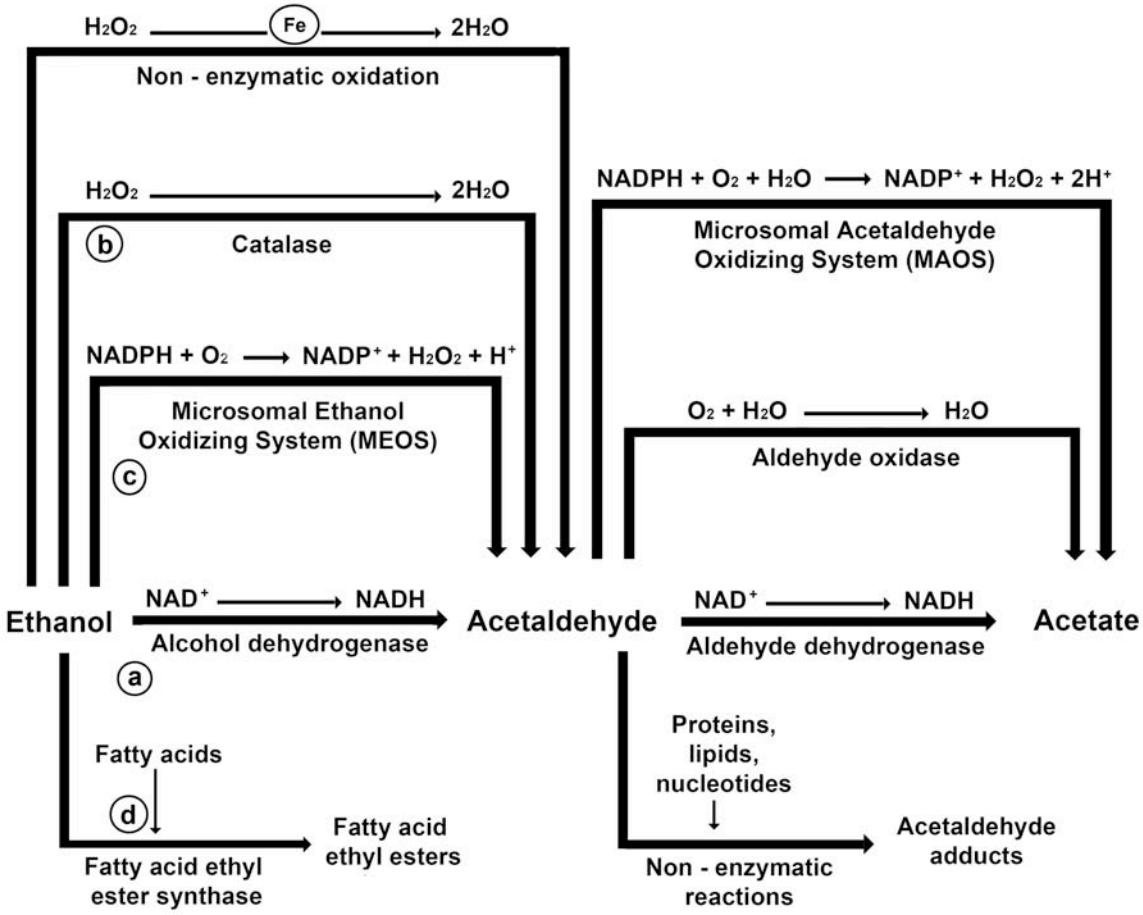


Figure 2. Principle metabolic pathways related to ethanol and acetaldehyde metabolism. Three metabolic systems are responsible for the ethanol oxidation in the liver. (a) The enzyme alcohol dehydrogenase promotes the oxidation of ethanol into acetaldehyde, at which a nicotinamide adenine dinucleotide (NAD^+) is reduced to NADH. (b) Hepatic cell peroxisome oxidation couples ethanol oxidation into acetaldehyde with the simultaneous degradation of hydrogen peroxide, which is catalyzed by the catalase enzyme. (c) The microsomal ethanol oxidizing system (MEOS) couples ethanol and nicotinamide adenine dinucleotide phosphate (NADPH) oxidation, which requires cytochrome P450 participation. MEOS is distinguished from alcohol dehydrogenase by its subcellular localization, its pH optimum, and its cofactor requirements. (d) Fatty acid ethyl ester synthase catalyzes the non-oxidative alcohol metabolism pathway. (Modified according to KOOB and LE MOAL 2006.)

1.2 Neurobiological effects of alcohol

Despite the generally held view that alcohol is an unspecific pharmacological agent, recent molecular pharmacology studies demonstrated that alcohol has several primary targets. These are the *N*-methyl-D-aspartic acid (NMDA), γ-aminobutyric acid (GABA), glycine, 5-hydroxytryptamine-3 (serotonin, 5-HT) and nicotinic acetylcholine receptors (nAChR) as well as L-type Ca^{2+} channels and G-protein-activated inwardly rectifying K^+ channels (VENGELIENE *et al.* 2008; WANG *et al.* 1994). In addition, alcohol intake also evokes several indirect effects on a variety of neurotransmitter/neuropeptide systems, which together lead to the common acute effects of alcohol. Once alcohol drinking becomes chronic, several changes in the

brain take place. Mainly, the balance between inhibitory and excitatory neurotransmission through different transmitter systems is altered in this phase of the disease. The specific neurotransmitter systems most studied in alcoholism research (at the cellular level) are probably GABA and glutamate (VENGELIENE *et al.* 2008) (Figure 3).

Alcohol exposure is known to enhance $GABA_A$ receptor function. Chimeric receptor constructs that combined sections of the GABA ρ1 (which are – although evolutionary related to ionotropic $GABA_A$ receptors - inhibited by ethanol) and glycine receptors (whose currents are enhanced by ethanol) were used to identify a small region of amino acids required for the enhancement of $GABA_A$ and glycine receptor function by alcohol (MIHIC and HARRIS 1996). Two amino acids in the $GABA_A$ receptor transmembrane segments (TM) 2 (Ser270) and 3 (Ala291) were shown to be critical for allosteric modulation of $GABA_A$ and glycine receptors by alcohols. It was suggested that Ser270 and Ala291 are part of a hydrophobic binding site in pockets formed between at least two TM domains. This hypothesis was supported by the fact that replacing either of these two amino acids resulted in loss of ethanol potentiation (LOBO and HARRIS 2008; MIHIC *et al.* 1997). Although the alcohol - $GABA_A$ interaction was confirmed in several electrophysiological studies (AGUAYO *et al.* 2002; SOLDO *et al.* 1998), there are also *in vivo* and *in vitro* studies that could not verify this interaction (DAVIES 2003; MARSZALEC *et al.* 1998). These disparate findings demonstrate that alcohol does not increase $GABA_A$-mediated inhibition in all brain regions, in all cell types in the same region, at all $GABA_A$ sites on the same neuron or across species in the same brain region, which may be due to differences in subunit composition of the receptor (KOOB and LE MOAL 2006).

Acute ethanol enhances the inhibitory tone of neurons containing $GABA_A$ receptors, but prolonged ethanol exposure leads to its reduction (GROBIN *et al.* 1998) showing that chronic but not acute alcohol use generally downregulates $GABA_A$ receptor function. The effects induced by chronic alcohol exposure are supposed to be mediated via a decrease in $GABA_A$ receptor density and brain region specific up- or downregulation of α and β subunit gene expression (GOLOVKO *et al.* 2002; SANCHIS-SEGURA *et al.* 2007; VENGELIENE *et al.* 2008). Behavioural studies using knockout mice that are deficient for different $GABA_A$ receptor subunits demonstrated that α1, α2, α5 and δ subunit deletion leads to lower alcohol consumption (CRABBE *et al.* 2006b; JUNE *et al.* 2007; VENGELIENE *et al.* 2008). Further, protein kinase C (PKC) activity appears to be a critical determinant of alcohol actions on $GABA_A$ receptors. Thus, mice lacking the ε isoform of PKC showed increased sensitivity of $GABA_A$ receptors to allosteric modulation by alcohol and are known to consume less alcohol (HODGE *et al.* 1999). In contrast, $GABA_A$ receptors are less sensitive to alcohol effects in mice lacking PKCγ (HARRIS *et al.* 1995) and accordingly PKCγ knockouts show increased voluntary alcohol consumption (HODGE *et al.* 1999). Hence, PKCε and PKCγ may mediate phosphorylation of $GABA_A$ receptors and thus regulate alcohol drinking in opposite ways.

Due to variations in PKC isoform expression in different brain regions, this regulation may depend on the regions involved (KOOB and LE MOAL 2006).

There is also evidence that metabotropic $GABA_B$ receptors might play a crucial role in controlling the levels of chronic alcohol intake. Studies with alcohol-preferring rat lines revealed that the $GABA_B$ agonist baclofen suppresses voluntary alcohol consumption. However, repeated use of baclofen might lead to the development of tolerance (COLOMBO *et al.* 2000). Another study referring to this showed that co-administration of a positive allosteric modulator of the $GABA_B$ receptor such as CGP7930 enhances the potency of baclofen and reduces the development of tolerance (ADAMS and LAWRENCE 2007).

Since the late 1980's and early 1990's it is known that ethanol also acts by inhibiting neuronal NMDA receptor function (HOFFMAN *et al.* 1989; LOVINGER *et al.* 1989) and the NMDA receptor is now considered as one of the primary molecular targets for the actions of ethanol in the brain. As NMDA receptors are heterotetrameric protein complexes that form ligand-gated ion channels, they are composed of at least one NR1 subunit (for which there are at least 8 splice variants) and a combination of NR2A-D and NR3A or 3B subunits (STEPHENSON 2006). The receptors containing NR2B subunits are particularly sensitive to inhibition by ethanol, whereas NR2C and NR3 subunit-containing receptors appear to be less sensitive (GASS and OLIVE 2008). As examined in cultured rat cortical neurons, the inhibition of NMDA receptor function is mediated by a non-competitive mechanism (WIRKNER *et al.* 2000). In the rat dorsal striatum, ethanol exposure produced an increase in NR2B subunit phosphorylation, and a corresponding increase in the activity of Fyn kinase, which phosphorylates NR2B (WANG *et al.* 2007). The influence of alcohol on glutamate signalling is robust and not as dependent upon brain region or cell type as the alcohol effects on the $GABA_A$ receptor for example. Alcohol-induced changes in glutamate signalling pathways are observed in most neuron types in brain regions like the cerebral cortex, nucleus accumbens, septum, amygdala, hippocampus, locus coeruleus, VTA and cerebellum (for review see GASS and OLIVE 2008). The ability of ethanol to inhibit NMDA receptor function is dependent on its heteromeric subunit composition. For example, the NR1-2b splice variant that is co-assembled with the NR2C subunit was shown to be most sensitive to ethanol-induced inhibition among 32 NR1/NR2 subunit combinations expressed in HEK293 cells (JIN and WOODWARD 2006). Further, the extracellular Mg^{2+} and glycine concentrations, intracellular Ca^{2+} concentrations, and the phosphorylation by proteins kinases such as Fyn, PKA, and PKC as well as by the phosphorylation regulator DAARP-32 impact on ethanol-induced receptor inhibition (for review see GASS and OLIVE 2008).

In response to constant inhibition of NMDA receptor function, chronic alcohol use and alcohol withdrawal leads to an upregulation of various NMDA receptor subunits including the

NR1, NR2A and NR2B subunits in the cerebral cortex and hippocampus (FOLLESA and TICKU 1995; TREVISAN *et al.* 1994). Induced by chronic ethanol, NR1 expression is also upregulated in the VTA and amygdala (ORTIZ *et al.* 1995), regions that are critical for the reinforcing effects of ethanol. Additionally, NMDA receptor conductance and cation influx as well as synaptic clustering is increased (BLEVINS *et al.* 1995; FLOYD *et al.* 2003; NAGY *et al.* 2003). As a result of ethanol-induced upregulation of NMDA receptor expression, the central nervous system enters a state of hyperexcitability upon acute withdrawal from ethanol exposure (TSAI and COYLE 1998; TSAI *et al.* 1995). Following prolonged ethanol withdrawal, NMDA receptor expression and functionality are reduced (ROBERTO *et al.* 2006). A similar action of alcohol was discovered for non-NMDA glutamate receptors like the kainate or the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (CARTA *et al.* 2003; MARTIN *et al.* 1995; ROBERTO *et al.* 2004), although these receptors appear to be less sensitive to inhibition by ethanol than NMDA receptors, requiring concentrations of 50 mM or greater (GASS and OLIVE 2008).

Other ionotropic receptors were also characterized as primary targets for the action of alcohol. Thus, glycine receptor activity is enhanced by alcohol and it potentiates 5-HT3 (LOVINGER 1999) and neuronal nAChR function (NARAHASHI *et al.* 1999). Other studies revealed that alcohol (and acetaldehyde) in intoxicating doses activates neurons in the ventral tegmental area (VTA) *in vivo* and *in vitro*, partly via a direct activation of dopaminergic neurons in the VTA. During ethanol withdrawal, a decrease in dopaminergic activity in the VTA was linked to the dysphoria of acute and protracted withdrawal (DIANA *et al.* 1993). This reduced dopaminergic transmission is prolonged, thus outlasting the physical signs of ethanol withdrawal (BAILEY *et al.* 2000; DIANA *et al.* 1996).

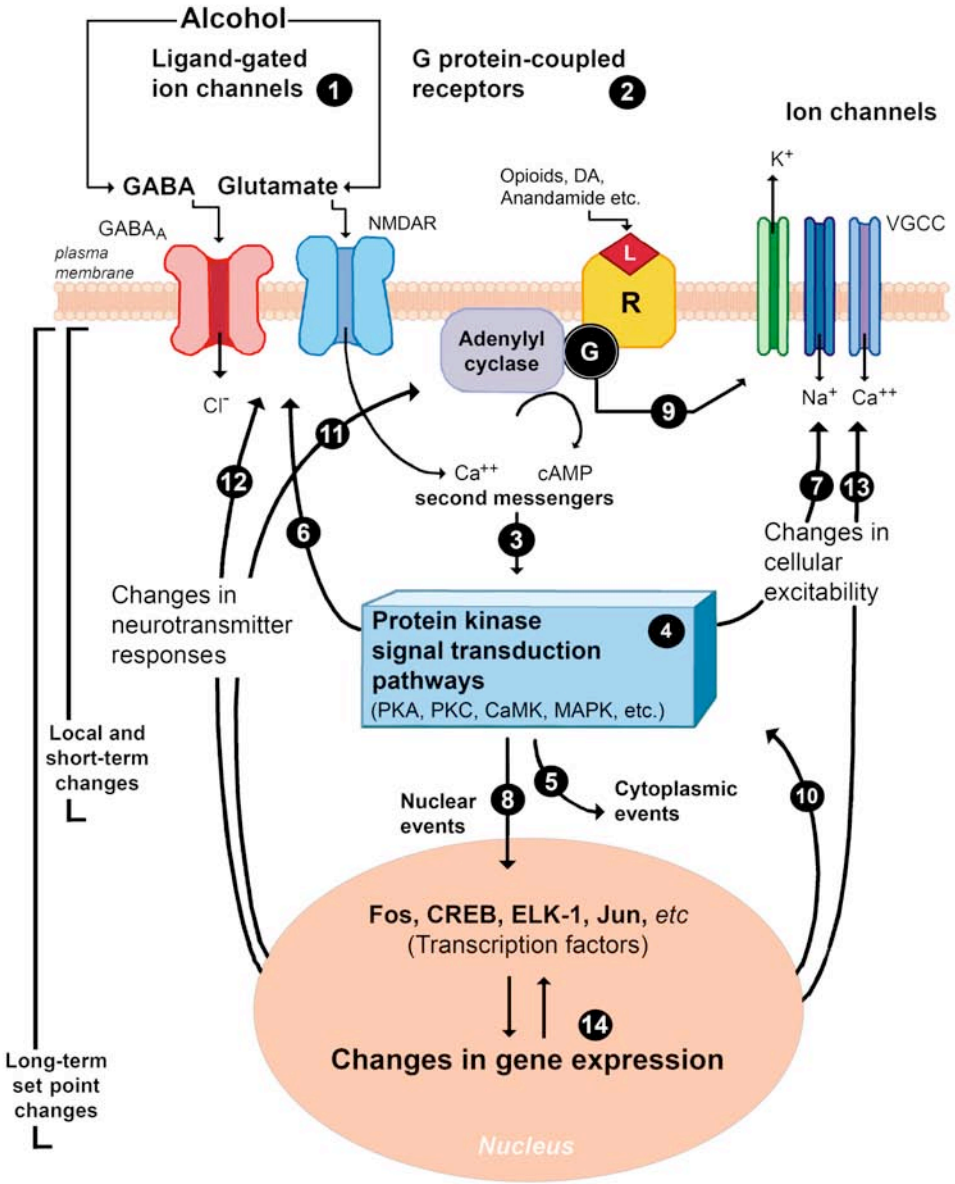


Figure 3. Molecular mechanisms of neuroadaptations in alcoholism. Alcohol, by acting on neurotransmitter systems, affects the phenotypic and functional properties of neurons through the general mechanisms outlined in the diagram. Shown are examples of ligand-gated ion channels (1) such as the $GABA_A$ and the NMDA receptor (NMDAR), G protein-coupled receptors (R) such as opioid, dopamine (DA), or the cannabinoid CB1 receptors (2). These receptors modulate the levels of second messengers like cAMP and Ca^{++} (3), which in turn regulate the activity of protein kinase transducers (4). Such protein kinases affect the functions of proteins located in the cytoplasm, the plasma membrane, and the nucleus (5–8). Among membrane proteins, ligand-gated and voltage-gated ion channels are affected (6 and 7). Alcohol has been proposed to affect the $GABA_A$ response via PKC phosphorylation. G_i and G_o proteins also can regulate potassium and calcium channels directly through their $\beta\gamma$ subunits (9). Protein kinase transduction pathways also affect the activities of transcription factors (8). While membrane and cytoplasmic changes may only be short-lasting, changes of transcription factor activity may result in long-term functional changes. These can include changes in gene expression of proteins involved in signal transduction (10) and/or neurotransmission (11–13), resulting in altered neuronal responses. Chronic exposure to alcohol has been reported to increase levels of PKA (10) and adenylyl cyclase (11) in the nucleus accumbens and to decrease levels of $G_{i\alpha}$ (11). Moreover, chronic ethanol induces differential changes in subunit composition in the $GABA_A$ and in the glutamate ionotropic receptors (12) and increases expression of voltage-gated

calcium channels (VGCC) (13). Chronic exposure to alcohol also alters the expression of transcription factors (14). CREB expression, for instance, is increased in the nucleus accumbens and decreased in the amygdala by chronic alcohol treatment. The receptor systems depicted in the figure may not coexist in the same cells. (Modified according to KOOB *et al.* 1998.)

Ethanol intake, preference and the severity of ethanol withdrawal in animal models have been shown to be reduced after selective pharmacological manipulations of some of the respective neurotransmitter systems (NEVO and HAMON 1995).

1.3 Animal models in alcoholism research

It is not possible to model alcoholism with all its complex facets in animals. Nonetheless, several animal models exist that mimic distinct aspects of the disorder, most of them using rodents as model organisms. Animal models provide the possibility of controlling the environmental conditions to a great extent. Furthermore, one can separate the genetic and environmental influences, so that all the variance in the phenotype can be referred to the respective aspect of interest (GRISEL 2000). To assure that an animal model mimics the human conditions as closely as possible, validity criteria have been determined that should be considered before establishing a disease model (Box 1).

Box 1. Criteria of validity

Construct validity: Assesses the accuracy with which a model parallels the human condition. Construct validity is difficult to assess and must continuously integrate additional data from both animal and human tests.

Aetiological validity: The causative phenomena of the model are similar or identical to those of the disorder. It is conceptually similar to construct validity, but is perhaps more difficult to assess because causal mechanisms for human psychopathologies are rarely known.

Face validity: The phenotype of the animal model resembles the human disorder. Superficial similarity between animal and human measures might indicate convergence of an underlying aetiology. This assessment is probably a good starting point for model development but requires additional validation.

Predictive validity: The model enables predictions about the human condition; determination necessitates development of appropriate measures in humans to assess the reliability of the model.

(Modified according to BENNETT *et al.* 2006.)

In alcoholism research, rats have become prominent organisms to model alcohol addiction-related behaviours like alcohol dependence, tolerance, withdrawal, preference or reinstatement (relapse) (SANCHIS-SEGURA and SPANAGEL 2006; SPANAGEL 2000). Rats are also often utilised for pharmacological manipulations to elucidate neurobiological pathways involved in the development of the disease and to examine the properties of potential agents for treatment.

With regard to the molecular and genetic basis of alcoholism, mice are the most commonly used vertebrate model organism, because they are readily amenable to a wide spectrum of genetic manipulations (ABUIN *et al.* 2007; BRAULT *et al.* 2006; GAVERIAUX-RUFF and KIEFFER 2007; SCHMIDT-SUPPRIAN and RAJEWSKY 2007). Although mice are not suitable for modelling all aspects of human behaviours, they have considerable genetic homology with humans (> 99% of mouse genes have a clear human homolog) (BENNETT *et al.* 2006). In hypothesis-free approaches (like quantitative trait loci analyses, see 1.5) wildtype mice are investigated for phenotypes with strong evidence of genetic control and subsequently examined for their genetic constitution. Mouse models have been developed to assess almost all aspects of alcohol addiction including acute effects (e.g. thermoregulation, motility), tolerance, somatic dependence, alcohol preference and reward as well as relapse to alcohol-seeking behaviour (Box 2).

Box 2. Behavioural paradigms in alcoholism research. The box presents a selection of behavioural paradigms commonly used to assess alcoholism-related behaviours. For a more extensive description see section 3 Methods and the following references (CRABBE and BELKNAP 1980; CRABBE *et al.* 1994a; FONT *et al.* 2006; KOOB 2000; RACZ *et al.* 2008; SELF and NESTLER 1998; SHEPHERD *et al.* 1994).

Acute and chronic ethanol effects: Thermoregulation after acute ethanol exposure (e.g. injection) provides evidence about acute ethanol effects. After chronic ethanol exposure, the direction of difference in thermoregulation indicates tolerance or sensitization effects.

Forced ethanol-drinking paradigm: Produces a state of somatic ethanol dependence by forcing the animals to consume an ethanol solution, supplied as their only drinking source over several weeks. Alcohol consumption and animal responses can be monitored during the experiment.

Two-bottle choice paradigm: Assesses ethanol preference in mice, since they have free choice between an ethanol solution and water. Alcohol consumption relative to total fluid intake is calculated as a preference ratio.

Handling-induced convulsions: Evaluates somatic ethanol withdrawal signs. Ethanol withdrawal-induced convulsions or seizures are easy to measure via gently turning and lifting the animals and are scored by severity.

Zero maze: In this maze experiment the behaviour of an animal in an anxiogenic environment can be used as a measure of anxiety-like behaviour in drug or non-drug states.

Open-field: Assesses the spontaneous locomotor activity of the animals in drug or non-drug states. The activity exhibited in a Plexiglas box that is larger than the home cage is automatically recorded by using photocell beams or a video-tracking system.

Stress responses: Exposure to a stressor (e.g. a mild foot-shock) can provoke relapse to alcohol-seeking behaviour in abstinent animals.

Conditioned place preference (CPP): Reveals motivational effects of drugs of abuse in experimental animals. The CPP paradigm uses a classical conditioning procedure to pair different environments with distinct drug or non-drug states. The time spent in drug- or non-drug associated environments provides information about positive and negative reinforcing effects of a drug.

1.4 Genetics of alcoholism

Several studies already stressed the impact of the (individual) genetic constitution on the development of alcohol addiction. Especially the results of human studies supported this hypothesis for many years, because it was shown that there is a fourfold enhanced risk of alcohol dependence in relatives of alcoholics and that identical twins of alcohol dependent subjects carry a higher risk than fraternal twins or full siblings. Additionally, adopted children of alcoholics have the same enhanced risk of contracting the disease as children raised by their alcohol dependent parent (MAYFIELD *et al.* 2008).

Alcoholism has a complex aetiology in which multiple genes and environmental factors are involved. Complex diseases lack the simple inheritance patterns characteristic of single (Mendelian) gene disorders, which makes it difficult to determine the individual's risk of inheriting or passing on the disease (RANNALA 2001). It is a rather daunting task to identify the underlying gene variants in complex diseases. The most successful studies so far have used candidate gene approaches where the biological evidence already suggested a possible involvement in the disease process. Only with the advent of chip-based technologies that permit the simultaneous detection of thousands of polymorphisms in each individual has it been possible to realize systematic whole-genome association studies (EBERLE *et al.* 2007; FAN *et al.* 2006; ROPERS 2007). Nevertheless, these studies are still discouraging and cost-intensive, because they ideally require very large sample sizes with more than 10.000 cases and controls. Currently, very little is known about complex genetics and how common gene variants contribute to disease aetiology. Common gene variants often do not affect the protein-coding capacity of a gene locus and thus are thought to change gene regulatory mechanisms. Indeed, disease-associated alleles are often unknown, because association studies only identify specific polymorphisms or haplotypes that are over- or underrepresented in the case versus the control sample. It than remains to be determined which of the observed polymorphisms is responsible for the increased genetic risk – a task that has seldom been tackled successfully so far. These small and subtle gene effects are difficult to address with genetically manipulated mouse strains. Although it is possible to generate "humanized" mice that carry gene regulatory alterations in disease-associated alleles, it remains to be shown how informative such mouse models will be (SHULTZ *et al.* 2007).

Previous research pointed towards a more general influence of genetic variations (polymorphisms) on repetitive alcohol use and associated problems. It is believed that disease-relevant genes influence a range of (genetically influenced) intermediate characteristics or endophenotypes, which subsequently affect the risk of developing alcohol dependence. One approach to study a complex disease is to dissect the heterogeneous disorder by using such intermediate (endo-) phenotypes and thus to reduce complex

behaviour to more homogeneous components. Endophenotypes have the advantage that the number of influential factors contributing to the disease should is fewer and more easily identifiable. Each of these endophenotypes is likely to reflect the actions of multiple genes and to relate to both the genetic and environmental influences (CRABBE *et al.* 2006b; HINES *et al.* 2005; SCHUCKIT *et al.* 2004).

1.4.1 Linkage versus association studies

Two basic strategies are commonly used to detect genes implicated in multifactorial disorders: linkage analysis and association mapping. Both approaches rely on similar principles and assumptions, namely on the co-inheritance of adjacent DNA variants. Linkage analysis tests for co-segregation of a gene marker and disease phenotype within closely related subjects, to determine if the marker and the disease gene are physically linked. On the other hand, association studies examine the co-occurrence of a marker and disease at the population level, usually by comparing marker frequencies in unrelated cases and controls. Linkage and association studies can use both 'reverse-genetics' (testing random, anonymous DNA markers) and 'forward-genetics' (using candidate gene polymorphisms with presumed functional significance for the disease), though 'forward-genetics' is the mainstay of association mapping (BARON 2001).

For human genome-wide association approaches, the case-control study design is often the method of choice for characterizing the genetic contributions to the disease. It has recently made a tremendous impact on the analysis of complex traits. These analyses benefit from a very large sample size; cases are readily obtained, efficiently genotyped and compared with control populations (LANDER and SCHORK 1994). One particular difficulty for association studies is the choice of control populations. The selection of a control population is often biased, due to the fact that they are retrospectively defined after the collection of the disease group. The problem resulting from wrongly defined controls may be stratification effects leading to the association of allele frequency differences with the disease, although these differences only reflect evolutionary or migratory history, gender differences or other independent processes. An important drawback of association studies is the fact that detailed phenotype information is relegated to a secondary role. In case–control studies, the main indicator for underlying genetic disease susceptibility is the binary categorization of individuals as affected or unaffected. But addictive disorders in particular combine complex processes, in which different aspects of the disease are usually regulated by completely different genes. The future direction may therefore be the combination of linkage and association studies to efficiently investigate the genetics of complex diseases (KRUGLYAK 2008).

1.5 Quantitative trait loci analysis

"*What does a QTL signify? A good QTL is a claim that a particular chromosomal region contains a causal source of variation in the phenotype. The importance of this hypothesis depends on the quality and relevance of the phenotype and the statistical strength of the QTL. As usual, test and be sceptical.*"
(webQTL; http://www.genenetwork.org)

Drug addiction-related behavioural phenotypes are influenced by multiple gene loci, each of which typically contributes a small effect size. Thus, the behaviours vary by degrees and constitute a quantitative trait. Genetic loci contributing to these quantitative traits (quantitative trait loci, QTL) are identified by quantitative genetic approaches (QTL analysis). In general, linking genetic variation with trait variation identifies QTL and a significant linkage of phenotype and genotype suggest that the DNA status helps to determine trait expression.

As stated above, mouse QTL studies provide distinct advantages over human studies in the examination of genetic causes of a quantitative trait (e.g. alcoholism), even in the absence of specific hypotheses regarding its aetiology or candidate genes. Since it is possible to control the experimental environment of laboratory mice and thus isolate genetic and environmental influences, one can attribute virtually all the variance in phenotype between different animal strains to genetic factors (GRISEL 2000). A common strategy in mouse QTL studies is based on the breeding of a suitable genetically divers population of mice, followed by phenotype and genotype analyses of the individuals. Typically, two or more inbred strains of mice are crossed for at least two generations. The progenitor mouse strains should have sufficient variation for the traits of interest and they should be genetically diverse enough to enable genetic mapping (BENNETT *et al.* 2006; FLINT 2003; GRISEL 2000). The sample size required for the identification of QTL depends largely on the effect size that a QTL contributes to phenotypes on interest. Inference about QTL can be made if one or more genetic markers are over- or underrepresented in the analysed individuals. Genotyping is often done by means of microsatellite markers, which contains mono, di-, tri-, or tetranucleotide tandem repeats flanked by specific sequences (Figure 4a). Microsatellites alleles at a specific locus can differ in the number of repeats among individuals (lengths polymorphism) - one characteristic that qualifies them as genetic markers. Furthermore, microsatellites are inherited in a Mendelian fashion, are widely dispersed in eukaryotic genomes, are highly variable, and based on polymerase chain reaction (PCR) (CULLIS 2002; SOUTHERN 1975), all of which are necessary conditions for mapping studies. In literature microsatellites are also known as simple sequence repeats (SSR), short tandem repeats

(STR), or variable number tandem repeats (VNTR) and have been characterized for most of the commonly used inbred mouse strains.

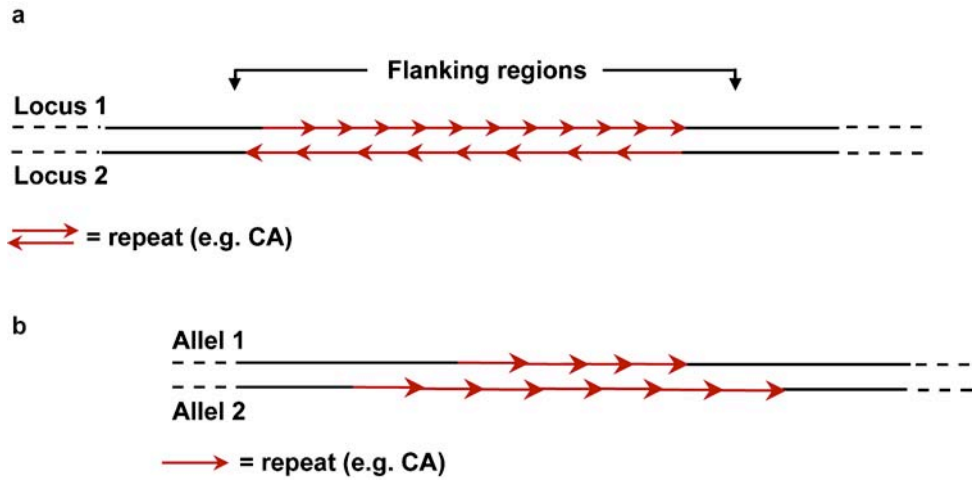


Figure 4. (a) Schematic illustration of a microsatellite locus. The red arrows indicate repeat units; the black lines represent the DNA strands. Exemplary, a dinucleotide tandem repeat is shown. (b) Fragment lengths polymorphism of a microsatellite locus between two alleles. C: Cytidine; A: Adenosine.

Another QTL strategy involves the phenotyping and genetic analysis of recombinant inbred (RI) strains, which have a unique combination of well-circumscribed genome contributions from two or more progenitor inbred strains. Traditionally, RI strains are derived by consecutive brother x sister mating (> 20 generations), starting from a first filial (F1) generation of an intercross of two inbred strains. More advanced RI strains, as those generated in the Collaborative Cross project, are derived from multiple inbred strains (CHURCHILL *et al.* 2004). In alcoholism research, previous studies already detected several QTL contributing to the different aspects of the disease (BENNETT *et al.* 2006; CRABBE 2002; MAYFIELD *et al.* 2008), whereby the specific aetiological factors underlying the susceptibility to alcoholism remain unknown.

1.6 Aim of the study

Previous studies already identified genes that increase or reduce the risk of becoming alcohol-dependent. The neural basis of alcohol dependence is also more and more clarified. But despite all the knowledge about alcoholism and its concomitant phenomena that has been achieved in years of research, the specific aetiological factors underlying susceptibility to alcoholism remain unknown. Especially the determination of genetic factors redounding to the disorder is an important direction alcoholism research should follow to further understand this common disease.

The aim of the present thesis therefore was the identification of gene loci or quantitative traits that contribute to the development and manifestation of alcohol addiction and related behaviours. To reach this aim, a QTL study design in mice was chosen. The second filial (F2) generation of a C57BL/6J and C3H/HeJ mice intercross was first phenotyped in paradigms related to alcohol addiction, whereas the development of a high-throughput phenotyping protocol allowed the serial analysis of several hundred animals. By choosing the phenotypes described in this work and the serial experimental setup, a wide spectrum of alcohol-related traits within the same F2 animal cohort is addressed, which allows to evaluate whether these traits are regulated by common or distinct QTL. Subsequently, the genetic constitution of these mice was analysed by microsatellite marker mapping. A further aim was the establishment of a highly informative microsatellite marker set for the high-throughput mouse genotyping. This set finally consisted of 264 primer pairs showing fragment lengths polymorphisms between the alleles of the parental mouse strains and were distributed throughout the mouse genome. Via subsequent statistical analyses the data obtained from pheno- and genotyping were linked to determine QTL contributing to alcohol dependence and related traits.

2 MATERIAL

2.1 Equipment

ActiMot	ActiMot, TSE Systems
Analytical balance	BP 121 S, Sartorius
CCD camera	KY-F75U, JVC
Centrifuges	Biofuge fresco, Heraeus Instruments
	Biofuge pico, Heraeus Instruments
	Biofuge stratos, Heraeus Instruments
	Sorvall Evolution RC, Kendro
Digital gel documentation	Chemi Doc Syst CCIR, Bio-Rad Laboratories
Drinking bottles	Cascade 5; Hagen, Holm
Electrophoresis chamber	Bio-Rad Laboratories
Genetic Analyzer	ABI 3130xl, Applied Biosystems
Liquid handling platform	Multiprobe II, PerkinElmer
Magnetic stirrer	MR 3001 K, Heidolph, Fisher
Microplate Analyzer	Fusion™ Universal Microplate Analyzer, PerkinElmer
PCR iCycler	iCycler, Bio-Rad Laboratories
pH meter	inoLab, WTW
Rectal thermometer	Portable Thermometer BAT-12, Harvard Apparatus
Spectral photometer	91-ND-1000 UV/Vis, Nanodrop
Startle response system	Startle Response System, TSE Systems
Sterilising oven	Varioklav 25T, H+P Labortechnik
Video-tracking system	Videomot, TSE Systems
Vortexer	Vortex-Genie 2, Scientific Industries
Zero maze	In-house workshop

2.2 Software

Data collection software	Applied Biosystems, Version 3.0
Fusion Instrument Control Software	PerkinElmer
GeneMapper	Applied Biosystems, Version 3.7
Microsoft Office 2008	Microsoft Germany
R software language	R, Version 2.7.2 (2008)

R/qtl	R/qtl, Version 1.09-43 (2008)
Statistika	StatSoft, Inc., Version 6 (2001)
WinPrep	Winprep for Multiprobe II, PerkinElmer

2.3 Chemicals and reagents

If not noted otherwise, all reagents used in this work were purchased from Invitrogen, Carl Roth, Merck or Sigma-Aldrich.

2.4 Enzymes

All enzymes used in this work were purchased from Qiagen (Hilden, Germany) and New England BioLabs (NEB).

2.5 Molecular weight standards

Molecular weight standards were used as lengths standard in polyacrylamide (PA) gel- and capillary electrophoresis.

Table 1. Molecular weight standards.

Molecular weight standards	Fragment size (bp)
DNA 100 bp ladder (Invitrogen)	2072, 1500, 1400, 1300, 1200, 1100, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100
DNA 25 bp ladder (Invitrogen)	500, 475, 450, 425, 400, 375, 350, 325, 300, 275, 250, 225, 200, 175, 150, 125, 100, 75, 50, 25
GS-500 ROX™ Size Standard (ABI)	35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, 500

2.6 Oligonucleotides

All oligonucleotides used in this work were synthesized and delivered by metabion international AG. The forward primer of each pair was labelled at the 5' end with the fluorescent dye FAM, HEX or TET. All reverse primers contained the sequence GTGTCTT (5'–3') at their 5' ends to promote nontemplate-directed nucleotide addition (+ A) to the PCR

products. Information about primer sequences, modifications and expected fragment lengths are provided in Supplement table S1.

2.7 Solutions

6x loading dye:

Glycerol	50 % (v/v)
EDTA	0.002 mM
Bromophenol blue	0.0025 % (w/v)
Xylene-Cyanol	0.0025 % (w/v)

10x TBE buffer:

EDTA	10 mM
Tris base	100 mM
Boric acid	85 mM

1x TAE buffer:

EDTA	0.5 mM
Tris-Acetat	40 mM

1x TE buffer:

Tris, pH 7.4	10 mM
EDTA	1 mM

2.8 Databases and programs

The human und murine genome sequence can be retrieved either via the *ensembl* browser (http://www.ensembl.org/), at UCSC genome bioinformatics (http://genome.ucsc.edu/) or at the *National Center for Biology and Information* (NCBI; http://www.ncbi.nlm.nih.gov/genome/guide/).

MGI (http://www.informatics.jax.org/):

MGI (mouse genome informatics) is the international database resource for the laboratory mouse, providing integrated genetic, genomic, and biological data to facilitate the study of human health and disease. The homepage contains information about genes, phenotypes, gene expression, functional annotations, biochemical pathways, marker polymorphism and information about orthology for mouse, human, rat, chimp, dog, and 12 other mammalian species.

NCBI Homepage (http://www.ncbi.nlm.nih.gov/):

The homepage of the NCBI offers numerous links to helpful databases and programs. The one used most in this work are given below:

Human-Mouse Homology Map (http://www.ncbi.nlm.nih.gov/Homology/):

This homology map opposes homologue chromosomal regions of human and mouse and provides the orthologous genes of these two species.

NCBI (http://www.ncbi.nlm.nih.gov/genome/guide/M_musculus.html):

All available data of the mouse genome sequencing project can be found at this webpage.

OMIM (http://www.ncbi.nlm.nih.gov/Omim/):

The OMIM (Online Mendelian Inheritance of Man) database includes an index of human genes and genetic disorders. Additionally, detailed descriptions, figures and reference are provided.

2.9 Mouse strains

In this work the C57BL/6J and C3H/HeJ mouse strains and offspring from crosses of these strains were used for analyses. The preference of C57BL/6J mice for alcohol was one important selection criterion for these mice. According to behavioural studies, C3H/HeJ mice show differences in ethanol addiction-related behaviours compared to C57BL/6J mice. In particular they differ substantially in their preference for ethanol, which is a basic requirement for mapping ethanol-related traits (CRABBE *et al.* 2006b; ELMOR and GEORGE 1995; LI *et al.* 2005). A caveat of the C3H/HeJ mice is the fact that they and all other Jackson sub-strains are homozygous for the retinal degeneration 1 mutation (Pde6brd1), which causes blindness by weaning age. The Pde6b (phosphodiesterase 6B) gene is located on mouse chromosome 5 at 57.0 cM (MGI). Furthermore, this strain is homozygous for an inversion on Chromosome 6 (symbol: In(6)1J) (AKESON *et al.* 2006). The inversion covers 20% of Chromosome 6, but it results in no reported phenotype.

Parents of a mapping population must have sufficient variation for the traits of interest at both the DNA sequence and the phenotypic level, which is true for the parental strains selected here. In addition to that, C57BL/6J and C3H/HeJ mice meet the condition of a low degree of relationship, as they represent different branches of the family tree of laboratory mice (WITMER *et al.* 2003).

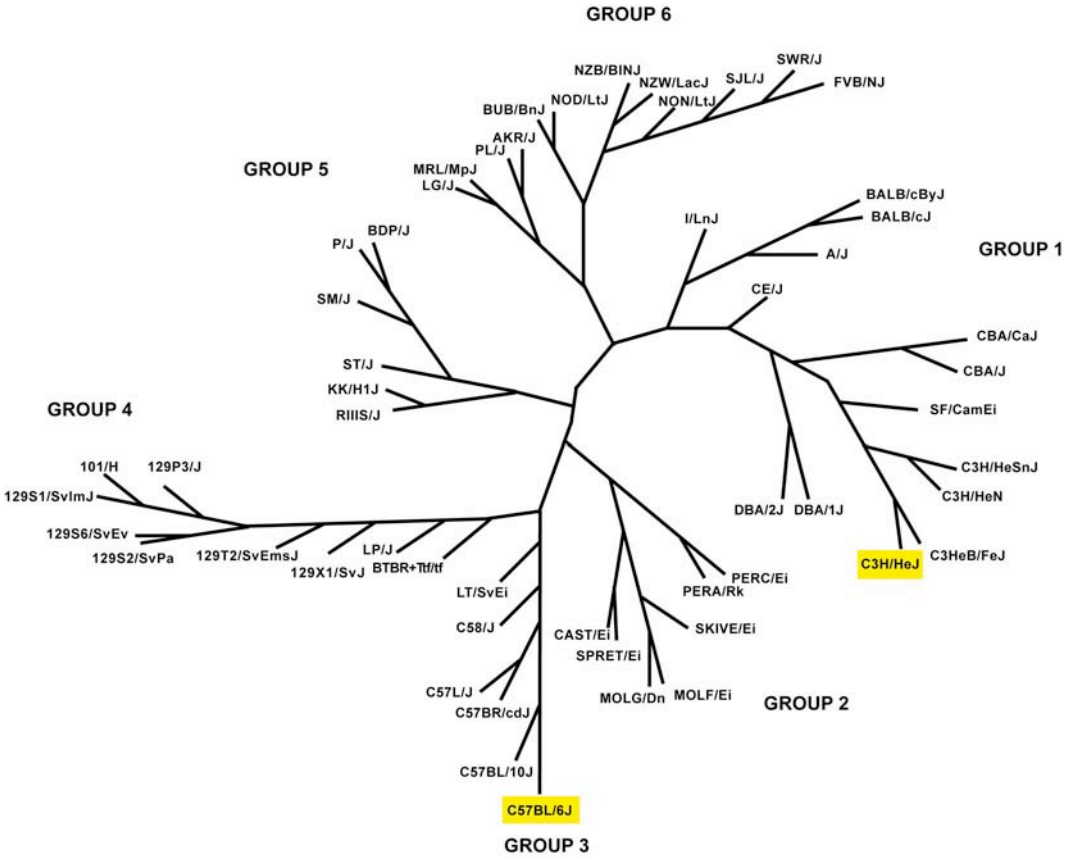


Figure 5. Mouse family tree. Mouse strains are organized into six major groups of branches. C57BL/6J mice belong to Group 3 of the family tree, C3H/HeJ mice to Group 1 (yellow boxes). The length and angles of the branches are optimized for printing and are not quantitative measures of evolutionary distance between strains. (Modified according to WITMER *et al.* 2003.)

Genetic variation of the trait between the parents is important to reach the goal of finding genes contributing to a particular phenotype. If the parental mice differ to a great extend at the phenotypic level, there is reasonable chance that genetic variation exist. Moreover, the more DNA sequence variation exists, the easier it is to find polymorphic informative markers (LIU 1998).

3 METHODS

3.1 Behavioural experiments

All behavioural experiments were conducted at the in-house animal facility of the university's clinical centre.

3.1.1 Animals

8-10 week old breeding animals were obtained from Janvier Laboratory (Janvier Breeding Center, France), which were mated to the second filial generation; first by crossing C57BL/6J x C3H/HeJ mice and subsequently by intercrossing the F1 generation. Eight to ten week old male and female mice were used in the studies.

The S1 (Sicherheitsstufe 1, §§ 4-7 GenTSV) animal facility provided standardised climatic conditions where all animals were housed under reversed light-dark conditions (lights on at 7:00 p.m. and lights off at 9:00 a.m.). Animal care and experiments described in this work were approved by legal authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW). All procedures were in compliance with national (Tierschutzgesetz v. 18.5.2006 (BGBl. I S. 1206, 1313), g v. 18.12.2007 (BGBl. I S. 3001; 2008, 47)) regulations and institutional guidelines. Cage allocation conformed the prescribed number of animals. Mice were housed individually if necessary for the respective experiment, otherwise in groups up to six animals. The animals had free access to food, drinking water or alternatively an ethanol solution when indicated by the experiment.

3.1.2 Experimental design

Behavioural assessments in F2 generation mice were performed in the following order: 1. Acute drug effect; 2. Forced ethanol drinking; 3. Withdrawal studies; 4. Ethanol preference; 5. Stress-induced ethanol drinking (Figure 6). Every animal conducted the whole test battery, but some animals died during the course of the experiments and thus their number abated over time. The parental and F1 generations of mice were also examined in all paradigms, whereas the behaviours were not assessed in a consecutive sequence, but separately for each paradigm using different animals. Behavioural data of these two generations thus also provided information about the possible impact of the serial experimental setup used for the F2 generation mice.

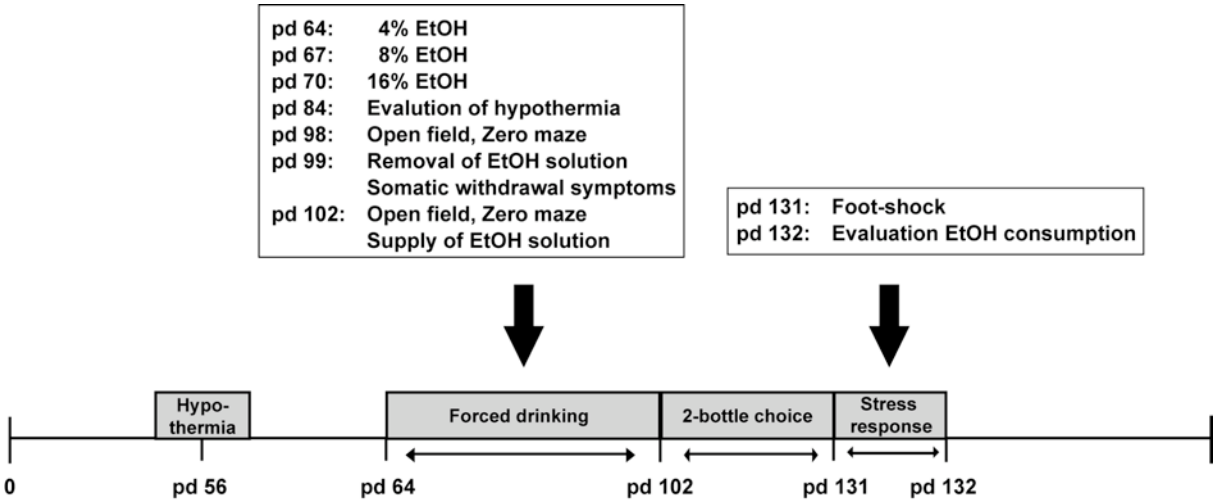


Figure 6. Schedule of the behavioural experiments. Phenotyping began with the determination of acute hypothermia on postnatal day (pd) 56. All subsequent experiments were conducted during pd 64 and 132 according to the order displayed in the scheme.

3.1.3 Alcohol-induced hypothermia

To determine acute alcohol effects and tolerance, parental and F1 animals received a single intraperitoneal (i.p.) injection of 1, 2 or 4 g/kg ethanol or saline (control). The animals' body temperature was measured with a rectal thermometer immediately before and 30 minutes after ethanol treatment to monitor the physiological response in terms of temperature differences (hypothermia). In the F2 generation, all mice received an i.p. injection of 2 g/kg ethanol to assure comparability. This dose was chosen because the parental strains previously showed significant differences in hypothermia after chronic ethanol treatment, without exhibiting severe symptoms of toxication (up to death), as it was observed after administration of 4 g/kg ethanol.

3.1.4 Chronic alcohol consumption and alcohol withdrawal

Ethanol tolerance was evaluated three weeks after the beginning of forced ethanol drinking. For the forced drinking paradigm, animals were supplied with an ethanol solution as their only drinking source (Figure 7a). The ethanol concentration in the drinking water was initially low and then gradually increased, as displayed in Figure 6. As a result of this protocol, the mice were habituated to the ethanol taste and the development of a potential taste aversion was avoided. During the whole experiment, ethanol consumption (g/kg), food consumption (g/day), and the body weight of the mice (g) was recorded twice a week. Evaluation of ethanol-induced hypothermia after chronic ethanol consumption was performed in the same manner as the measurement for acute alcohol effects (see 3.1.3).

Changes in body temperature evaluated for acute and for chronic effects were compared to determine tolerance effects, whereby a reduction in temperature difference (reduced hypothermia) indicates the development of tolerance, an increase (increased hypothermia) is a sign of sensitization.

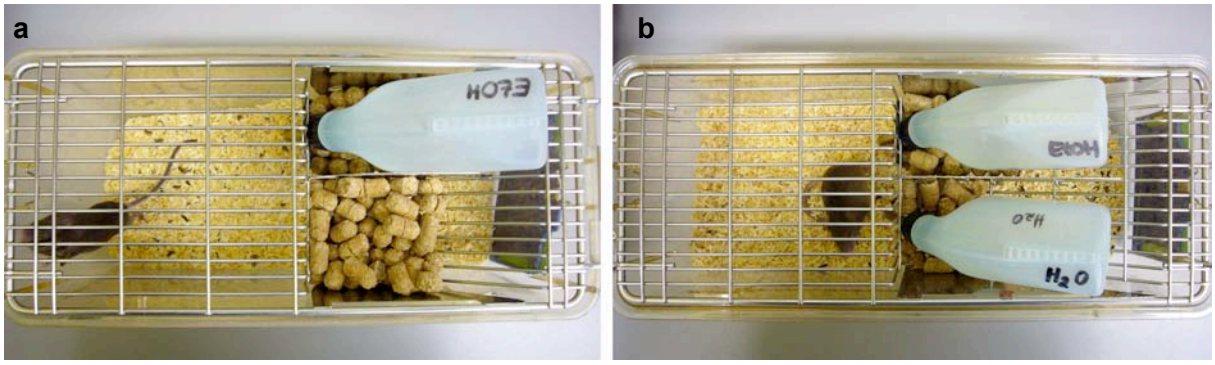


Figure 7. Top view on a mouse cage during the forced drinking (a) and the two-bottle choice paradigm (b).

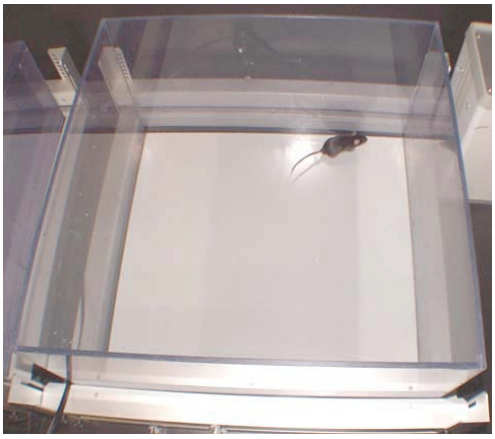
For ethanol withdrawal studies, animals were maintained on a 16% ethanol solution for two more weeks to produce a state of somatic dependence. The ethanol solution was then replaced with tap water and withdrawal symptoms were determined using handling-induced convulsions (GOLDSTEIN and PAL 1971; WATSON *et al.* 1994). The procedure was performed 3 hours after replacing ethanol with water by an experimenter, who was blind to the prior treatment. Each mouse was lifted carefully by the tail, held under a 60 Watt light bulb and was next gently rotated. The ensuing behaviour of the animals was scored for withdrawal symptoms according Table 2.

Table 2. Evaluation scale of somatic withdrawal symptoms for handling-induced convulsions.

Behaviour	**Score**
No behavioural change	0
Mild tremor on lifting and turning	1
Continuous severe tremor on lifting and turning	2
Clonic forelimb extensor spasm on lifting	3
Clonic forelimb extensor spasm on lifting, continuing after placing mouse on cage top	4

To determine ethanol- and withdrawal-induced locomotor activity and anxiety the open field and the zero maze test were conducted. Testing was done during the forced drinking paradigm before and seventy-two hours after ethanol withdrawal.

Locomotor activity was measured in an infrared-beam-operated open field arena (45 x 45 x 23 cm, ActiMot, TSE Systems; Figure 8) for 20 minutes.



The open field box was kept in a soundproof enclosure. At the beginning of the test session, each mouse was placed in the centre of the testing chamber and subsequently the distance travelled (m) was recorded. Testing arenas were cleaned with a 70% ethanol solution between subjects.

Figure 8. Open field arena.

The zero maze apparatus used for anxiety measurements consisted of an annular white platform (inner diameter of 46 cm, 5.6 cm width) elevated 40 cm above the ground level (Figure 9). It was divided into four equal quadrants, whereas non-transparent walls enclosed the two opposite quadrants. Each mouse was placed into the open area of the maze and the animals' behaviour was videotaped using a camera fixed above the maze and analyzed with a video-tracking system (VideoMot; TSE Systems). Between subjects the apparatus was cleaned with a 70% ethanol solution. Distance travelled in the open parts of the maze in relation to the total distance (OD%) was evaluated as a parameter for anxiety.

Figure 9. Zero maze apparatus.

3.1.5 Alcohol preference

Ethanol preference was measured via the two-bottle choice test, which followed the forced drinking paradigm. The animals had the free choice between two drinking bottles (with a metal ball in the sipper tubes to stop the dropping of fluids) (Figure 7b) during the whole experiment. One of these bottles contained an 8% v/v ethanol solution, while the other contained tap water. Bottle positions were changed daily to exclude the development of side preferences. The ratio of alcohol to total fluid consumption (%), the amount of consumed ethanol (g/kg), the body weight (g), and the food consumption (g) were determined twice a week.

3.1.6 Stress-induced alcohol drinking

The influence of stress on ethanol preference and consumption was calculated with animals that had been in the two-bottle choice test since four weeks and thus exhibited a stable ethanol intake. The mice received a mild electric foot-shock through a grid floor located in a dark chamber (Startle Response, TSE Systems) with a continuous background white noise (65 dB). A warning signal (sound and light) was presented a few seconds before the shock. Five intermittent electric foot shocks (intensity, 0.5 mA; duration, 100 msec; interval between shocks, 55 - 60 sec) were delivered computerised through the grid floor. The chamber was cleaned with a 70% ethanol solution between subjects. The ratio of alcohol to total fluid consumption (%) and the amount of consumed ethanol (g/kg) was determined before (mean week 2-4) and 24 hours after the shock.

3.1.7 Statistical data analysis of behavioural data

All statistical analyses were performed using the STATISTICA software package. Acute ethanol effects and tolerance in the parental and F1 generation were evaluated using two-way analysis of variance (ANOVA). To evaluate ethanol-induced hypothermia in naïve and ethanol-experienced mice, as well as for the comparison of acute and chronic ethanol effects (tolerance) in the F2 generation, a two-way repeated measures ANOVA was performed. Significance of ethanol withdrawal symptoms was calculated using a non-parametric Kruskal-Wallis ANOVA. A two-way ANOVA was used to analyze locomotor activity and anxiety-levels in all generations. Ethanol preference and consumption, as well as stress-induced ethanol drinking in the parental mice, were evaluated using a three-way repeated measures ANOVA. Analyses of these parameters in F1 and F2 animals were achieved via a two-way repeated measures ANOVA. Overall, significant ANOVA were followed by a *post hoc* Tukey test for pair-wise comparison. A value of $p < 0.05$ was considered to represent a significant effect.

3.2 Biomolecular experiments

The biomolecular experiments described in this work were conducted in laboratories of the Life & Brain Center at the university's clinical centre. Statistical calculations for the QTL analysis were performed with the help of Professor Dr. Wienker and Dr. Diaz Lacava from the Institute of Medical Biometry, Informatics and Epidemiology, University of Bonn.

3.2.1 Mouse tissue preparation

Liver tissue of all mice was removed for further analysis. Therefore, mice were sacrificed via cervical dislocation and the isolated organ was immediately shock-frozen in isopentan bedded on dry ice. Tissue samples were stored at -80°C.

3.2.2 Isolation of genomic DNA

Genomic DNA was isolated from frozen liver tissue. The isolation was conducted using the Qiagen DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol.

DNA concentration was evaluated photometrically at a wavelength of 260 nm using the *Fusion™ Universal Microplate Analyzer*. For nucleic acid quantification, the Beer-Lambert ($A = \varepsilon * b * c$) equation is modified to use an extinction coefficient with units of $M^{-1} cm^{-1}$. Using this extinction coefficient gives a manipulated equation:

$$c = (A * \varepsilon) / b$$

c: Nucleic acid concentration (ng/µl)
A: Absorbance (absorbance units, AU)
ε: Wavelength-dependent extinction coefficient ($M^{-1} cm^{-1}$)
b: Path length (cm)

The generally accepted extinction coefficient for double-stranded DNA is 50 $M^{-1} cm^{-1}$.

Isolated and purified DNA was stored in a fridge at 4 °C until further use. Remaining tissue was kept at -80°C for long-term storage.

3.2.3 Normalization of genomic DNA

High-throughput DNA normalization was achieved using the liquid-handling platform *Multiprobe II*. Normalization was necessary to assure the use of equal DNA amounts for

subsequent polymerase chain reactions (PCR), a prerequisite for equal signal strengths on the genetic analyzer (see 3.2.5). All samples were normalized to a concentration of 3 ng/µl DNA. Normalized DNA was stored at 4 °C until further use.

3.2.4 Selection of the microsatellite marker set

The selection and design of the microsatellite markers used for genotyping was based on the dataset from the Whitehead Institute/MIT Center for Genome Research (WICGR). High marker polymorphism between the two parental strains and an equal distribution throughout the mouse genome were main criteria for selection. With the help of different mouse genome browsers (see 1.1), strain specific allele fragments lengths resulting from microsatellite amplification were compared to find microsatellites that were polymorphic between the parental mouse strains. The resulting markers were then selected for genome distribution, with a spacing of one marker each 5 cM. Thus, a dense microsatellite marker map was established to provide high-resolution QTL mapping.

Microsatellites were amplified with primer sequences complementary to their flanking regions, whereas the required primer sequences were also taken from the MGI database. All primers used in this study were initially tested to confirm DNA amplification using PCR with DNA samples isolated from parental and offspring mouse liver. PCR products were primarily separated on agarose- and polyacrylamide gels. Agarose- and polyacrylamide gel electrophoreses were performed according to Sambrook and Russell (SAMBROOK and RUSSELL 2001). PCR conditions for all markers were finally adjusted according to the electrophoresis specifications of the genetic analyzer (see also 3.2.5). The resulting microsatellite marker set consisted of 264 markers that were spaced at a mean distance of 5.5 cM throughout the mouse genome (Supplement table S1). DNA amplification resulted in PCR products with a length between 74 and 360 bp.

3.2.5 Genotyping and fragment length analysis

To determine the genetic constitution of F2 generation mice at a specific microsatellite locus genotyping of genomic DNA was performed via PCR. Subsequent analyses of the PCR products were done with a genetic analyzer, providing electrophoretic separation in a resolution up to one base pair lengths difference (Figure 10).

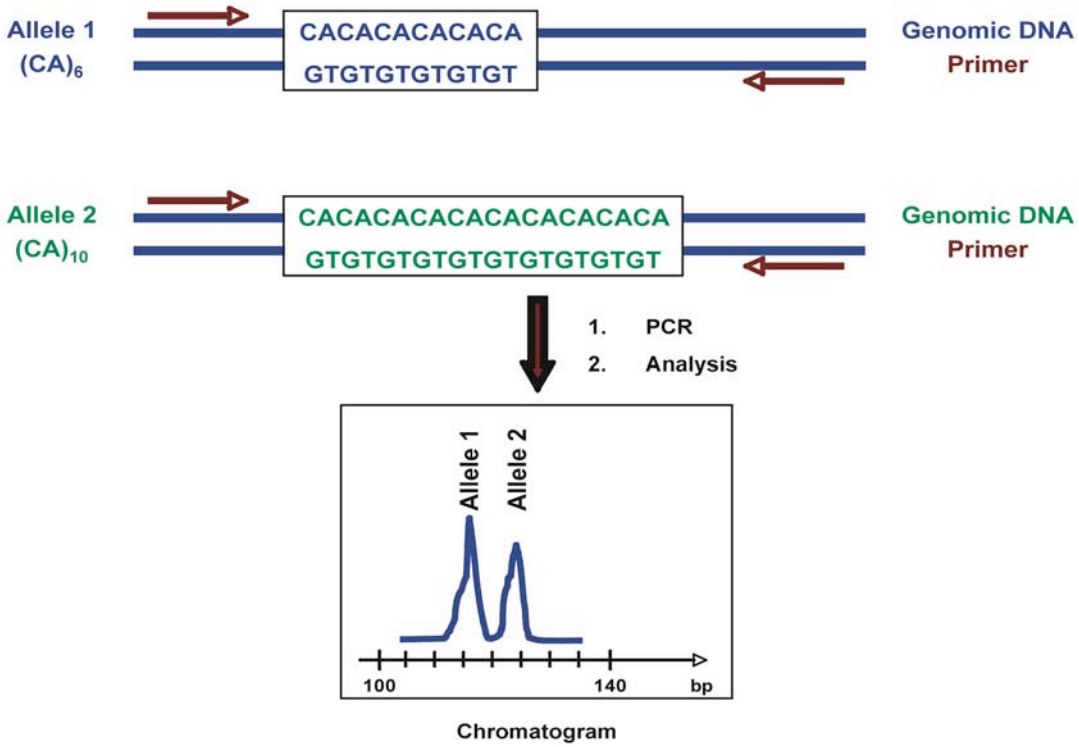


Figure 10. Workflow of DNA genotyping and fragment length analysis. $(CA)_n$: Number of dinucleotide tandem repeats; C: Cytidine, A: Adenosine. See text below for details.

3.2.5.1 Polymerase chain reaction

Specific amplification of DNA fragments was achieved using PCR. The standardised PCR reaction and temperature profiles (Table 3 and Table 4) were specifically adjusted according to the thermodynamic properties of the oligonucleotide primer and the lengths of the amplified PCR products. Of great importance is the specific annealing temperature of the primer pairs with the DNA strands. To calculate the annealing temperature the following simplified formula can be used:

$T_{Annealing} = 2x\ n_{A+T} + 4x\ n_{G+C}$ [°C]

n_{A+T}: Number of A- and T- nucleotides

n_{G+C}: Number of G- and C- nucleotides

Depending on the primer pair used for PCR either the HotStar Taq (Qiagen) or the NEB Taq was applied. PCR was carried out in 10 µl reactions.

Table 3. Standard PCR reaction (10 µl):

Component	Final concentration
Genomic DNA	3 ng/µl
Reverse primer	0.2 µM
Forward primer	0.2 µM
10x HotStar Taq buffer	1x
$MgCl_2$	2.25 mM
dNTP mix	0.2 mM
HotStar Taq polymerase	0.125 U
Water (HPLC gradient grade) to 10 µl	

Table 4. Standard temperature profile:

	I	Initial activation step	95 °C	15 min	
35 cycles	II	Denaturation	94 °C	45 s	} 35 cycles
	III	Annealing	60 °C	45 s	
	III	Extension	72 °C	45 s	
	IV	Final extension	72 °C	10 min	
	V	Cooling	4 °C	∞	

To efficiently genotype all F2 mice, a high-throughput PCR setup was established. Primer pairs were not multiplexed in one reaction batch, but were assembled according to their annealing temperature and the required Taq polymerase. Using 96-well microtiter plates, 96 single PCR reactions could thus be conducted at once. Following PCR, products of different allele sizes and fluorescent dye labelling were pooled with up to four PCR products in one well of a microtiter plate. This way of sample handling allowed the integration of four 96-well plates into one 384-well plate, which was next loaded on a genetic analyzer. The high-throughput PCR setup and associated sample processing was achieved by using the liquid-handling platform *Multiprobe II*.

3.2.5.2 Fragment lengths analysis

To determine fragment lengths, PCR products were loaded on a ABI 3130*xl* Genetic Analyzer (supplied with the *Data collection software*®, Figure 11) and analysed with the *ABI GeneMapper*® software according to the manufacturer's protocol. The genetic analyzer platform was provided with 16 capillaries with a length of 36 cm each. For internal DNA size standard, *GeneScan™ 500 ROX™ Size Standard* was used, which is a dye-labelled size

standard for the reproducible sizing of fragment analysis data. The standard contained 16 ROX dye-labelled, single-stranded DNA fragments. As there is an internal DNA size standard in each capillary, the alleles could be sized accurately in base pairs. Alleles were visualised in a graphic format, with a peak representing an allele.

Figure 11. ABI 3130*xl* Genetic Analyzer.

Before capillary electrophoresis, PCR products were diluted with HPLC-purified water (1:2) and subsequently with deionised formamide (Applied Biosystems). This mixture was then denatured in a thermocycler at 96 °C for 5 minutes.

Each sequencer reaction batch was composed as follows:

Deionised formamide	9.7 µl
ROX™ Size Standard	0.3 µl
Each PCR product (diluted)	1 µl

3.2.6 Detection and mapping of quantitative traits

The probability of linkage between behaviour and marker is defined by the logarithm of the odds (LOD) score. The LOD score is a statistical estimate of whether two loci are likely to lie near each other on a chromosome and are therefore expected to be inherited together. The LOD score indicates the strength of evidence for the presence of a QTL, with larger LOD scores corresponding to greater evidence (BROMAN 2001).

Mapping of QTL was done using the software package R/qtl (BROMAN *et al.* 2003), which is an extensible, interactive environment for mapping quantitative trait loci in experimental crosses. It is implemented as an add-on package for the freely available and widely used

statistical language/software R (R DEVELOPMENT CORE TEAM 2008). The core of R/qtl is a set of functions that make use of the hidden Markov model (HMM) technology to calculate QTL genotype probabilities, to simulate from the joint genotype distribution and to calculate the most likely sequence of underlying genotypes (all conditional on the observed marker data) (BROMAN *et al.* 2003). R/qtl also calculates several functions that are useful for a quality control of the input data used for mapping. Also these functions offer the possibility to scrutiny the reliability of the resulting outputs. The results provide information about the proportion of missing genotypes for each individual or each marker in the cross, about the recombination fractions (the proportion of meiotic products, which are non-parental – recombinant - at the loci) for all pairs of markers and the LOD scores for tests of linkage between pairs of markers. Furthermore, a genetic map of marker locations for all chromosomes is evaluated, which is then compared to that estimated with the observed data. This comparison gives information about the reliability of the observed genotype information: The more the marker locations differ between the two maps (which signifies variation in marker positions), the higher the possibility of genotyping errors.

QTL mapping was done in several stages to identify loci acting individually and QTL that interacted, either additively or epistatically. To determine individually-acting QTL, a single-QTL genome scan was conducted with the function *scanone*. For this function, R/qtl uses the maximum likelihood via the EM algorithm (LANDER and BOTSTEIN 1989), the Haley-Knott regression (HALEY and KNOTT 1992), or the multiple imputation method of Sen and Churchill (SEN and CHURCHILL 2001). In the present study, the multiple imputation (IMP) algorithm was chosen to substitute missing data points, albeit preliminary analyses using all three algorithms showed only minor differences between the results. Here, R/qtl uses the pseudomarker algorithm described by Sen and Churchill (SEN and CHURCHILL 2001). This algorithm simulates multiple versions of complete genotype information on a genome-wide grid of locations using information in the marker genotype data. Weights are assigned to the simulated genotypes to capture information in the phenotype data. The weighted complete genotypes are used to approximate quantities needed for statistical inference of QTL locations and effect sizes.

As an additional tool, R/qtl provides the opportunity to calculate *Bayesian credible intervals* (confidence intervals) for a single-QTL on a particular chromosome. This interval was calculated with the function *bayesint*, whose output is a chromosomal interval in centimorgan (cM) that contains the QTL with a probability of 95%. Significance thresholds for single-QTL were based on 10.000 permutations.

For interacting QTL, a two-QTL genome scan was performed using the R/qtl function *scantwo*, again via the imputation method described above. Therefore, the maximum LOD score is calculated for every pair of position for the full model (lod.full; two QTL plus interaction) and for the additive model (lod.add; two QTL but no interaction). Furthermore, a LOD score for a test of epistasis (lod.int) is calculated by taking the difference between the maximum full LOD and the maximum additive LOD for each pair of chromosomes. Epistasis describes the phenotypic effect of interaction among alleles at multiple loci in addition to the direct effect of individual loci or, in other words, when the action of one gene is modified by one or several other genes. In a summary function, each pair of chromosomes is considered and the maximum LOD score for the full model and the maximum LOD score for the additive model is calculated, whereby these two models are allowed to be maximized at different positions.

A significant lod.full in combination with a significant lod.int now indicates epistasis between the two respective chromosomal positions, whereby a significant lod.full in combination with a significant lod.add implies that the interaction of the two loci is additive. In the present study, the 5% permutation-derived significance threshold level based on 100 permutations, giving significance levels for the full, the interaction and the additive LOD scores. In the results, genome-scan-adjusted p-values are given. To estimate the appropriate LOD threshold, the phenotype data are permuted, whereby the genotype data stay intact. Now interval mapping is performed and the maximum LOD score across the genome is identified. This process is repeated *n* times. The observed LOD score (with the phenotypes in the correct order) is compared to the *n* LOD scores obtained from permuted versions of the data. The proportion of these *n* LOD scores that exceed the actual, observed LOD score, is reported as an approximate p-value (BROMAN 2001).

4 RESULTS

Mice of the parental, the first and the second filial generation were tested in paradigms modelling different aspects of alcohol addiction and related behaviours including alcohol-induced hypothermia, alcohol tolerance, withdrawal-induced anxiety and locomotor effects, somatic withdrawal symptoms, alcohol preference and stress-induced changes in alcohol preference. Evaluation of behaviour in C57BL/6J and C3H/HeJ mice was conducted to give evidence for the different ethanol-related behaviour patterns in these mice. Subsequent quantitative trait mapping was conducted using the data obtained from pheno- and genotyping of the heterogeneous F2 mice.

4.1 Behavioural analysis of the parental mouse strains

The C57BL/6J and C3H/HeJ strains were selected, because they are genetically distinct and represent different branches of the family tree of laboratory mice (WITMER *et al.* 2003). In addition, it has been reported that they differ substantially in their preference for ethanol (LI *et al.* 2005). First, the behavioural and physiological responses to ethanol were characterized in these parental strains.

4.1.1 Acute ethanol-induced hypothermia and tolerance

Injection of ethanol to drug-naïve animals produced a dose-dependent reduction in body temperature in both parental strains, albeit with different response magnitudes, as revealed by significant main effects for *strain* ($F_{1,61}$ = 20. 57, $p < 0.0001$), *dose* ($F_{3,61}$ = 92.76, $p < 0.0001$) and *strain x dose* interaction ($F_{3,61}$ = 9.52, $p < 0.0001$). Both strains showed a significant reduction in body temperature with a dose of 2 and 4 g/kg ($p < 0.05$). Administration of 4 g/kg ethanol produced a markedly stronger effect in C57BL/6J compared to C3H/HeJ mice ($p < 0.05$) (Figure 12a). A gender effect was not observed.

To investigate the development of tolerance after chronic ethanol exposure, the animals were restricted to an ethanol solution (16%) as their only drinking source for a period of two weeks and subsequently ethanol-induced hypothermia was examined. A two-way ANOVA revealed again significant main effects for *strain* ($F_{1,74}$ = 18.43, $p < 0.0001$), *dose* ($F_{3,74}$ = 62.89, $p < 0.0001$) and *strain* x *dose* interaction ($F_{3,74}$ = 7.56, $p < 0.001$). Both strains exhibited significantly enhanced hypothermia after administration of 2 and 4 g/kg ethanol compared to saline controls ($p < 0.05$), but again only the highest dose of ethanol resulted in a significant difference between the two strains ($p < 0.05$).

When comparing the results before and after chronic ethanol exposure, no significant interaction between the main factors *strain*, *dose*, and *drug experience* ($F_{3,135} = 0.06$, p = n.s.) was found. Thus, the parental strains did not develop tolerance to the hypothermic effects of ethanol (Figure 12b).

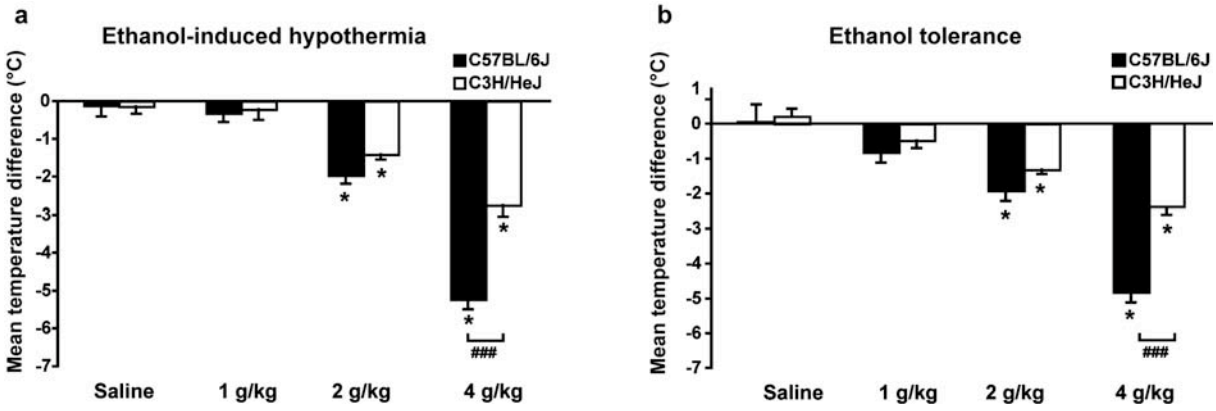


Figure 12. Ethanol-induced hypothermia and tolerance. (a) Ethanol-naïve C57BL/6J and C3H/HeJ mice showed a significant reduction in body temperature after i.p. injection of 2 and 4 g/kg ethanol compared to saline controls. A significant strain difference was observed with a dose of 4 g/kg. C57BL/6J mice: saline, n = 5; 1 g/kg, n = 8; 2 g/kg, n = 8; 4 g/kg, n = 6. C3H/HeJ mice: saline, n = 11; 1 g/kg, n = 5; 2 g/kg, n = 21; 4 g/kg, n = 5. (b) After two weeks of forced drinking, both strains still exhibited significantly enhanced hypothermia after administration of 2 and 4 g/kg ethanol compared to controls. Administration of 4 g/kg ethanol resulted in a significant difference between the two strains. The parental strains did not develop tolerance to the hypothermic effects of ethanol. C57BL/6J mice: saline, n = 5; 1 g/kg, n = 6; 2 g/kg, n = 6; 4 g/kg, n = 6. C3H/HeJ mice: saline, n = 11; 1 g/kg, n = 11; 2 g/kg, n = 28; 4 g/kg, n = 12. Values represent mean ± SEM. Between strain comparison: ### $p < 0.001$. Within strain comparison: *$p < 0.05$.

4.1.2 Ethanol dependence and withdrawal

The forced drinking paradigm was conducted to produce a state of somatic ethanol dependence in the mice (Figure 13a and b). During four weeks of testing the parental strains exhibited significantly different drinking behaviour ($F_{1,53} = 11.28$, $p < 0.01$). Also, there was a significant main effect for *strain* x *gender* interaction ($F_{1,53} = 13.92$, $p < 0.001$). A *post hoc* Tukey test for this interaction revealed that overall C3H/HeJ females differed significantly from C3H/HeJ male mice ($p < 0.05$). Within factor analysis revealed significant differences over the time course of the experiment ($F_{3,159} = 5.71$, $p < 0.001$) and a significant *time* x *strain* interaction ($F_{3,159} = 9.84$, $p < 0.0001$).

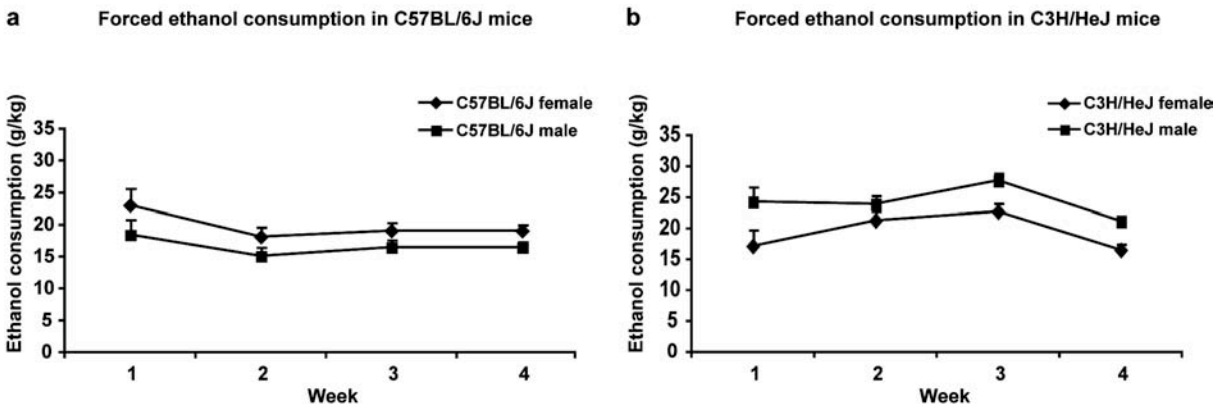


Figure 13. (a) Forced ethanol consumption. Comparison of mean ethanol consumption data revealed significant strain differences between C57BL/6J and C3H/HeJ mice. C57BL/6J males: n = 20; C57BL/6J females: n = 17; C3H/HeJ females: n = 9; C3H/HeJ males: n = 11. Values represent mean ± SEM.

After the forced drinking paradigm, withdrawal studies were performed. Handling-induced convulsions were evaluated three hours after replacing the ethanol solution with tap water. Mice of both parental strains exhibited significantly more tremors or forelimb extensor spasm after ethanol withdrawal and thus were physically dependent on ethanol (Kruskal-Wallis ANOVA, $p < 0.001$; Table 5).

Table 5. Somatic ethanol withdrawal symptoms. Three hours after ethanol withdrawal the frequencies of high handling-induced convulsion (HIC score) were significantly increased in both strains. C57BL/6J: n = 160; C3H/HeJ: n = 61.

		HIC score frequencies (%)				
		0	1	2	3	4
C57BL/6J	EtOH	42.5	56.3	1.3	0	0
	Withdrawal	1.3	17.5	45.0	33.8	2.5
C3H/HeJ	EtOH	55.7	41.0	3.3	0	0
	Withdrawal	4.9	32.8	32.8	21.3	8.2

Locomotor activity was not changed, as mice of both strains were similarly active in the open-field arena before and after withdrawal ($F_{1,62} = 0.22$, p = n.s. for *strain* x *withdrawal* interaction; Figure 14a). Overall, C3H/HeJ mice travelled a significantly longer distance in this test ($F_{1,62} = 6.48$, $p < 0.05$).

In the zero maze test there was evidence for higher anxiety-levels 72 hours after ethanol removal ($F_{1,66} = 9.84$, $p < 0.01$) (Figure 14b). C3H/HeJ mice were generally more active in the open area compared to C57BL/6J animals ($F_{1,66} = 74.73$, $p < 0.0001$) thus displaying lower anxiety-levels. A significant *strain* x *withdrawal* interaction was not found ($F_{1,66} = 0.299$, p = n.s.).

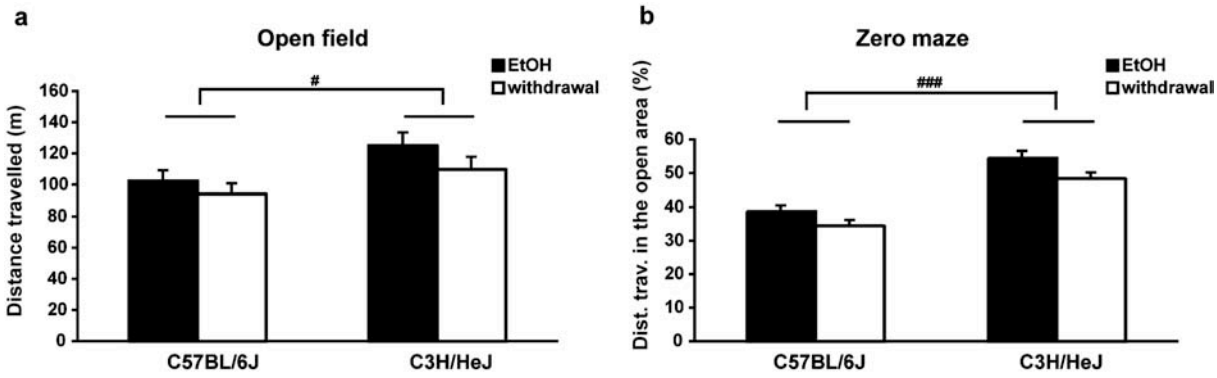


Figure 14. Open field and zero maze behaviour. (a) C57BL/6J and C3H/HeJ mice differed significantly in their open field locomotor activity. C57BL/6J: n = 41; C3H/HeJ: n = 25. (b) C3H/HeJ animals showed a higher locomotor activity in the open area of the maze than C57BL/6J mice. After withdrawal of ethanol, both strains showed a significant decrease in open area locomotion indicative of elevated anxiety levels. C57BL/6J: n = 41; C3H/HeJ: n = 29. Values represent mean ± SEM. Between strain comparison: # $p < 0.05$, ### $p < 0.001$.

4.1.3 Ethanol preference and consumption

Ethanol preference values were higher in C57BL/6J compared to C3H/HeJ mice (Figure 15a). A three-way repeated measure ANOVA revealed a significant *strain* effect ($F_{1,224} = 25.05$, $p < 0.0001$) and a significant interaction between *strain* and *gender* ($F_{1,224} = 24.58$, $p < 0.0001$). Female C57BL/6J mice showed a higher preference for ethanol than males, while the opposite was true in the C3H/HeJ strain. Thus, gender effects seem to be strain specific. There was also a significant effect for the within factor *time* ($F_{3,672} = 11.21$, $p < 0.0001$), reflecting an increase in ethanol preference values during the course of the experiment.

Similar results were obtained for the ethanol consumption data, since C57BL/6J and C3H/HeJ mice differed significantly in ethanol consumption ($F_{1,328} = 58.68$, $p < 0.0001$) (Figure 15b). Additional significant effects were found for *gender* ($F_{1,328} = 5.41$, $p < 0.05$) and for *strain* x *gender* interaction ($F_{1,328} = 20.94$, $p < 0.0001$). As for ethanol preference, significant differences for the within factors *time* ($F_{3,984} = 11.15$, $p < 0.0001$) as well as for *time* x *strain* interaction ($F_{3,984} = 4.32$, $p < 0.01$) was observed. There was no difference in the amount of consumed food, nor was there any difference in body weight.

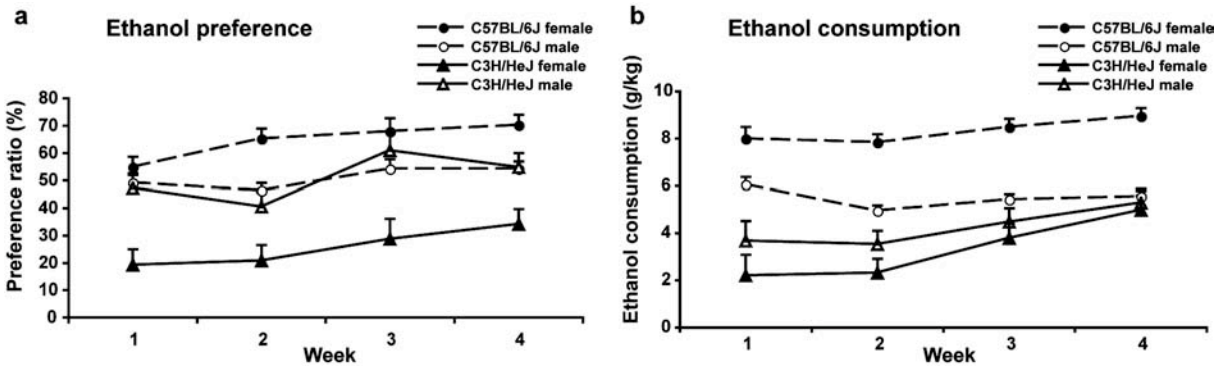


Figure 15. Ethanol preference and consumption. (a) Ethanol preference was highest in female C57BL/6J mice and lowest in female C3H/HeJ mice. C57BL/6J males: n = 112; C57BL/6J females: n = 60; C3H/HeJ females: n = 26; C3H/HeJ males: n = 30. (b) Ethanol consumption values differed significantly between the two parental strains. C57BL/6J males: n = 192; C57BL/6J females: n = 84; C3H/HeJ females: n = 27; C3H/HeJ males: n = 29. Values represent mean ± SEM.

4.1.4 Stress-induced ethanol drinking

In order to evaluate changes in ethanol preference and consumption after stress exposure, the animals received a mild foot-shock after they had performed the two-bottle choice paradigm for four weeks. For ethanol preference, data analysis revealed significant effects for *strain* ($F_{1,114} = 38.35$, $p < 0.0001$), *strain x gender* interaction ($F_{1,114} = 11.6$, $p < 0.001$) and *stress x strain x gender* interaction ($F_{1,114} = 5.13$, $p < 0.05$). Generally, C3H/HeJ mice exhibited lower preference ratios before and after foot-shock compared to C57BL/6J mice.

In C57BL/6J female mice there was no change in ethanol preference (p = n.s.), whereas in C57BL/6J male mice there was a significant increase in ethanol preference 24 hours after foot-shock ($p < 0.05$). In contrast, ethanol preference in C3H/HeJ mice did not change after foot-shock stress (p = n.s., Figure 16a). Overall, analysis of the ethanol consumption data revealed an increase in consumption after foot-shock ($F_{1,114} = 4.30$, $p < 0.05$). Furthermore, the two parental strains differed significantly in ethanol consumption ($F_{1,114} = 41.48$, $p < 0.0001$). Significant main effects were also detected for *gender* ($F_{1,114} = 5.32$, $p < 0.05$) and *gender* x *strain* interaction ($F_{1,114} = 5.14$, $p < 0.001$) (Figure 16b).

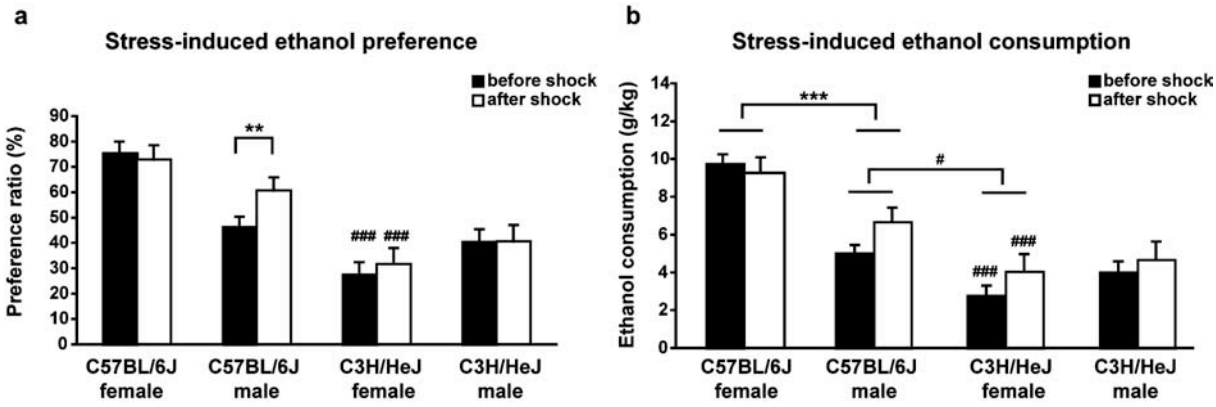


Figure 16. Stress-induced ethanol preference and consumption. (a) C3H/HeJ mice exhibited lower preference ratios before and after foot-shock compared to C57BL/6J mice. In C57BL/6J female mice there was no change in ethanol preference, whereas C57BL/6J male mice exhibited a significant higher ethanol preference after foot-shock. C57BL/6J males: n = 38; C57BL/6J females: n = 31; C3H/HeJ females: n = 25; C3H/HeJ males: n = 24. (b) Ethanol consumption was higher in C57BL/6J mice in stress-free and stressful conditions. An increase in ethanol consumption after foot-shock was observed in all mice except female C57BL/6J. C57BL/6J males: n = 39; C57BL/6J females: n = 31; C3H/HeJ females: n = 25; C3H/HeJ males: n = 23. Between strain comparison: # $p < 0.05$; ### $p < 0.001$. Within strain comparison: **$p < 0.01$; ***$p < 0.001$. Values represent mean ± SEM.

4.2 Behavioural analysis of the first filial generation

Breeding of C57BL/6J and C3H/HeJ mice led to a genetically homogeneous F1 generation, which was also phenotyped for ethanol-related behaviours.

4.2.1 Acute ethanol effects and tolerance

Drug-naïve mice of the F1 generation received an i.p. injection of three different doses of ethanol, which produced a similar dose – dependent reduction in body temperature as in the parental mice. Two-way ANOVA revealed a significant main effect for *dose* ($F_{3,52} = 57.32$, $p < 0.001$), but not for *gender* ($F_{1,52} = 0.58$, p = n.s.) or *gender x dose* interaction ($F_{3,52} = 1.09$, p = n.s.). A significant increase in hypothermia was seen after administration of 2 and 4 g/kg ethanol ($p < 0.001$; Figure 17a).

Chronic exposure to an ethanol solution (16%) again led to a dose – dependent hypothermic effect in F1 mice ($F_{3,78} = 51.86$, $p < 0.0001$ for factor *dose*; $F_{1,78} = 0.41$, p = n.s. for factor *gender*; $F_{1,78} = 0.77$, p = n.s. for *dose x gender* interaction). Still, there was a significant increase in hypothermia after administration of 2 and 4 g/kg ethanol compared to saline controls ($p < 0.001$) (Figure 17b).

Three-way ANOVA did not reveal significant main effects for *dose* x *gender* x *drug experience* interaction ($F_{3,130} = 1.57$, p = n.s.). Thus, F1 mice did not develop tolerance to the hypothermic effects to ethanol.

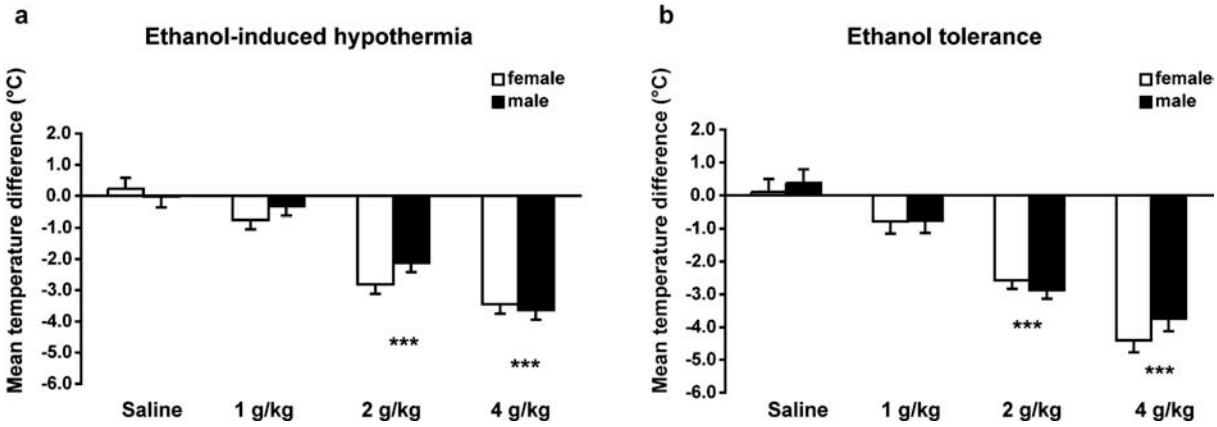


Figure 17. Ethanol-induced hypothermia and tolerance. (a) Administration of ethanol dose - dependently decreased the body temperature of female and male F1 mice, which was significant for 2 and 4 g/kg ethanol compared to saline controls. Saline, n = 6; 1 g/kg, n = 8; 2 g/kg, n = 8; 4 g/kg, n = 8. (b) After chronic ethanol drinking, hypothermia was still significantly increased after injection of 2 and 4 g/kg ethanol compared to saline controls, whereby no tolerance effect was observed. Females: Saline, n = 8; 1 g/kg, n = 9; 2 g/kg, n = 18; 4 g/kg, n = 9. Males: Saline, n = 7; 1 g/kg, n = 9; 2 g/kg, n = 18; 4 g/kg, n = 8. Values represent mean ± SEM. ***p < 0.001.

4.2.2 Ethanol dependence and withdrawal

The forced drinking paradigm was used to monitor ethanol consumption in F1 animals (Figure 18). Female and male mice exhibited a significantly different drinking behaviour ($F_{1,53}$ = 22.25, p < 0.0001), whereby females consumed higher amounts of ethanol. In both gender, the initial variation in ethanol consumption ($F_{3,159}$ = 52.72, p < 0.0001) stabilized during the 4-week testing period.

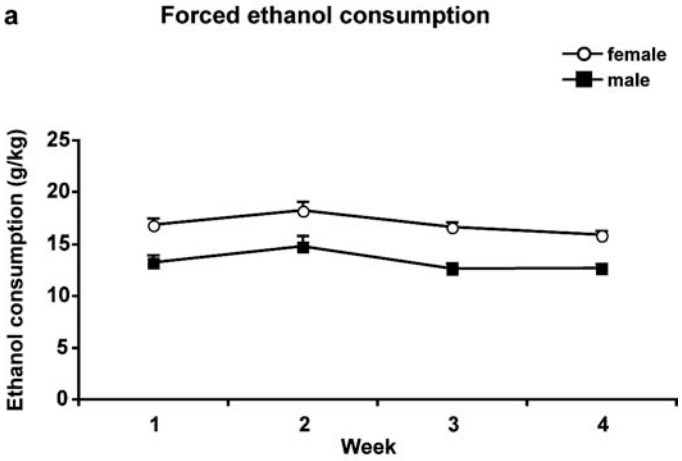


Figure 18. Forced ethanol consumption. Female F1 mice consumed significantly higher amounts of ethanol compared to their male counterparts. After two weeks of testing, all mice displayed a stable alcohol intake. Females: n = 30; Males: n = 25. Values represent mean ± SEM.

Withdrawal symptoms were evaluated three hours after replacing the ethanol solution with tap water as described for the parental strains. Mice of both gender exhibited significantly more withdrawal symptoms after ethanol removal as seen by increased tremors and stronger forelimb extensor spasm (p < 0.0001; Table 6).

Table 6. Somatic ethanol withdrawal symptoms. Three hours after ethanol withdrawal the frequencies of high handling-induced convulsion (HIC score) were significantly increased in both gender. Females: n = 30; Males: n = 25.

		HIC score frequencies (%)				
		0	1	2	3	4
Female	EtOH	23.3	53.3	23.3	0	0
	Withdrawal	3.3	10.0	26.7	46.7	13.3
Male	EtOH	44.0	40.0	16.0	0	0
	Withdrawal	8.0	16.0	20.0	48.0	0

Withdrawal-induced changes in locomotor activity were not observed in the open field arena ($F_{1,78}$ = 2.52, p = n.s.; Figure 19a), whereas in the zero maze mice of both gender covered significantly less distance in the open area 72 hours after ethanol withdrawal ($F_{1,78}$ = 24.44, p < 0.0001 for *withdrawal* ; Figure 19b).

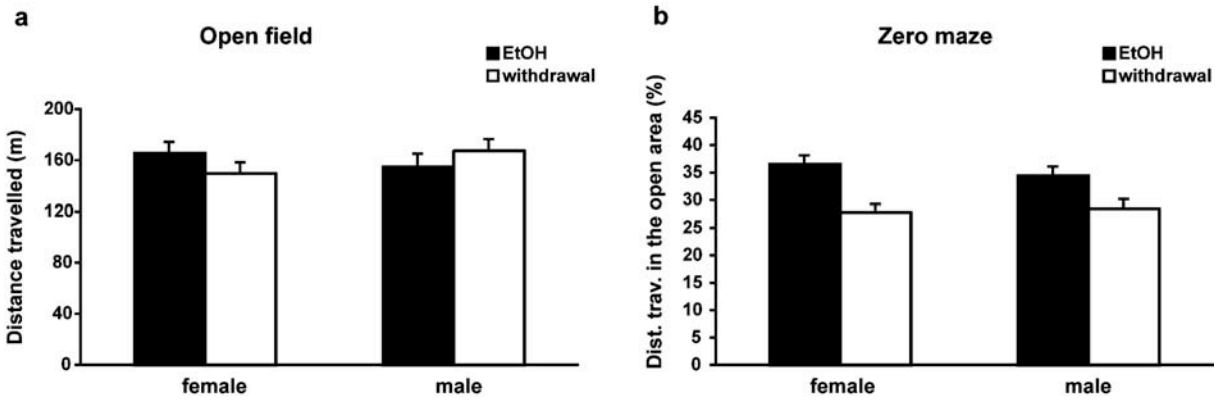


Figure 19. Open field and zero maze behaviour. (a) Locomotor activity of F1 mice remained unchanged after ethanol withdrawal. (b) Overall, F1 mice showed significantly elevated anxiety levels after ethanol withdrawal, represented by the increase in distance travelled. Females: n = 23; Males: n = 20. Values represent mean ± SEM.

4.2.3 Ethanol preference and consumption

Ethanol preference and consumption were calculated in the two-bottle choice paradigm over a period of four weeks (Figure 20a and b, respectively). The preference values slightly varied over the course of the experiments ($F_{3,132} = 3.07$, $p < 0.05$). This effect was similar in both gender ($F_{3,132} = 0.53$, p = n.s for *week* x *gender* interaction). Analysis of the ethanol consumption data did not reveal a significant main effect in F1 animals ($F_{1,51} = 2.42$, p = n.s. for factor *gender*; $F_{3,153} = 2.25$, p = n.s. for factor *week*; $F_{3,153} = 0.23$, p = n.s. for *gender x week* interaction). F1 animals showed no difference in food consumption, nor were there any differences in body weight.

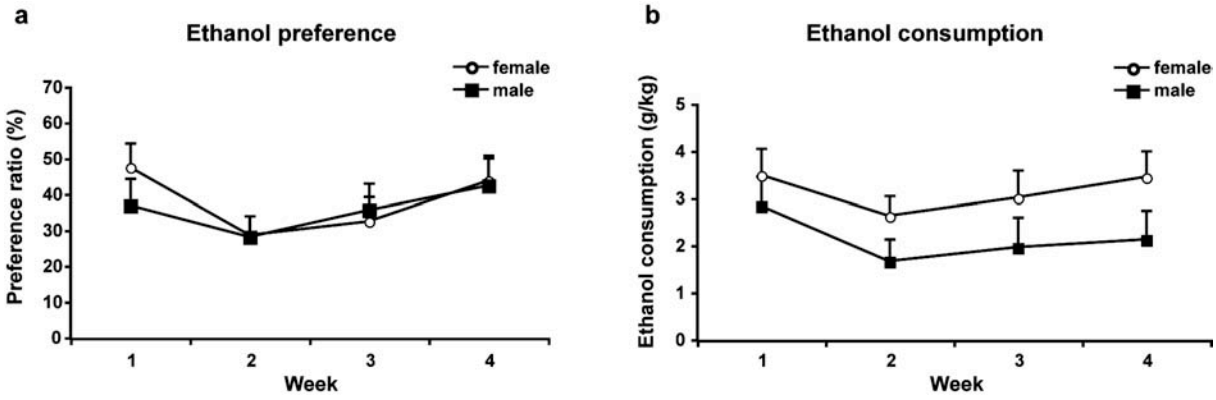


Figure 20. Ethanol preference and consumption. (a) Ethanol preference ratio did not differ between male and female F1 animals, but it slightly varied over the experiment. Females: n = 25; Males: n = 21. (b) As for the preference values, ethanol consumption did not differ between females and males. Females: n = 29; Males: n = 24. Values represent mean ± SEM.

4.2.4 Stress-induced ethanol drinking

Foot-shock stress did not provoke higher ethanol preference values in neither of the gender. Two-way repeated measure ANOVA revealed no significant main effects ($F_{1,24} = 0.93$, *p* = n.s. for factor *gender*; $F_{1,24} = 0.21$, *p* = n.s. for factor *stress*; $F_{1,24} = 0.16$, p = n.s. for *gender x stress* interaction; Figure 21a). In addition, no significant change in ethanol consumption was found, despite a tendency of higher alcohol intake after foot- shock stress in female mice ($F_{1,51} = 2.23$, p = n.s. for factor *gender*; $F_{1,51} = 2.29$, p = n.s. for factor *stress*; $F_{1,51} = 0.67$, p = n.s. for *gender x stress* interaction; Figure 21b).

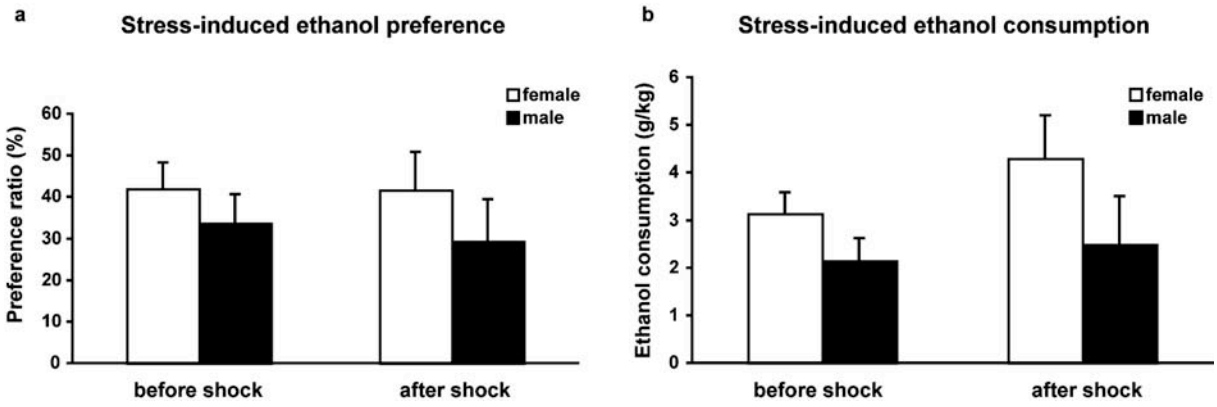


Figure 21. Stress-induced ethanol preference and consumption. (a) Ethanol preference was not affected by the foot-shock stress in neither of the gender. Females: n = 14; Males: n = 12. (b) The amount of consumed ethanol did not reach significance in the F1 mice. Females: n = 29; Males: n = 24. Values represent mean ± SEM.

4.3 Analysis of the second filial generation

F2 generation mice obtained from the C57BL/6J x C3H/HeJ mice intercross were first phenotyped for several ethanol responses and subsequently genotyped to assess their genetic constitution at microsatellite loci throughout the mouse genome. QTL mapping was done via linkage analysis of the pheno- and genotyping data using two different approaches. Given below are the combined results for the F2 pheno- and genotyping.

4.3.1 Data quality control

In a first step the quality of input data was controlled to assure reliable mapping results. The current version of R/qtl (v2.7.2) includes functions for plotting marker distributions and estimating genetic maps. The final genetic map used in the present study consisted of 264 microsatellite markers with an average marker distance of 5.56 cM, which represents high-density whole genome coverage (Figure 22a). The side-by-side comparison of the genetic map obtained from databases to that estimated with the observed data revealed a minor shift in marker positions, as seen by the extended chromosome lengths in Figure 22b. Overall, this shift is tolerable, as marker positions can vary to a certain extend depending on differing experimental factors or different databases used to define the location of marker. In Figure 22c, missing genotypes calculated from the input data are plotted. For each chromosome, individual mice are plotted vertically, the marker horizontally. Loss of genotype information in the present population lies within normal range. Nevertheless, the IMP algorithm was chosen for subsequent QTL mapping to substitute the missing data points (see 3.2.6 Detection and mapping of quantitative traits). As a last quality control tool, R/qtl provides the function of estimating recombination fractions, which gives information necessary for the construction of linkage maps. In Figure 22d, red-coloured pixels indicate a large LOD score or a small recombination fraction, while blue-coloured ones indicate the opposite.

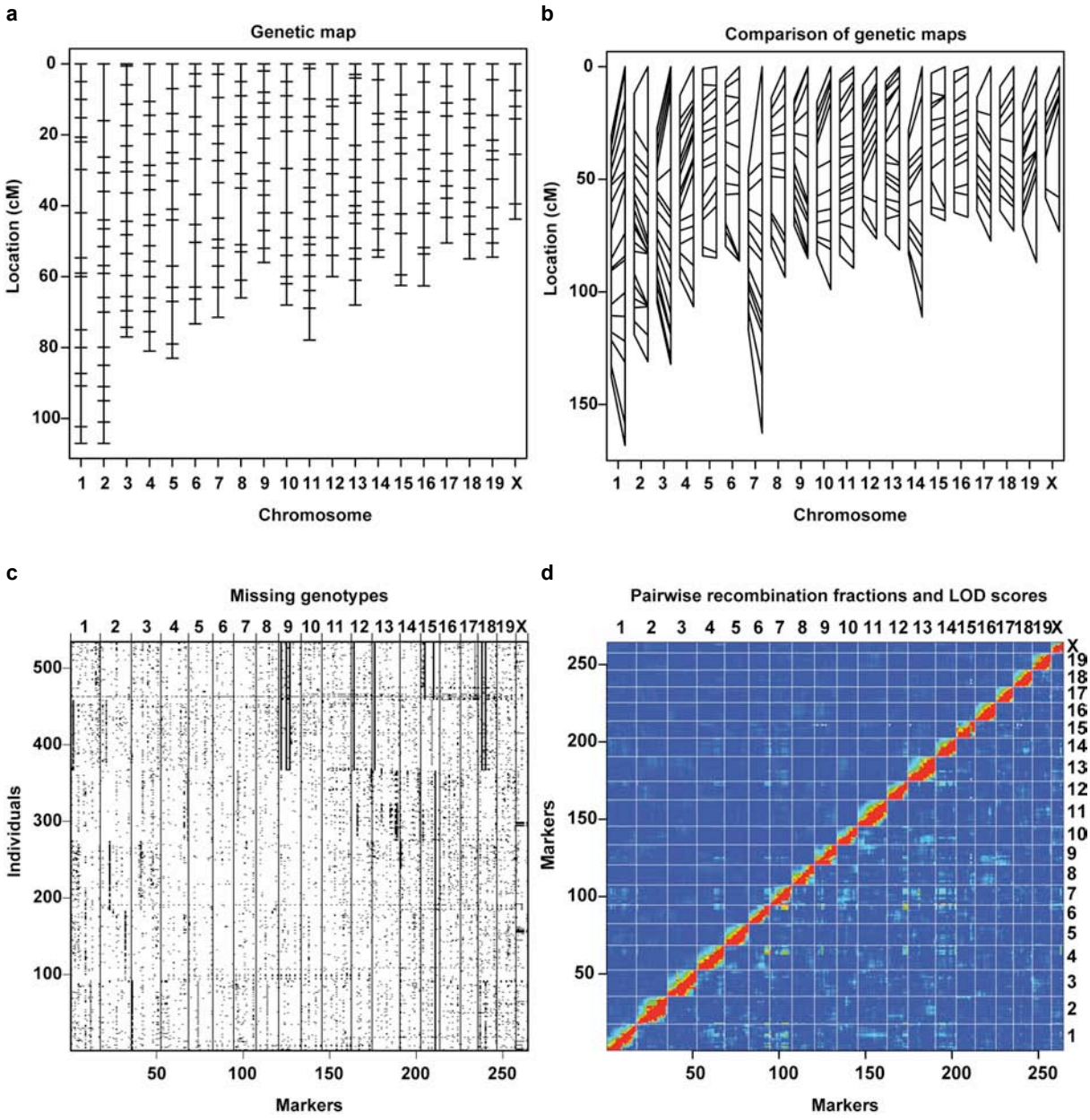


Figure 22. (a) Marker locations on the genetic map used in the present study. (b) Side-by-side comparison of the genetic map obtained from the MGI database (left) to that estimated with the observed data (right). (c) Pattern of missing genotype data in the cross. Black pixels indicate missing genotypes. (d) Estimated recombination fractions and LOD scores for all pairs of markers (The recombination fractions are in the upper left triangle; LOD scores are in the lower right triangle).

4.3.2 Mapping of quantitative traits

In the followings sections, the results calculated with the function *scanone* are described, which maps QTL acting individually. Subsequently, possible QTL interaction (either additively or epistatically) was calculated using the function *scantwo* of R/qtl.

4.3.2.1 Acute ethanol-induced hypothermia and tolerance

F2 animals received an i.p. injection of 2 g/kg ethanol to determine acute ethanol effects and ethanol tolerance. As shown in Figure 23a, administration of ethanol produced a reduction in body temperature that was similar in both gender ($F_{1,550}$ = 2.21, p = n.s.). The single-QTL genome scan revealed one locus associated with this hypothermic effect on chromosome 1 at 85 cM (Figure 23b). The 95 % confidence interval for this QTL covered the 81 to 87 cM region (Table 10). Additionally, the 10 cM position on chromosome 7 was associated with ethanol-induced hypothermia. In contrast to the parental strains, chronic exposure to an ethanol solution (16%) resulted in a reduced hypothermic effect of ethanol ($F_{1,550}$ = 116.19, p < 0.0001). This result demonstrated that mice from the F2 generation developed tolerance after four weeks of forced ethanol drinking that was present in both gender ($F_{1,550}$ = 1.17, p = n.s. for *drug experience x gender* interaction). After chronic ethanol exposure a completely different set of QTL was found on chromosomes 3, 6 and 13, with the highest LOD scores at 55 cM, 24.7 cM and 39 cM, respectively (Figure 23c).

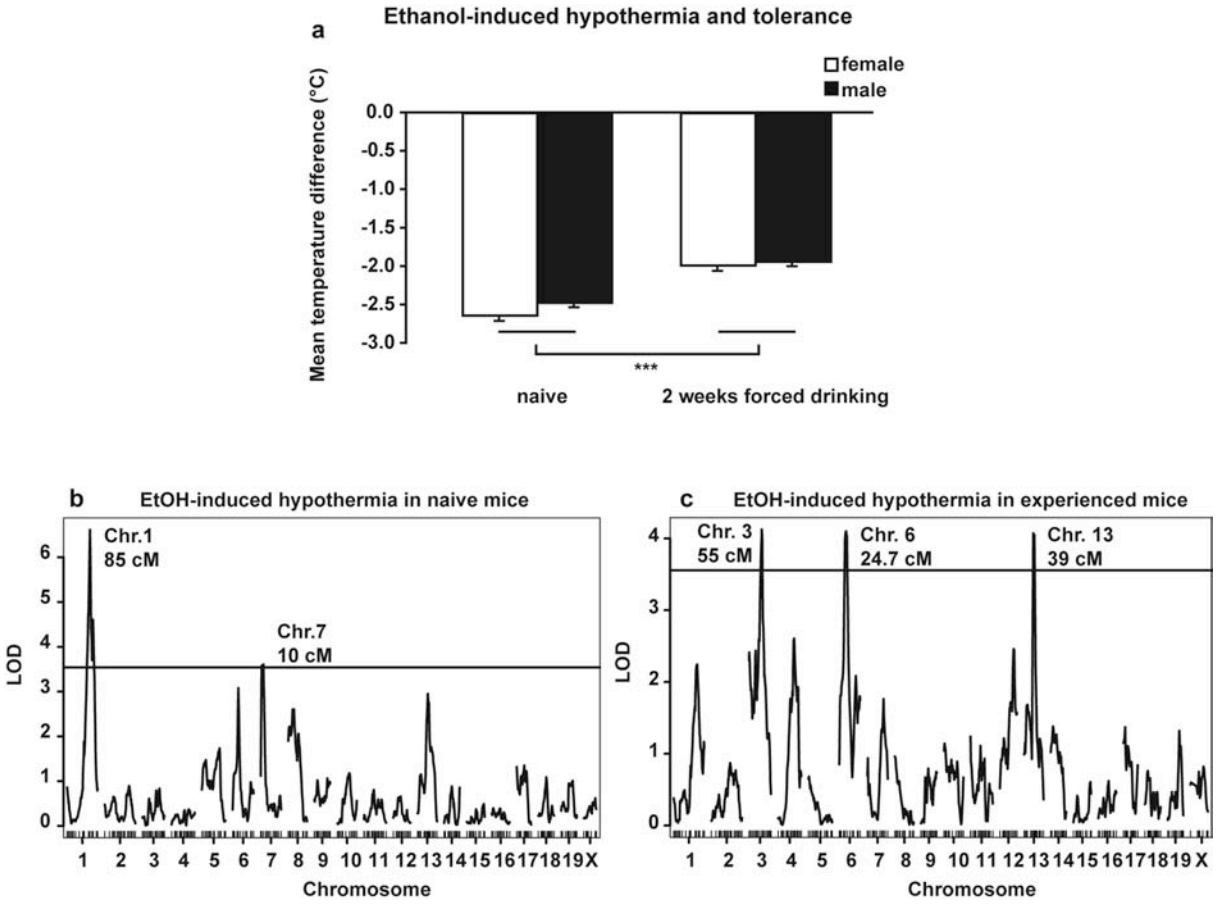


Figure 23. Ethanol-induced hypothermia in naïve and ethanol-experienced mice. (a) Two weeks of ethanol drinking caused the development of ethanol tolerance in both female and male F2 mice, as indicated by the significantly reduced hypothermia after the second injection of 2 g/kg ethanol. Females: n = 280, males: n = 272. Values represent mean ± SEM, ***p < 0.0001. (b) A single-QTL genome scan revealed loci associated with the hypothermic effect on chromosome 1 and 7. (c) After chronic ethanol exposure we found QTL on chromosomes 3, 6 and 13. The horizontal line represents the threshold for significant LOD scores (p < 0.05).

Possible interaction of chromosomal loci was analysed in a two-dimensional genome scan for the thermal response of the mice. To assess the nature of existing interaction, three different LOD scores are required. The LOD score for the full model indicates if there actually is any interaction, whereby the additive and the epistasis LOD score denote the type of the interaction - either additively or epistatically. In Figure 24, LOD scores for the full and either the additive model (a, b) or for epistasis (c, d) are plotted against each other for EtOH-induced hypothermia in naïve mice. There was evidence for interaction between loci on chromosome 1 and several loci on other chromosomes (Figure 24a), with highest LOD scores and significant genome-scan-adjusted p-values for an interaction with chromosomes 6, 7, and 8 (p < 0.05, Figure 24b). To evaluate the nature of interaction for EtOH-induced hypothermia in naïve mice, the epistasis LOD score has to be taken into account.

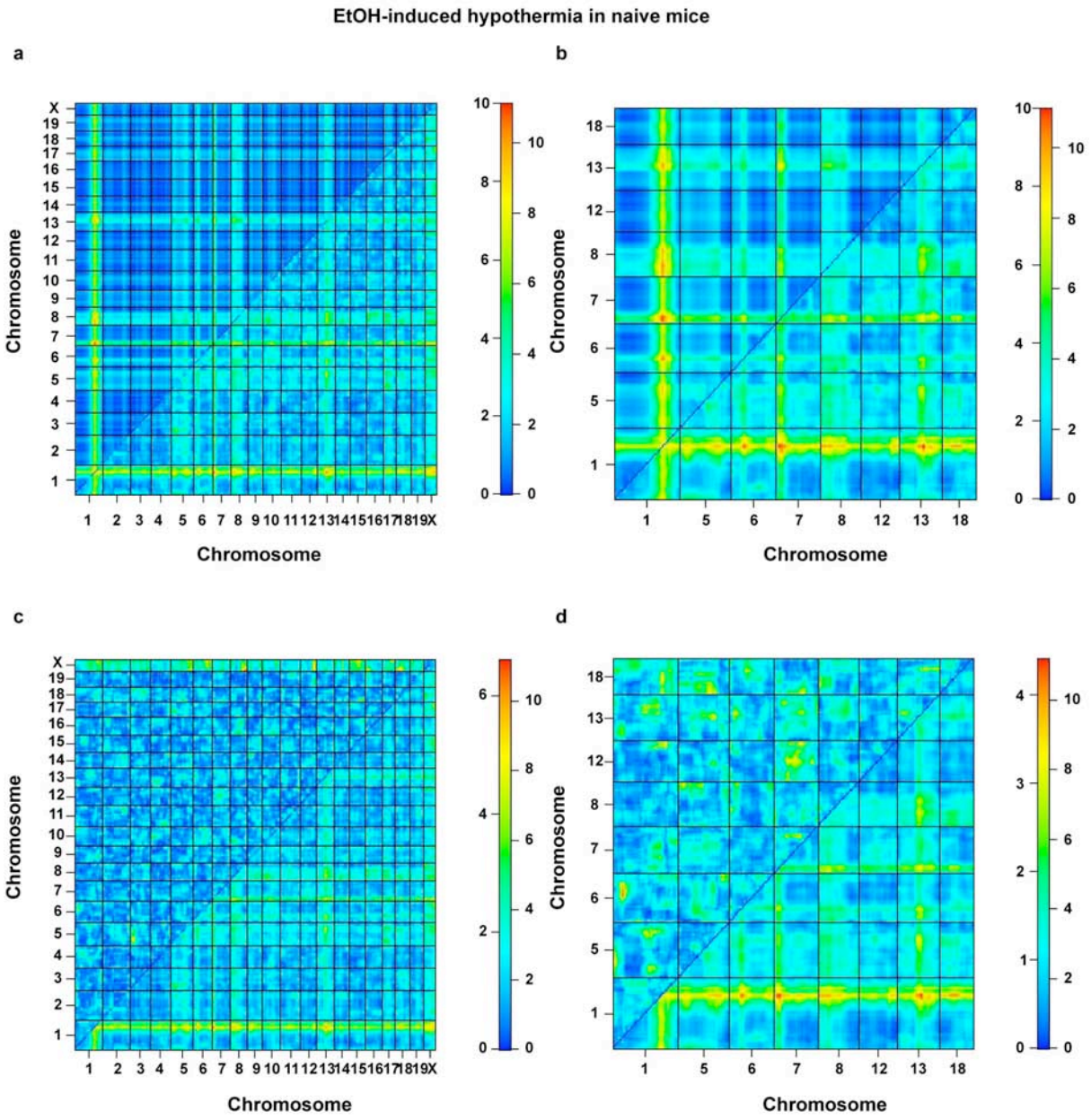


Figure 24. Results of the two-dimensional scan for hypothermia measurements in EtOH-naïve mice. In (a), LOD scores for a two-QTL model with interactions are plotted below the diagonal (full model), whereby LOD scores for the additive model are shown above the diagonal. In (c), the full model (below) is plotted against the model for epistasis (lod.int, above). Close-up of the two-dimensional scan on selected chromosomes for the full vs. either the additive (b) or the epistasis model (d). In the colour scale, the numbers to the right and left correspond to the values below and above the diagonal, respectively. 5% permutation derived threshold level based on 100 permutations. The tick marks correspond to the ends of the labelled chromosomes.

As shown in Table 7, the *scantwo* - analysis revealed no significant epistasis LOD score for this trait ($p > 1$), but p-values for the additive LOD score were found to be significant ($p < 0.01$). These results indicated that the chromosomal interaction is of additive nature.

Table 7. Two-dimensional genome scan for EtOH-induced hypothermia in naïve mice. LOD scores, chromosomal positions and genome-scan-adjusted p-values for the full and the additive model as well as for the epistasis LOD score.

Trait	Chr.1	Chr.2	Pos1 (full)	Pos2 (full)	lod.full	p (lod.full)	lod.int	p (lod.int)	Pos1 (add)	Pos2 (add)	lod.add	p (lod.add)
Ethanol-induced hypothermia in naive mice	1	6	85.0	20.68	10.98	0.02*	0.97	> 1	85	20.68	10.0	< 0.001*
	1	7	85.0	4.50	11.35	0.01*	1.40	> 1	85	6.5	9.95	< 0.001*
	1	8	85.0	8.0	10.39	0.03*	0.82	> 1	85	18.0	9.57	< 0.001*

Abbreviations: Chr.: Chromosome; Pos: chromosomal position in cM; p: p-value; lod.full/int/add = LOD score for the full, epistasis and additive model, respectively. * = Significant p-value.

For EtOH-induced hypothermia in experienced mice (Figure 25), the two-dimensional genome scan at first suggested a possible interaction between loci on chromosome 3 and 13 as well as between loci on chromosome 3 and 6, but after calculating 100 permutations this assumption failed, because the LOD score for the full model turned out to be not significant (genome-scan-adjusted p-value > 0.05).

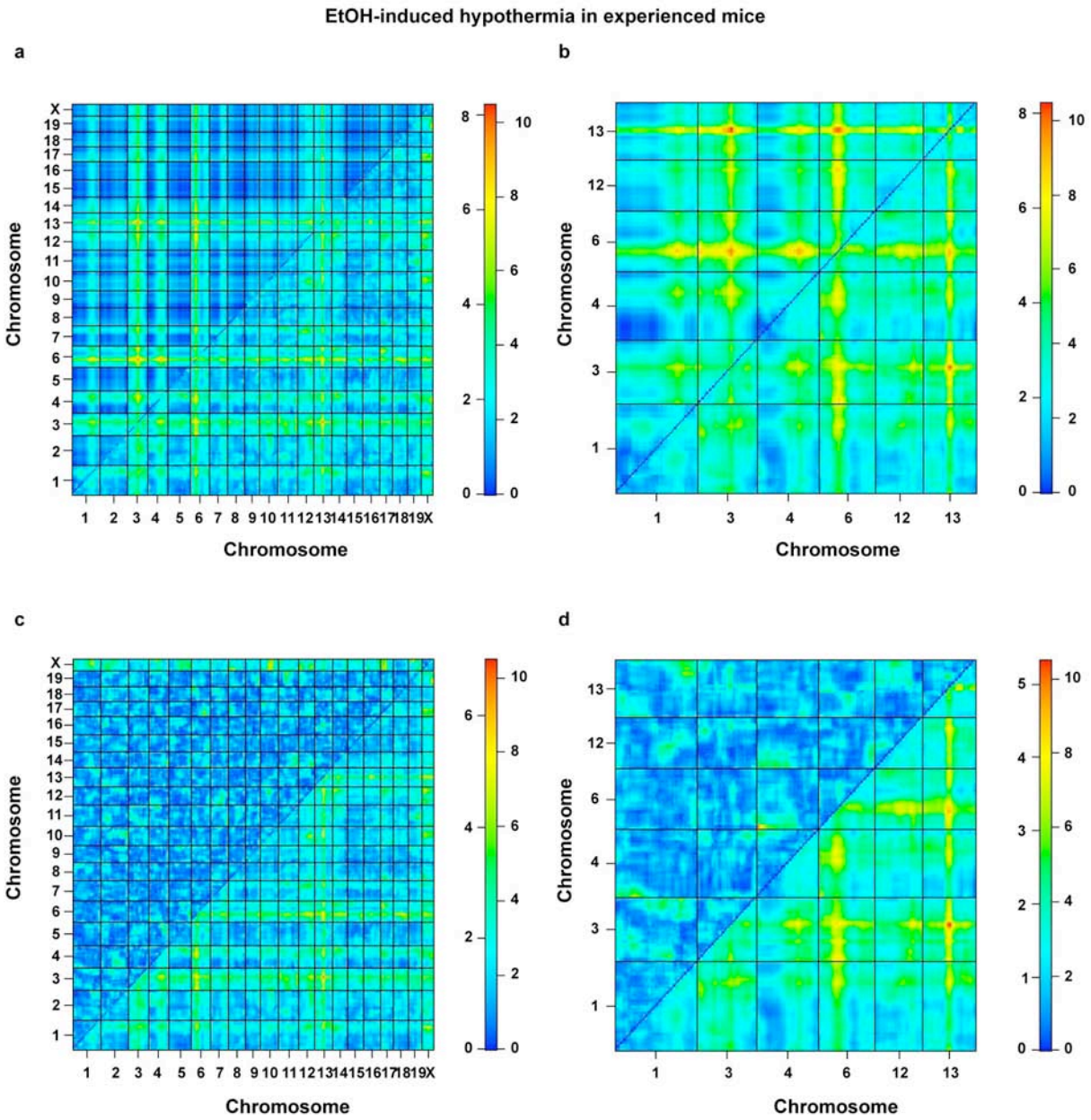


Figure 25. Results of the two-dimensional scan for hypothermia measurements in experienced mice. LOD scores for a two-QTL model with interactions are plotted below the diagonal (full model). In (a), the full model is plotted against the additive model, in (c) against the difference of variance explained by the full model and the additive model (lod.int, epistasis). Close-up of the two-dimensional scan on selected chromosomes for the full vs. either the additive (b) or the epistasis model (d). In the colour scale, the numbers to the right and left correspond to the values below and above the diagonal, respectively. 5% permutation derived threshold level based on 100 permutations. The tick marks correspond to the ends of the labelled chromosomes.

The significant p-value for the additive LOD score does not denote an additive interaction, because the p-value for the full model, which calculates general interaction effects between two positions, did not exceed the significance threshold (Table 8).

Table 8. Two-dimensional genome scan for EtOH-induced hypothermia in experienced mice. LOD scores, chromosomal positions and genome-scan-adjusted p-values for the full and the additive model as well as for the epistasis LOD score.

Trait	Chr.1	Chr.2	Pos1 (full)	Pos2 (full)	lod.full	p (lod.full)	lod.int	p (lod.int)	Pos1 (add)	Pos2 (add)	lod.add	p (lod.add)
Ethanol-induced hypothermia in experienced mice	3	13	54.6	43.0	10.26	0.08	2.38	> 1	50.6	41.0	7.88	0.03*
	3	6	56.6	22.7	8.71	0.40	1.14	> 1	54.6	22.7	7.56	0.01*
	6	13	22.7	39.0	9.32	0.26	1.16	> 1	22.7	39.0	8.16	< 0.01*

Abbreviations: Chr.: Chromosome; Pos: chromosomal position in cM; p: p-value; lod.full/int/add = LOD score for the full, epistasis and additive model, respectively. * = Significant p-value.

4.3.2.2 Ethanol withdrawal

After ethanol removal, F2 mice exhibited significantly more tremors or forelimb extensor spasm during lifting and turning and thus received significantly higher withdrawal scores (Kruskal-Wallis ANOVA, $p < 0.001$; Table 9).

Table 9. Somatic ethanol withdrawal symptoms. Frequencies of high handling-induced convulsion (HIC score) were significantly increased 3 hours after ethanol withdrawal. Females: n = 278; Males: n = 274.

		HIC score frequencies (%)				
		0	1	2	3	4
Female	EtOH	41.1	37.3	18.6	3.0	0
	Withdrawal	10.5	25.4	34.3	24.6	5.2
Male	EtOH	39.1	41.4	17.6	2.0	0
	Withdrawal	10.1	25.9	27.5	25.9	10.5

The single-QTL genome scan detected no significant QTL regions for the handling-induced convulsions as determined before ethanol withdrawal or after withdrawal (Figure 26a and b).

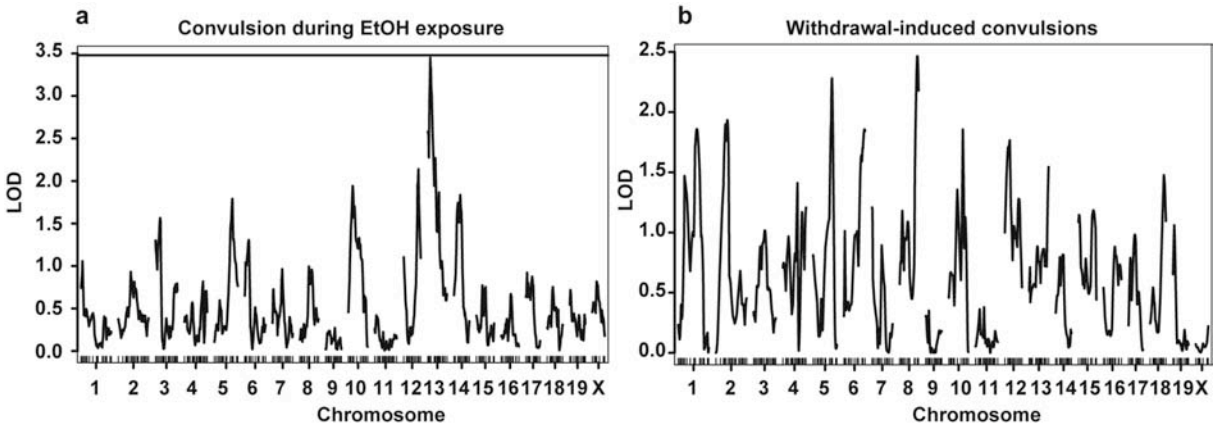


Figure 26. Somatic ethanol withdrawal symptoms. During ethanol exposure (a) and after ethanol withdrawal (b) no significant single-QTL were identified. The horizontal line represents the threshold for significant LOD scores ($p < 0.05$).

Ethanol withdrawal also caused a decrease in locomotor activity in the open field arena ($F_{1,546} = 16.26$, $p < 0.0001$) in male ($F_{1,546} = 5.27$, $p < 0.05$), but not in female mice (Figure 27a). A highly significant QTL for the trait *locomotion* was found on chromosome 1 with a peak LOD score at 65 cM (Figure 27b). After ethanol withdrawal, the peak at 65 cM was also detected on chromosome 1. Additional QTL were identified on chromosome 7 (50 cM) and 11 (43.1 cM) after withdrawal (Figure 27c). Due to the significant gender interaction, a QTL analysis was performed separately for each gender. The QTL found on chromosome 1 in the combined analysis was also seen in female and male mice before and after ethanol removal. For withdrawal-associated locomotion, a significant locus on chromosome 2 (1.4 cM) in male mice was detected. In females, a region on chromosome 11 (44.8 cM) was found to be significantly associated with this trait (Table 10). The QTL on chromosome 7 was no longer observed in the gender-specific analysis.

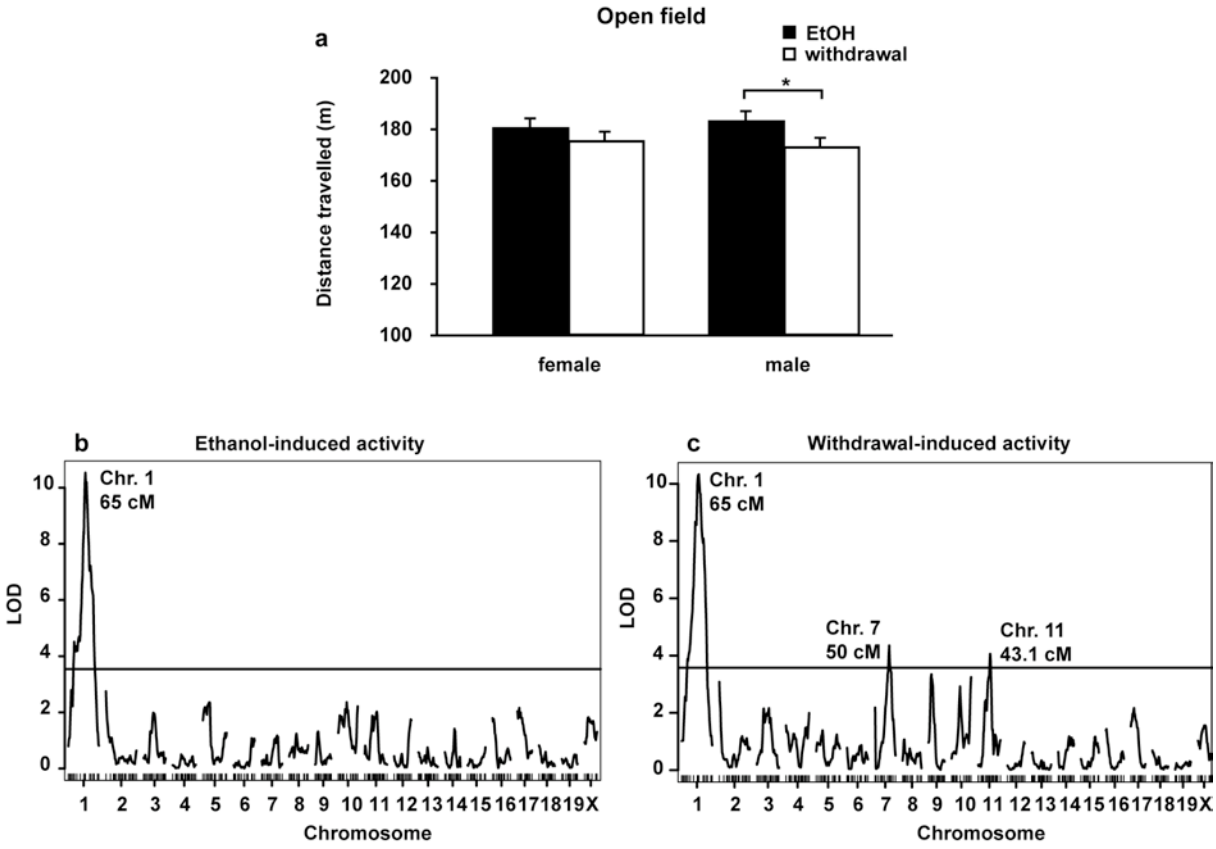


Figure 27. Open field. (a) Distance travelled in the open field arena was significantly reduced after ethanol removal in male animals. Females: n = 275, males: n = 273. Values represent mean ± SEM, *p < 0.05. (b) Under alcohol-drinking conditions, a highly significant QTL was found on chromosome 1 at 65 cM. (c) After ethanol withdrawal, QTL were identified on chromosome 1 (65 cM), 7 (50. cM) and 11 (43.1 cM). The horizontal line represents the threshold for significant LOD scores ($p < 0.05$).

Ethanol withdrawal induced significantly more anxiety behaviour in the zero maze as displayed by a reduced activity in the open area of the maze ($F_{1,545} = 513.49$, $p < 0.0001$). This effect was observed in both gender ($F_{1,545} = 0.05$, p = n.s.; Figure 28a). Two positions on chromosome 5 were significantly associated with zero maze behaviour, whereas the more proximal section of the chromosome (42 cM) was related to the activity during ethanol exposure, the distal region (59 cM) to withdrawal-related zero maze behaviour. Furthermore, a QTL on chromosome 12 at 18 cM was found for ethanol-induced anxiety and at 21 cM for anxiety behaviour during withdrawal. Only after ethanol withdrawal a QTL on chromosome 1 was identified, with a peak correlated region at 79 cM (Figure 28b and c).

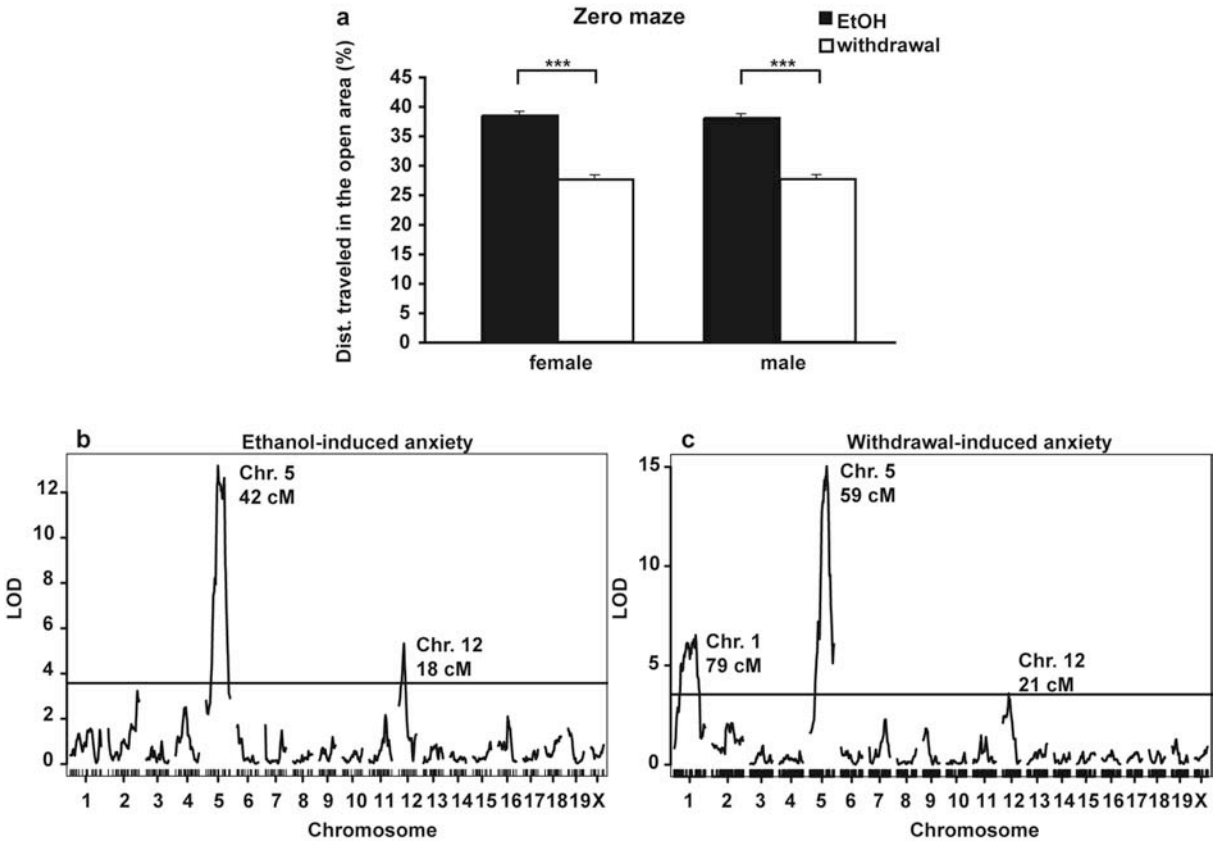


Figure 28. Zero maze. (a) After ethanol withdrawal, mice covered a significantly shorter distance in the open area of the maze, indicative of elevated levels of anxiety. Females: n = 277, males: n = 270. Values represent mean ± SEM, ***p < 0.0005. (b) Two loci were significantly associated with ethanol-induced anxiety, one on chromosome 5 (42 cM) the other on chromosome 12 (18 cM). (c) Under alcohol-free conditions a QTL on chromosome 1 (79 cM) and again a significant loci on chromosome 5 (59 cM) and 12 (21) were found. The horizontal line represents the threshold for significant LOD scores (p < 0.05).

4.3.2.3 Ethanol preference and consumption

F2 animals displayed an overall lower preference for the ethanol solution than the parental strains (Figure 29a). In the two-bottle choice test, the preference values showed a slight but significant reduction during the four-week experiment ($F_{3,1590}$ = 3.02, p < 0.05) in both gender ($F_{1,530}$ = 1.16, p = n.s.). Measurement of ethanol consumption also revealed a significant decrease in consumption from week 1 to 4, which then slightly increased after the fourth week ($F_{3,1605}$ = 7.78, p < 0.0001). Again no gender difference was found ($F_{1,535}$ = 0.05, p = n.s.; Figure 29b).

The QTL genome scan revealed a locus on chromosome 16 linked with the mean preference ratio (31.4 cM) and the mean ethanol consumption (19.4 cM), calculated as the mean values over the four-week experiment (Figure 29c and d).

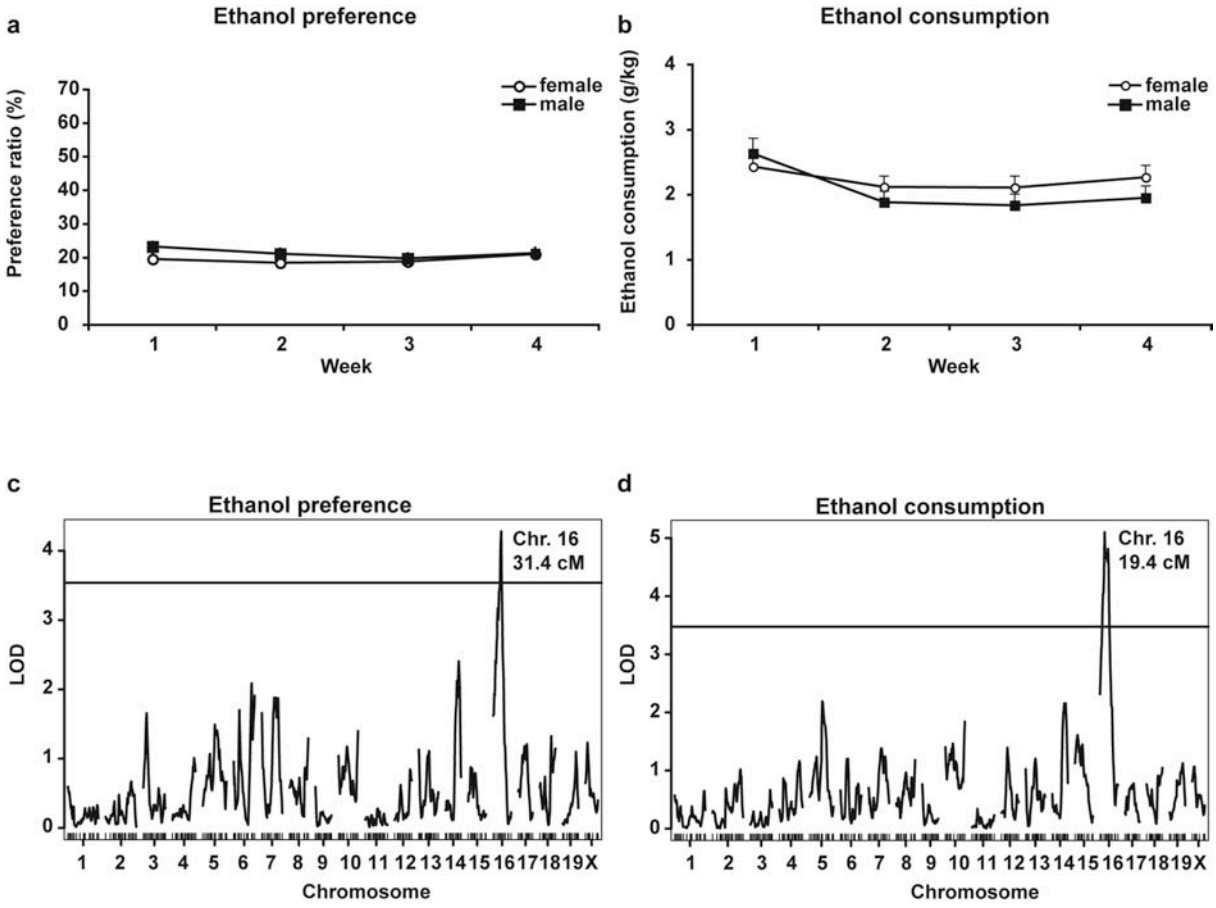


Figure 29. Ethanol preference and consumption. Mice of both gender showed a similar ethanol preference (a) and consumption (b) over four weeks in the two-bottle choice test. Females: n = 267, males: n = 265. QTL genome scans revealed loci on chromosome 16 linked with the mean preference ratio at 31.4 cM (c) and the mean ethanol consumption at 19.4 cM (d). The horizontal line represents the threshold for significant LOD scores ($p < 0.05$).

4.3.2.4 Stress-induced ethanol drinking

Stress produced by a mild foot-shock resulted in an increased ethanol preference ($F_{1,490} = 14.32$, $p < 0.001$). Again this increase was similar in male and female F2 mice ($F_{1,490} = 0.93$, p = n.s) (Figure 30a). Before exposure to the foot-shock stress, the single-QTL genome scan revealed one region on chromosome 16 above the significance threshold (33 cM) (Figure 30c). After the stressor, however, no significant QTL was found for this trait.

Ethanol consumption was also increased after foot-shock stress ($F_{1,491} = 6.28$, $p < 0.05$) in female mice, while males consumed similar amounts of ethanol (g/kg) before and after the foot-shock ($F_{1,491} = 10.37$, $p < 0.01$) (Figure 30b). A QTL for ethanol consumption before exposure to a foot-shock stress was mapped on chromosome 16 at 29.4 cM (Figure 30d). After the shock, no significant association was found (Figure 30e, f). In a subsequent gender specific QTL scan we could not identify any significant QTL for the phenotype or the gender effect.

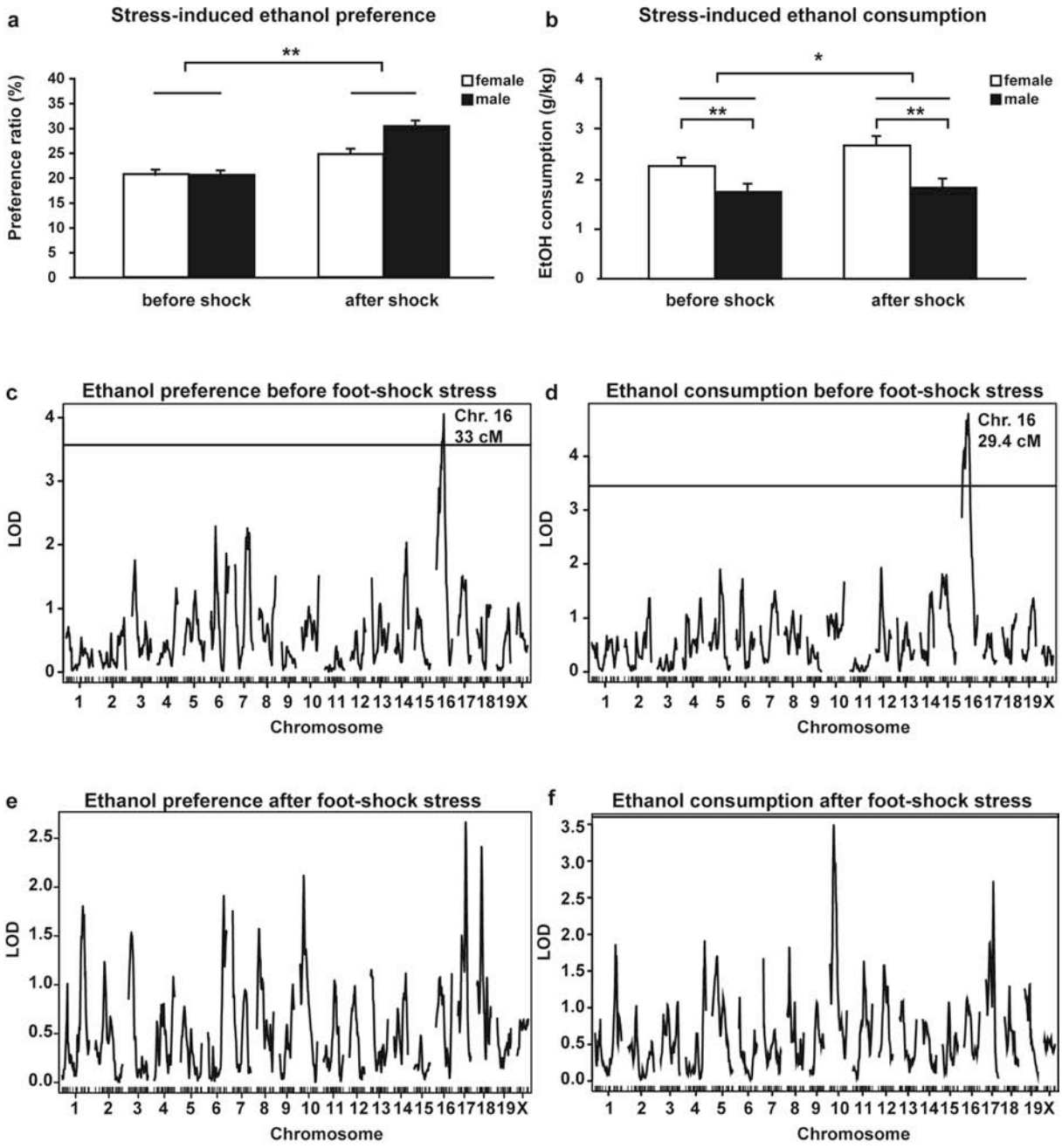


Figure 30. Stress-induced ethanol drinking. Mice of both gender exhibited a higher ethanol preference (a) and consumption (b) after the foot-shock. In contrast to their ethanol preference, female mice consumed significantly more ethanol (g/kg) before and after foot-shock compared to males. Females: n = 245, males: n = 247. Values represent mean ± SEM. *p < 0.05; **p < 0.001. (c, d) Before the foot-shock stress, QTL genome scans revealed loci on chromosome 16 for both traits (33 and 29.4 cM). (e, f) After the shock, no significant association was found. The horizontal line represents the threshold for significant LOD scores (p < 0.05).

Table 10a. Position, p-values and LOD scores of all significant QTL revealed via single-QTL analysis combined for both gender.

Trait	All (n = 534)			95 % CI (cM)
	Chr.	Location (cM)	p-value (LOD score)	
EtOH-induced hypothermia	1	85	< 0.001 (6.6)	81 - 87
	7	10	0.04 (3.6)	3.4 - 10.5
EtOH-induced hypothermia in experienced mice	3	55	0.013 (4.1)	11.2 - 62.6
	6	24.7	0.014 (4.1)	18.7 - 30.7
	13	39	0.014 (4.1)	39 - 45
EtOH -induced activity	1	65	< 0.001 (10.5)	63 - 71
Withdrawal-induced activity	1	65	< 0.001 (10.3)	59 - 71
	7	50	0.007 (4.4)	42.5 - 57.5
	11	43.1	0.02 (4.1)	33 - 47.1
Anxiety during EtOH exposure	5	42	< 0.001 (13.2)	39 - 64
	12	18	< 0.001 (5.3)	13 - 22
Withdrawal-induced anxiety	1	79	< 0.001 (6.5)	39 - 83
	5	59	< 0.001 (15.0)	49 - 63
	12	21	0.04 (3.6)	3 - 32
Mean EtOH pref. (TBC)	16	31.4	0.009 (4.3)	17.4 - 35.4
Mean EtOH cons. (TBC)	16	19.4	0.002 (5.1)	15.4 - 33.4
EtOH pref. before shock	16	33	0.02 (4.1)	13.4 - 36.5
EtOH cons. before shock	16	29.4	0.002 (4.8)	7.4 - 35.4
Mean EtOH cons. (FD)	1	109	0.02 (3.9)	97 - 112
	2	102	0.01 (4.3)	80.9 - 108
	5	29	0.03 (3.9)	20 - 43
	10	2	0.003 (5.0)	2 - 21
	15	49	0.001 (5.2)	6.7 - 56.7

Table 10b. Position, p-values and LOD scores of all significant QTL revealed via single-QTL analysis for female mice.

Trait	Female (n = 271)			95 % CI (cM)
	Chr.	Location (cM)	p-value (LOD score)	
EtOH-induced hypothermia in experienced mice	6	26.7	0.014 (4.1)	20.5 - 74
EtOH -induced activity	1	67	< 0.001 (7.0)	63 - 71
Withdrawal-induced activity	1	51	0.001 (5.3)	29 - 71
	11	44.8	0.002 (5.0)	35.1 - 45.1
EtOH -induced anxiety	5	64	< 0.001 (6.3)	42 - 71
Withdrawal-induced anxiety	1	64	0.015 (4.1)	41 - 80
	5	51	< 0.001 (5.6)	45 - 67
Mean EtOH cons. (FD)	2	92	0.003 (4.9)	79 - 99
	10	2	0.048 (3.6)	2 - 66
	15	32.7	0.028 (3.8)	6.7 - 52.7
	2	92	0.003 (4.9)	79 - 99

Table 10c. Position, p-values and LOD scores of all significant QTL revealed via single-QTL analysis for male mice.

	Male (n = 263)			95 % CI (cM)
	Chr.	Location (cM)	p-value (LOD score)	
EtOH-induced hypothermia	1	85	0.012 (4.2)	81 - 99
EtOH-induced hypothermia in experienced mice	1	85	0.04 (3.6)	75 - 91
EtOH -induced activity	1	69	0.023 (3.9)	25.7 - 95.8
Withdrawal-induced activity	1	69	< 0.001 (5.9)	57 - 79
	2	1.4	0.011 (4.2)	1 - 5
EtOH -induced anxiety	5	43	< 0.001 (8.9)	41 - 51
Withdrawal-induced anxiety	5	57	< 0.001 (10.3)	45 - 64
	12	13	0.023 (3.9)	1 - 23

Chr: chromosome number. 95% CI: the R/qtl 95% confidence interval for the QTL (in cM). A p-value < 0.05 represents significant effects.

4.3.2.5 Summary of the two-dimensional-QTL analysis

As described in detail for the *hypothermia* trait (see 4.3.2.1), a two-dimensional genome scan was also performed for all other alcohol-related traits using the function *scantwo* of R/qtl. Table 11 summarizes the results obtained from these analyses, presenting the significant interaction LOD scores and p-values for the respective phenotype.

R/qtl did not calculate significant p-values for the *ethanol-induced activity* phenotype as well as for two parameters measured during the two-bottle choice procedure (mean ethanol preference, ethanol preference before shock; Table 11). For *ethanol consumption after shock*, the genome-scan-adjusted p-value was 0.06 and thus not significant. Nonetheless, the LOD score for the additive model reached the 5% permutation derived threshold, indicating that there likely exists additive interaction between two respective QTL. No epistatic interactions were detected for the primary loci with significant linkages, but we found chromosomal loci that might contain genes encoding for products that make additive contributions to the respective phenotype.

Table 11. Position, p-values and LOD scores of QTL revealed via two-dimensional QTL analysis combined for both gender.

Trait	Chr.1	Chr.2	Pos1 (full)	Pos2 (full)	lod.full	p (lod.full)	lod.int	p (lod.int)	Pos1 (add)	Pos2 (add)	lod.add	p (lod.add)
Ethanol-induced activity	1	1	63.0	93.0	13.96 #	n.a.	1.87	n.a.	63.0	99.0	12.1 #	n.a.
	1	2	63.0	103.0	14.21 #	n.a.	1.52	n.a.	63.0	1.0	12.69 #	n.a.
	1	3	63.0	70.6	12.87 #	n.a.	0.70	n.a.	63.0	46.6	12.17 #	n.a.
	1	4	63.0	64.0	13.66 #	n.a.	2.26	n.a.	63.0	32.0	11.4 #	n.a.
	1	5	63.0	23.0	14.48 #	n.a.	1.04	n.a.	63.0	23.0	13.44 #	n.a.
	1	6	63.0	46.68	13.59 #	n.a.	2.05	n.a.	63.0	62.68	11.54 #	n.a.
	1	7	63.0	52.5	14.18 #	n.a.	2.66	n.a.	63.0	8.5	11.52 #	n.a.
	1	8	63.0	50.0	16.40 #	n.a.	4.10	n.a.	63.0	32.0	12.31 #	n.a.
	1	9	63.0	69.0	12.68 #	n.a.	0.96	n.a.	63.0	23.0	11.72 #	n.a.
	1	10	63.0	70.0	14.27 #	n.a.	1.28	n.a.	63.0	30.0	12.99 #	n.a.
	1	11	63.0	45.1	14.29 #	n.a.	2.40	n.a.	63.0	45.1	11.9 #	n.a.
	1	12	63.0	3.0	13.05 #	n.a.	0.85	n.a.	63.0	61.0	12.2 #	n.a.
	1	13	63.0	41.0	13.60 #	n.a.	1.87	n.a.	63.0	59.0	11.73 #	n.a.
	1	14	63.0	35.5	14.16 #	n.a.	2.10	n.a.	63.0	39.5	12.06 #	n.a.
	1	15	63.0	68.7	12.27 #	n.a.	0.83	n.a.	63.0	68.7	11.45 #	n.a.
	1	16	63.0	7.4	13.63 #	n.a.	1.13	n.a.	63.0	5.4	12.5 #	n.a.
	1	17	63.0	10.1	14.00 #	n.a.	1.27	n.a.	63.0	8.1	12.73 #	n.a.
	1	18	63.0	56.0	12.29 #	n.a.	0.87	n.a.	63.0	2.0	11.42 #	n.a.
	1	19	63.0	48.5	12.44 #	n.a.	1.08	n.a.	63.0	50.5	11.36 #	n.a.
	1	X	63.0	57.5	14.98 #	n.a.	2.29	n.a.	63.0	55.5	12.69 #	n.a.
Withdrawal-induced activity	1	7	63.0	50.5	13.96 #	< 0.001*	0.18	> 1	63.0	50.5	13.77 #	< 0.001*
	1	9	63.0	25.0	14.41 #	< 0.001*	0.84	> 1	63.0	23.0	13.57 #	< 0.001*
Anxiety during ethanol exposure	2	5	103.0	61	18.24 #	< 0.001*	1,29	> 1	103.0	61.0	16.96 #	< 0.001*
	5	12	53.0	19	19.64 #	< 0.001*	1,15	> 1	53.0	19.0	18.5 #	< 0.001*
Withdrawal-induced anxiety	1	5	53.0	57.0	27.53 #	< 0.001*	2.47	> 1	59.0	57.0	25.06 #	< 0.001*
	1	12	61.0	3.0	11.98 #	< 0.001*	2.17	> 1	69.0	21.0	9.8 #	< 0.001*
	5	12	51.0	27.0	20.05 #	< 0.001*	1.61	> 1	55.0	23.0	18.44 #	< 0.001*
Two-Bottle Choice												
Mean ethanol preference (%)	14	16	51.5	31.4	12.28 #	n.a.	5.26	n.a.	51.5	29.4	7.02 #	n.a.
Ethanol preference before shock (%)	6	16	32.68	31.4	10.43 #	n.a.	3.43	n.a.	22.68	31.4	7.0 #	n.a.
	14	16	51.5	33.4	10.44 #	n.a.	3.8	n.a.	51.5	31.4	6.64 #	n.a.
Ethanol consumption after shock (g/kg)	19	19	22.5	24.5	68.23	0.06	17.01	0.9	22.5	24.5	51.22 #	< 0.001*
Forced drinking												
Mean ethanol consumption (g/kg)	1	15	103.0	52.7	10.26 #	0.01*	1.38	> 1	107.0	44.7	8.88 #	< 0.001*
	2	10	101.0	2.0	10.15 #	0.01*	0.48	> 1	101.0	2.0	9.67 #	< 0.001*
	2	15	103.0	44.7	10.07 #	0.02*	0.60	> 1	101.0	44.7	9.46 #	< 0.001*
	5	15	29.0	50.7	11.57 #	< 0.001*	1.68	> 1	29.0	44.7	9.89 #	< 0.001*
	10	15	14.0	48.7	10.62 #	< 0.001*	0.51	> 1	2.0	48.7	10.11 #	< 0.001*

Abbreviations: Chr.: Chromosome; Pos: chromosomal position in cM; p: p-value; n.a.: not assessed; lod.full/int/add = LOD score for the full, epistasis and additive model, respectively. * = Significant p-value, # = Significant LOD score.

Noticeable are the results for the ethanol-induced activity phenotype, because there is strong evidence for an additive interaction of chromosome 1 with all other chromosomes. In the single-QTL analysis, only the QTL on chromosome 1 was detected, whereby no other chromosomal region reached significance. Overall, the two-dimensional genome scan revealed additive interaction between QTL that also act individually on the trait, but running the *scantwo* function also exposed interacting QTL that were not revealed by the single-QTL analysis. Furthermore, not all individually acting chromosomal regions were found to interact with each other in the expression of the respective trait.

5 DISCUSSION

Many studies have shown that virtually all drug responses are under genetic control (CRABBE *et al.* 2006b), but the complex genetic nature of drug addiction has made it difficult to identify specific susceptibility genes. Here a consecutive analysis of behavioural and physiological responses to ethanol in combination with QTL mapping was used to investigate the complex genetic regulation of behaviours relevant to ethanol addiction in a mouse intercross population. Several genetic loci contributing to physiological and behavioural responses to ethanol were identified, including several novel chromosomal regions where no obvious candidate genes have yet been mapped. Some of these loci are specifically associated with responses in ethanol-experienced animals. Additionally, several QTL that have already been published were confirmed by the findings of the present study, thus validating the approach and supporting the significance of these loci on the development and manifestation of alcoholism.

The parental and the F1 generations of mice were analyzed in a non-serial design, which means that unrelated behaviours were assessed in different groups of animals. These data showed that the parental strains differed significantly for most phenotypes and that F1 mice generally display intermediate phenotypes.

5.1 Alcohol effects on body temperature regulation

Acute alcohol intake exerts influence on several physiological responses, one of which is body temperature alteration. Being out in the cold, we tend to believe a "little sip" will help take away the chill - and it seems to work. The face flushes and the skin becomes warm, regardless of the temperature outside. However, these misleading signs of warmth hide the facts about drinking alcohol. While alcohol may make us feel hotter, it actually causes a decrease in core body temperature. In animal studies, low doses of alcohol have been demonstrated to increase release of nitric oxide and augment endothelium-mediated vasodilation (DENG and DEITRICH 2007). This state of an increased calibre of the vessels (vasodilatation) reflects an increase in surface area entailing temperature loss and increased blood flow (PUDDEY *et al.* 2001). Thus, the increasing blood flow into the skin, giving that warm feeling and making the face flush, induces a rapid decrease in body temperature, which in turn brings about an increased danger of hypothermia. Higher acute doses impair endothelium-dependent relaxation responses (MAYHAN and DIDION 1995). In contrast, chronic administration of alcohol to rats has generally been associated with tolerance to the

acute inhibitory effects of high-dosage alcohol on endothelium-mediated vasodilatation and may even result in augmentation of such responses (PUDDEY *et al.* 2001; SUN *et al.* 2008; WILLIAMS *et al.* 1990).

Tolerance to the hypothermic effects induced by acute alcohol treatment (BROWMAN *et al.* 2000; COLLINS *et al.* 1996; CRABBE 1994; CRABBE and BELKNAP 1980; CRABBE *et al.* 1982; RACZ *et al.* 2003) is partly due to enhanced metabolism and reduced CNS sensitivity (CHANDLER *et al.* 1998; COLLINS *et al.* 1996; DENG and DEITRICH 2007). Measurements of alterations in body temperature changes after chronic ethanol consumption therefore provide a useful tool to determine tolerance effects as an adaptive physiological response to prolonged alcohol exposure.

In mice, administration of 2-4 g/kg ethanol at ambient temperatures results in a profound hypothermia in some mouse strains. Data of the present study revealed a dose-dependent reduction in body temperature with a maximum effect of approximately 5°C in C57BL/6J mice, compared to 3°C in C3H/HeJ mice, which is in good agreement with similar results from previous studies (BECKER and LOPEZ 2004; CRABBE *et al.* 2006a) and thus reflects the genetic contribution to this effect. Furthermore, these differences in body temperature changes demonstrate the phenotypic variation in ethanol tolerance within the two parental strains, on which this mapping study is based. Most mice develop tolerance to the hypothermic effects of ethanol after two weeks of forced ethanol drinking (CRABBE *et al.* 2006a; CRABBE *et al.* 2006b). This was also the case in the F2 population mice, although some animals also exhibited sensitization to the hypothermic effects of ethanol. This demonstrates that opposite adaptive responses to chronic alcohol exposure can occur within the genetic spectrum represented in the intercross used here.

To determine QTL acting on ethanol-induced hypothermia before and after chronic ethanol drinking, data obtained from the pheno- and genotyping of the heterogeneous F2 generation mice were statistically linked using R/qtl. The locus with the highest linkage for acute responses was mapped to chromosome 1 at 85 cM. This finding is consistent with the Htas1 (hypothermia due to alcohol sensitivity) QTL (CRABBE *et al.* 1994b) that was linked to ethanol hypothermia after an i.p. injection of 3 g/kg ethanol (20% v/v in saline). This QTL contains the Prdx6 (peroxiredoxin 6) locus, which is associated with ethanol acceptance drinking and with amphetamine hypothermia (GOLDMAN *et al.* 1987), as well as with the severity of withdrawal from nitrous oxide (BELKNAP *et al.* 1993). Another potential candidate gene in this region is the TBX19 (T-box transcription factor, 86.6 cM), which regulates the expression of the proopiomelanocortin (POMC) gene and the terminal differentiation of the pituitary corticotroph lineage (PULICHINO *et al.* 2003). A recent genetic study showed that POMC is involved in ethanol addiction using knockout mice and human case-control

samples. Thus, ß-endorphin, which is derived from the POMC precursor protein, modulates ethanol consumption and dependence in animals and humans (RACZ *et al.* 2008).

The other region that was linked to ethanol-induced hypothermia is located on chromosome 7 (10 cM). For this chromosome, only a QTL for ethanol preference is known (Ap7q at 25 cM). To date, no other alcoholism-related QTL within the confidence interval found here was mapped in mice so far.

Overall, prolonged ethanol exposure in the forced drinking paradigm led to a reduced hypothermia. But in contrast to the mean values, the individual data of the F2 mice showed large variations. Some of the animals even exhibited a sensitization to ethanol, indicated by an increased hypothermia after chronic ethanol drinking. The distribution of individual values demonstrates the presence of informative outliers in the heterogeneous F2 population. For the hypothermic response QTL on chromosomes 3, 6 and 13 were found. On chromosomes 6 and 13, two more Htas QTL were already described (Htas6 on chromosome 6 at 60 cM, Htas5 on chromosome 13 at 63 cM) (CRABBE *et al.* 1994b) and two interesting genes were linked to ethanol-related behaviours within these regions. One is the syntaxin binding protein 3A gene (Stxbp3a) mapped to chromosome 3 F3 in the mouse genome. A variant of this gene, Stxbp1, is located on mouse chromosome 2 and was already identified as a candidate locus for ethanol preference (FEHR *et al.* 2005). Stxbp1 and Stxbp3a have syntaxin binding activity and are involved in glucose and protein transport. Both proteins have a Sec1-like protein domain, thus implying that the protein is involved in vesicle docking during exocytosis (MGI; http://www.informatics.jax.org/). Another interesting candidate gene is located at 41 cM within the QTL confidence interval on chromosome 13 - the DAT1 gene encoding for the dopamine transporter 1. Dopamine is one of the crucial neurotransmitter involved in drug reward. It was shown that administration of methamphetamine inhibits DAT transporter function and binding of DAT ligands, and that it produces a hyperthermic response (SANDOVAL *et al.* 2000). Furthermore, DAT expression influences thermal responses to psychostimulant drugs (JANOWSKY *et al.* 2001). After administration of ethanol, dopamine uptake by DAT in *Xenopus* oocytes is potentiated and transporter binding-sites are increased (RIHERD *et al.* 2008). The inclusion of the DAT1 gene within the QTL could point to the influence of dopamine in the development of tolerance after forced ethanol drinking and to a possible role of the dopamine transporter in the regulation of ethanol-induced hypothermia.

Interestingly, the QTL on chromosome 1 and 7 did not influence hyperthermia after chronic ethanol exposure. Thus the acute ethanol effects on body temperature and the adaptive mechanisms after chronic ethanol exposure are regulated by different sets of genes.

The results obtained from the single-QTL analysis discussed above demonstrate the complex regulation of even an individual trait related to alcoholism. The two-dimensional genome scan conducted for the hypothermia phenotype revealed no epistatic interaction of the QTL described. Nevertheless, additive interactions of some of the QTL found for ethanol-induced hypothermia in naïve mice demonstrate that three or more genes contribute to the trait in an additive or cumulative manner. The uncovering of genes that are involved in this interaction and their role in the genetic regulation of ethanol-induced hypothermia or the development of tolerance will be a task for future studies.

5.2 Alcohol and anxiety

Already 2000 years ago Hippocrates recommended drinking a mixture of wine and water as a remedy for anxiety (cited according to BAVING and OLBRICH 1996). Also the German neurologist Westphal, who first described the disease pattern of agoraphobia, named three ways to abate the phobia's symptoms in his work in 1871: a close companion, a walking stick or the consumption of alcohol. Since then a number of publications described the comorbidity of anxiety and alcoholism in humans ((BAVING and OLBRICH 1996; BRADY and LYDIARD 1993; KUSHNER *et al.* 2000; SCHUCKIT and HESSELBROCK 1994), but also in animals (COOK *et al.* 2002; KLIETHERMES *et al.* 2004; MOLLER *et al.* 1997). Typically, a prevalence of anxiety disorders in patients suffering from alcoholism or the incidence of alcohol misuse in individuals being under treatment because of anxiety disorders was found. Between 16% to 25% of the patients suffering from anxiety disorders, alcohol-drinking problems were diagnosed (WOODRUFF *et al.* 1972). In patients treated for alcohol dependence, even 23 % to 70 % exhibited dysfunctions in anxiety behaviour (MULLANEY and TRIPPETT 1979). Evidence for a causal relation of alcohol misuse and anxiety disorders was established by the examination of the temporary occurrence either diseases. The question is, whether the existence of an anxiety disorder abets the prevalence of alcohol dependence, or whether alcohol misuse can result in anxiety disorder development. Both hypotheses are currently discussed, whereby the former emanates from alcohol being a kind of self-medication for anxiety. The latter is based on findings showing adaptations in the nervous system after chronic alcohol use (see 1.2 Neurobiological effects of alcohol), which especially after ethanol withdrawal elicits anxiety states (BECKER and LOPEZ 2004; KLIETHERMES *et al.* 2004; RASMUSSEN *et al.* 2001). As already suggested by Kushner et al. (KUSHNER *et al.* 2000), the comorbidity between anxiety and alcoholism is possibly explained best by the consolidation of both hypotheses – namely the interaction of the anxiolytic and the anxiogenic effects of alcohol. Accordingly, short-term alcohol consumption causes anxiolysis, whereas long-term alcohol use contributes to the development of anxiety symptoms in terms of withdrawal-

induced anxiety, which in turn can lead to relapse even after prolonged abstinence.

5.2.1 Chronic alcohol consumption and withdrawal

Overall, the C57BL/6J strain displayed significantly lower activity levels in the open field compared to C3H/HeJ mice, but showed higher anxiety-levels in the zero maze. The anxiogenic situation in a new environment (open field arena) possibly explains the downward trend in locomotion after withdrawal and thus rather reflects anxiety than locomotion. Increased anxiety levels after withdrawal as seen for both strains in the zero maze is a well-known phenomenon that was previously shown by several studies (ABREU-VILLACA *et al.* 2008; JASOVA *et al.* 2007; KLIETHERMES 2005; KLIETHERMES *et al.* 2004; SANTUCCI *et al.* 2008; WILLS *et al.* 2008). The observed differences for anxiety measurements in the two strains are in line with other studies that also reported overall lower anxiety in C3H/HeJ compared to C57BL/6J mice (http://phenome.jax.org; (COOK *et al.* 2001; GRIEBEL *et al.* 2000), and reflect the phenotypic diversity of the parental strains in this paradigm. In the F2 mice, ethanol withdrawal caused a decrease in locomotor activity, an effect that was more profound in male mice. As expected and similar to the parental strains, the anxiety level after ethanol withdrawal was increased.

The QTL with the highest linkage for distance travelled in the open field - in the presence or absence of ethanol - was found on chromosome 1. This locus is already known as an *Activity-distance travelled* QTL (Actd1, 65 cM) (GILL and BOYLE 2005b; KOYNER *et al.* 2000), and thus the data presented here strengthen the evidence for this locus to play a role in locomotor behaviour. Several previous studies also demonstrated a location of an open field activity QTL on chromosome 1 (GERSHENFELD *et al.* 1997; KELLY *et al.* 2003; SINGER *et al.* 2005; TURRI *et al.* 2004), which indicates that there is more than one QTL for this trait on this chromosome (BOLIVAR *et al.* 2001; TURRI *et al.* 1999).

Furthermore, preceding studies identified QTL on chromosome 1 that mediate the psychomotor stimulant response to ethanol. The QTL for *activity response to ethanol* (Actr1) is mapped to chromosome 1 with a peak correlated region at 85 cM (MGI, http://www.informatics.jax.org/). A study of Downing and colleagues (DOWNING *et al.* 2003) revealed a QTL influencing ethanol-induced locomotor activation on chromosome 1 at the 88-100 cM interval. This region was also observed as a QTL mediating ethanol-activation (DEMAREST *et al.* 1999; HITZEMANN *et al.* 2000; HITZEMANN *et al.* 2002). Overall, several QTL have been localized in this region on chromosome 1 (83–102 cM) influencing behavioural and physiological responses to alcohol, which include alcohol preference, acute alcohol withdrawal, alcohol-induced hypothermia, sensitivity and tolerance to alcohol-induced ataxia and alcohol conditioned taste aversion (CRABBE *et al.* 1994b; GALLAHER *et al.* 1996; RISINGER and CUNNINGHAM 1998). The findings that QTL regions for open field activity and

for ethanol-induced activation are distinct from each other are consistent with the observation that these two traits are under an independent genetic control (DEMAREST *et al.* 1999; HITZEMANN *et al.* 2002). This conclusion also implies that the QTL on chromosome 1 observed in the present study reflects the general locomotor activity of the mice rather than alcohol-related locomotion. This assumption is supported by the two-dimensional QTL analysis for open field behaviour, because the primary QTL on chromosome 1 seems to interact additively with loci on all other mouse chromosomes. This again may point to a gene lying within this QTL coding for a protein that is involved in multiple physiological circuits like it is the case for transcription factors or hub proteins, which are implicated in complex protein networks (HE and ZHANG 2006).

After ethanol withdrawal, two QTL were found on chromosome 7 (50 cM) and chromosome 11 (43.1 cM) that were not detected for ethanol-induced activity. Furthermore, the examination of QTL-interaction showed an additive effect for the chromosome 7 and chromosome 1 QTL. As mentioned above, analysis of chromosome 7 revealed the ethanol preference QTL Ap7q (25 cM), but no other alcoholism-related mouse QTL. But, a locus for an activity phenotype alone was detected at 52 cM within the present confidence interval (TURRI *et al.* 2001a). Also noteworthy in this context is the Ofca2 (open field activity and conditioned avoidance 2) QTL mapped to 53 cM on chromosome 7 by Steinberger et al. (STEINBERGER *et al.* 2003). In the same study they also found an association with anxiety in the open field (entries into centre) at 54 cM on chromosome 7. This QTL may therefore reflect the alcohol withdrawal-induced anxiety in the open field.

The QTL on chromosome 11, which only impacts on alcohol withdrawal-induced activity, also suggests a candidate locus in this region. Although there is the Alcw3 (alcohol withdrawal 3) QTL mapped to chromosome 11 (BERGESON *et al.* 2003; CRABBE *et al.* 1996), it is located at 18 cM and thus outside the present confidence interval.

For zero maze behaviour a highly significant QTL on chromosome 5 was found, in both alcohol-drinking and alcohol-free conditions. This strong QTL additively interacts with all other primary QTL detected via the function *scanone*. A previously identified QTL for anxiety-related behaviour lying within the confidence interval is Elmaz1 (elevated maze behaviour, 57 cM). A second QTL for elevated maze behaviour, Elmaz2 (76 cM), is located outside this confidence interval (COHEN *et al.* 2001). Additionally in line with the QTL found on chromosome 12 (18 and 21 cM), an anxiety-related QTL has been mapped to this chromosome (Axtrb2, syntenic) (TURRI *et al.* 2001a). For withdrawal-induced anxiety behaviour a correlated locus on chromosome 1 (79 cM) was found. Since this QTL was completely absent before the withdrawal, this finding suggests the involvement of genes lying inside the confidence interval on chromosome 1 in alcohol-mediated anxiety. Because the confidence intervals for withdrawal-induced locomotor activity and withdrawal-induced

anxiety on chromosome 1 reflect different regions and are therewith genetically independent, a possible locomotor component in anxiety measures can be ruled out. A mouse QTL study looking for anxiety alone also discovered a chromosome 1 region without any drug treatment using the open-field test (TURRI *et al.* 2001b). In 2004, a QTL analysis verified a region spanning the 74-83 cM interval on chromosome 1 to be related to locomotor activity in the central (threatening) area of the open field. The authors suggested that the influence of the chromosome 1 QTL appears to be that of suppressing activity in most novel situations (HENDERSON *et al.* 2004). To date, two chromosome 1 QTL are known to influence anxious behaviour in mice and are annotated as Axtex (Anxiety-exploratory behaviour, syntenic) and Axtq1 (Anxiety QTL, 95 cM) (TURRI *et al.* 2001a; WILLIS-OWEN and FLINT 2006).

Another possible explanation for the highly significant QTL on chromosome 5 in the present study may be the fact that C3H/HeJ mice and all other Jackson sub-strains are homozygous for the retinal degeneration 1 mutation (Pde6brd1), which causes blindness by weaning age. Two mutations have been identified for retinal degeneration 1 (rd1) mice. In all mouse strains with the rd1 phenotype a murine leukaemia virus (Xmv-28) insertion in reverse orientation in intron 1 is found. Further, a nonsense mutation (C to A transversion) in codon 347 that truncates more than half of the predicted encoded protein has also been identified in all rd1 strains of mice. Despite the absence of rods, the mice exhibit normal photopotentiation (defined as a 50% augmentation in pupillary light response (PLR) compared to pre-bright light PLR during a one minute dim blue light exposure after bright light exposure). Although the entire outer retina of C3H/HeJ mice is destroyed, the inner retina remains intact (MGI Phenotypic Allele Detail, http://www.informatics.jax.org/). The Pde6brd1 mutation was mapped to chromosome 5 at 57 cM, a locus that lies within the QTL that was detected for zero maze behaviour in this study. Mice that are homozygous for this mutation are still able to differentiate between light and dark conditions and mice in general orientate mostly via their whiskers. However, it is certainly possible that the mutation contributed to the high LOD score in this QTL analysis.

5.3 Alcohol preference and consumption

The C57BL/6J mouse strain is known for its voluntary consumption of alcoholic solutions (CRABBE and BELKNAP 1980; TREADWELL 2006) and, unlike many other strains, C57BL/6J mice will readily drink ethanol solutions when they are presented together with tap-water. In contrast, it has been reported that female and male C3H/HeJ animals showed a much lower ethanol preference than C57BL6/J mice (LI *et al.* 2005). However, results of the two-bottle choice paradigm conducted in this study showed a significant difference between the two sexes. Female C57BL/6J mice showed a higher ethanol preference than male mice, whereas

the opposite was the case in the C3H/HeJ strain. Here, males displayed higher preferences ratios than females. Indeed, ethanol consumption of C3H/HeJ males was comparable to that of C57BL/6J male mice. In a study of Li *et al*. they demonstrated that F2 mice obtained from a C57BL/6J x C3H/HeJ intercross also revealed a sex difference with higher ethanol preference ratio in female mice compared to their male counterparts (LI *et al.* 2005). In the present study ethanol preference in the F2 population was determined at the end of the behavioural phenotyping after the forced drinking period. F2 animals now exhibited a significantly lower preference than the parental strains, and also showed no sex differences. It is noteworthy that the F2 mice in the present study exhibited a relatively low ethanol preference ratio of only 20%, which is comparable to the ethanol-naïve C3H/HeJ mice. One possible explanation for this finding may be the serial analysis approach used here. The mice are already alcohol-experienced after four weeks of forced ethanol drinking, which may have provoked an aversion for the ethanol solution. However this assumption is contrasted by results of an additional experiment conducted in our laboratory. Here, an individual group of F2 C57BL/6J x C3H/HeJ intercross mice displayed similar preference ratios in the two-bottle choice test, although they did not pass through the forced drinking paradigm before.

Ethanol preference and consumption in the two-bottle choice test are often evaluated in parallel, and it is known that these traits are influenced by several genetic factors (SABA *et al.* 2006; VADASZ *et al.* 2000a). Analysis of the second-generation mice revealed an overlapping peak correlated region on chromosome 16 (preference: CI 17.4 – 35.4 cM; consumption: CI 15.4 – 33.4 cM) for these behaviours. This concordance indicates that this region acts on both ethanol preference and consumption and may reflect a common genetic control. The results of the two-dimensional genome scan support such a common genetic control, because the QTL on chromosome 16 interacts additively with chromosome 14 in both traits. Interestingly, Gill et al. found a suggestive QTL influencing ethanol preference in male recombinant inbred (RI) mice on this chromosome within the confidence interval we describe here (29 cM) (GILL and BOYLE 2005a). The dopamine receptor 3 (Drd3) gene maps in the interval (23.3 cM) of the QTL area that appears to be implicated in the motivation to self-administer drugs under schedules where the response requirements are high. The possible role of Drd3 in the control of responding by conditioned incentive stimuli remains unknown, but it has been shown that the Drd3 receptor density increases in the nucleus accumbens during conditioning. In several paradigms, the involvement of the Drd3 in drug reward has been confirmed by using Drd3-deficient mice. In contrast, reactivity to stimuli associated with natural reinforcers, such as food, appears unaffected by modulation of the receptor (BENINGER and BANASIKOWSKI 2008; LE FOLL *et al.* 2005). Another male specific alcohol preference locus (Alcp21) (GILL *et al.* 1998) was mapped to chromosome 16 at 45.6 cM, thus about 10 cM distant from our QTL region.

5.4 Alcohol and stress

Even older references than for the comorbidity of alcoholism and anxiety exist for the relation between alcoholism and stress. For example the Greek lyricist Alcaeus of Mytilene already stated 2500 years ago, that the best defence against grief of the mind would be drinking plenty of wine. Similarly, Shakespeare wrote in his play *Julius Caesar* (Act IV, Scene III): “Speak no more of her. Give me a bowl of wine. In this I bury all unkindness. . . .” (cited according to SAYETTE 1999). The idea of stress-relief by alcohol has led to many investigations in order to elucidate the mechanism(s) of interaction between stress and alcohol and of the stress-reducing effect of alcohol as a motivation for alcohol consumption.

Many different forms of stress (physiological and psychological) are believed to increase addiction vulnerability like e.g. deleterious effects of early life stress, child maltreatment, the experience of violence or work-related stressors (BRADY and SONNE 1999). The exposure to such stressors can result in neuroendocrine, physiological, behavioural and subjective changes that are often long lasting and affect the corticostriatal-limbic motivational-, learning-, and adaptation - systems including mesolimbic dopamine, glutamate, and GABA pathways. These adaptations represent the underlying pathophysiology associated with the stress-related risk of addiction (BREESE *et al.* 2005; SINHA 2008).

Stress also increases the risk of mood and anxiety disorders that are highly comorbid with alcohol addiction. Accordingly, it is also important to examine whether there are specific stress-related factors that contribute to the development of mood and anxiety disorders or to addiction risk. Animal models of stress-induced increases in voluntary ethanol consumption provide a valuable mean to examine the (neuro-) biological principles of stress–alcohol interactions. For alcohol consumption, studies revealed increases (HILAKIVI-CLARKE and LISTER 1992; NUNEZ *et al.* 1999), decreases (VAN ERP and MICZEK 2001) as well as an unchanged intake (FIDLER and LOLORDO 1996) after stress. A deeper understanding of how stress may be responsible for drug abuse will likely have a significant impact on both prevention and treatment development in the field of alcohol addiction (SINHA 2001).

F2 animals showed an increased preference ratio after foot shock, whereby mice with a lower preference exhibited a more pronounced increase in stress-induced ethanol drinking than mice with a higher preference. Although the QTL data of the study at hand imply a similar genetic regulation of ethanol preference and stress-induced ethanol preference, there are also studies from knockout mice that suggest the at least partially independent genetic regulation of these behaviours: Mice with a genetic mutation of the corticotropin-releasing hormone receptor 1 showed enhanced long-term ethanol preference after stress exposure (SILLABER *et al.* 2002), while cannabinoid receptor 1 knockout mice showed no effects of

stress on ethanol consumption, although the ethanol preference of both strains were either unchanged or only mildly affected by the mutations (RACZ *et al.* 2003; SILLABER *et al.* 2002; WANG *et al.* 2003). Such differing results once again stress the complex genetics underlying ethanol-related traits.

Just under the significance threshold, a locus on chromosome 10 for stress-induced ethanol consumption was found. This locus at 17 cM lies within a syntenic QTL named Ssrq4 (stress response QTL) specified by Thifault and colleagues (THIFAULT *et al.* 2008). The Ssrq4 QTL showed significant linkage to thermogenesis during stress response and was identified in AcB RI strains (derived from A/J and C57BL/6J progenitors). Potential candidate genes for Ssrq4 include Cirbp (cold inducible RNA binding protein, 44 cM) and Igf1 (insulin-like growth factor 1.48 cM). Following GO (gene ontology) classification, Cirbp is localized in the nucleus, has RNA binding activity, and is involved in the response to stress. Another interesting result was revealed by the two-dimensional genome scan. Although not significant ($p = 0.06$), the data point towards additive QTL-QTL interaction between to different loci on chromosome 19. These QTL were completely absent in the single-QTL analysis, demonstrating that these QTL only act on the trait when occurring together.

5.5 Somatic alcohol dependence

The forced drinking paradigm was employed to produce a state of somatic ethanol dependence. For mice of the F2 generation, the amount of ethanol consumed during the procedure was linked to five chromosomal regions (chromosomes 1, 2, 5, 10 and 15) that all showed additive interaction in the two-dimensional analysis. In 2002, the Alcdp1 (alcohol dependency 1, 95.8 cM) QTL was mapped within the region of the confidence interval on chromosome 1 (BUCK *et al.* 2002). This locus was then confirmed by a study integrating functional genomics (gene expression) into the analysis strategy (HITZEMANN *et al.* 2004). In follow-up studies the authors finally identified promising candidate genes for loci on mouse chromosome 1 that affect alcohol physical dependence (DENMARK and BUCK 2008). Additionally, these QTL were shown to be syntenic with human chromosome 1q23.2-23.3 (KOZELL *et al.* 2008).

Several previous studies also showed that loci on chromosome 2 act on ethanol preference and consumption, which has been annotated as Etohc1 (ethanol consumption, 28.0 cM) (BELKNAP *et al.* 2001; FEHR *et al.* 2005; PHILLIPS *et al.* 1998; SABA *et al.* 2006). In the present study, the QTL for the ethanol consumption phenotype was mapped at 102 cM on chromosome 2, which is consistent with findings of Vadasz et al. (VADASZ *et al.* 2000b) using *Recombinant QTL Introgression* (RQI) strains. Analysis of their data indicated QTL with largest effects on alcohol consumption at 105 and 107 cM on chromosome 2. This QTL is in

close proximity to the gene coding for the α4 nicotinic acetylcholine (nACh) receptor subunit (108 cM). It has been shown that alcohol exposure is able to increase mRNA levels for the α4 subunit in M10, in SH-SY5Y neuroblastoma and in PC12 cells (DOHRMAN and REITER 2003; GORBOUNOVA *et al.* 1998). In 2001, the association between a functional polymorphism in the α4 nACh receptor subunit and alcohol consumption was confirmed (TRITTO *et al.* 2001). Also interesting is the proximity of our peak correlated region to the Oprl (opioid receptor – like, 110 cM) gene, coding for a receptor that is activated by nociceptin/orphanin FQ (CHIOU *et al.* 2007; PAN *et al.* 1996). As already mentioned above, there is significant experimental evidence implicating the endogenous opioid system in the processes of reward and reinforcement. Also in line with the results at hand, a study with AXB / BXA RI strains showed significant association between a marker at 107 cM (D2Mit74) and alcohol preference in both gender (GILL *et al.* 1996).

The QTL on chromosome 10 (2 cM) identified in this study is in close proximity to a QTL for ethanol preference identified by Gill et al. (at 4 cM). Interestingly, the mu opioid receptor gene (Oprm), which has been implicated in ethanol preference, maps to 8 cM on chromosome 10. The 6 cM distance between the mapping result here and the location of Opmr is within the resolution of the analysis.

There are two affirmed QTL close to the highest peak that was found on chromosome 15, which may be of relevance for alcoholism: The Drb7 (dopamine receptor binding 7, 45 cM) and the Lore5 (ethanol loss of righting reflex, 46 cM) QTL (MGI, http://www.informatics.jax.org/). Several overlapping regions on chromosome 15 were identified as QTL acting on ethanol consumption in previous studies (GEHLE and ERWIN 1998; PHILLIPS *et al.* 1998; TARANTINO *et al.* 1998; VADASZ *et al.* 2000a; VADASZ *et al.* 2000b).

6 SUMMARY AND OUTLOOK

The genotype-phenotype analysis presented here identified a number of novel QTL linked to a variety of physiological and behavioural responses to alcohol, and confirmed several previously identified ones. These findings demonstrate that the genetic analysis of alcohol responses evaluated in single endpoint analyses used in most pervious studies and the serial analysis protocol used here leads to convergent results. Thus, although one behavioural test can often influence the outcome of another test performed with the same animal, the genetic control of ethanol responses seems to be sufficiently robust to permit significant modifications to the experimental protocol. The present study clearly demonstrates independent genetic control of different physiological and behavioural responses to alcohol that are relevant for addiction using a broad spectrum of phenotypic information. The different traits observed in alcoholic patients and in animal models of alcoholism are obviously linked to numerous chromosomal regions and thus the findings stress the importance of examining the whole entity of a complex disease to identify its genetic basis. This study also provides first data showing interaction patterns between alcohol-related traits. Since all loci interactions found for primary QTL were of additive nature, the results underscore the conclusion made from the single-QTL analyses, namely that of an independent genetic control of alcohol responses. Obviously, no epistasis exists between the candidate loci presented here.

The knowledge of alcoholism-related chromosomal loci gained within the scope of this thesis provides the basis for subsequent experiments aiming at the identification of specific candidate genes that are involved in the genetic regulation of alcoholism and its related characteristics. The comprehensive comparison with existing literature and databases will be extended, also including findings from human studies, to further narrow down the QTL regions and thus the number of possible candidate genes. Fine mapping studies using single nucleotide polymorphisms (SNPs) are one option to follow up the present studies. Interesting findings will be validated using transgenic and/or knockout animals to elucidate the function of the respective gene. Brain tissues from the behaviourally and genetically well-characterized and diverse animals presented here have also been archived and will be an important resource for the downstream analysis of candidate genes for drug addiction.

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8 SUPPLEMENT

8.1 Supplemental material

Supplement table S1. Identifier, primer sequences, fluorescent label and expected fragment lengths of all microsatellite markers used for genotyping.

				Expected fragment lengths	
Marker name	**Forward primer**	**Reverse primer**	**5' - Label**	**C57BL/6J**	**C3H/HeJ**
D1Mit64	AGTGCATTATGAAGCCCCAC	TCAAATTTTAAAACAACCCATTTG	FAM	126	121
D1Mit430	TATTAATGTTGAAGCCAGAAGCC	CTTTAATCATCTCTGTGGCAAGG	HEX	121	131
D1Mit169	CGCTGACTGCTACTTTATTATATTCC	TCTGATTTACTGTCAATCAAGAGACC	TET	150	156
D1Mit245	TGGTTACACAAGTCCAATACCG	GGCCCAGGTCTATAAATAAGCC	FAM	151	163
D1Mit236	ATACCCACCTAGCCTTTGTATAGG	GGAAGAAGGCTCAGCAAGTG	HEX	150	143
D1Mit161	ACCAGCCCTCCTTTTTTGTT	CTTGCCTCTTCAGGCACCT	TET	121	123
D1Mit303	GGTTTCTATTTCGGTTCTCGG	TCTGTGCTGCAAAACAGAGG	FAM	131	125
D1Mit251	TCTGTCCTTTCTGATGATTACTTCA	ATGGTGAAATATCACATGGCA	HEX	195	207
D1Mit132	TATTGTTTATGGAAATTGGACCC	CATCTCTGAAGGAAAAAGTGCA	TET	155	169
D1Mit215	GAGCAGAGTGTGAGAAGGG	CCAGTGTGAGCCCATTCC	FAM	156	164
D1Mit149	AAAGAGAATCTGACTTACCCATGG	TGTGAGGGAGAAGAATTATGTCTG	HEX	135	129
D1Mit135	TTGATGACTTAAAAATGTCAATACTGA	ACACCCCTGCCTTAAAATATTT	TET	184	174
D1Mit94	CGACTTCCCTTGATGTCCAT	TTTGTGTTGTGCAGTCTGTCTG	FAM	163	223
D1Mit139	CGACATTATCACTTCAGAGTTTGA	GAGTTCCAGCCCACTGAGAG	HEX	249	237
D1Mit446	TGAGTATATCATGAAGACAGCAACC	ACGTATTTACCTTGTTCTGAATTTTG	TET	175	141
D1Mit538	CCCCAACTTGGTCATGATGT	CCTCAGAGGAGGTAGGTGAGG	FAM	149	147
D1Mit200	GCCATGTTCATGTACATAGGTAGG	ATGGATGGATGGTTTTCCTG	HEX	212	304
D1Mit399	TTAGGGTATGGGAAGGGGAG	TCATTTCCCAGTCATTGTGTG	TET	145	142
D1Mit353	TACACTATGGGTATATGCTCACTATGC	ACACATGAACATACTCATATGCACA	FAM	125	109
D1Mit206	TGAGGCACCTTTGTATTCAGC	CCAGATGTCTTTGAACATTCTCC	HEX	130	125
D1Mit221	GGATTGCTATAACAGGAGCAGG	ATGGCAGAAGATGGATGAGG	TET	139	128
D1Mit292	GAACTGGAGGTTTGCTACTGC	GGACATTGTTATCTCAGTTTTCTTC	FAM	208	205
D1Mit155	ATGCATGCATGCACACGT	ACCGTGAAATGTTCACCCAT	HEX	259	223
D2Mit1	CTTTTTCGTATGTGGTGGGG	AACATTGGGCCTCTATGCAC	TET	131	127
D2Mit117	CCCAAAGAACATACATCAATGTG	TGGAGATGCATGTTTAAAACTCA	FAM	181	177
D2Mit365	GAGATCCCACTGATGATACAAGC	AGATGTGCCCAAGGGTCC	HEX	109	113
D2Mit369	GCCTCCATCAAAGGAAGACA	TTTCTTCCCTGTCTATGTGATAAGG	TET	136	117
D2Mit458	GTAGTTGAGGAAGACAATTGACACA	AGTGCTGTCCTCTGGGCTTA	FAM	129	105
D2Mit90	TCTTTTGTAAAGATTTGTTTCGTG	TATGTCTAGGGTGTCCGATGC	HEX	93	107
D2Mit92	TGTATGCACAGGTATTTCCCC	TGAGGAAAGGGGATAAAATTTG	TET	155	117
D2Mit37	TGTGCAAGCCAGAAAAGTTG	GAAGGGGATTGTAAATTGGTACC	FAM	181	187
D2Mit100	GTGTTCCTAAGGTTGTATTTTGGC	GAAATTTGACAATTGCTAGGTGC	HEX	121	133
D2Mit101	ATAATTCCTGATTTGCTGTTTGTG	ACATGAAGCCTAGAGGGTGC	TET	199	227
D2Mit398	GTACCTCTGGCTCCTGAGG	TATTTTAAAAGTATAGGTGTGTGCCG	FAM	153	139
D2Mit305	CTCAGAAAACATGCAATTGAGG	ATGAGTGCAAACCAAATAAAATTG	HEX	144	108
D2Mit395	AGGTCAGCCTGGACTATATGG	AGCATCCATGGGATAATGGT	TET	135	129
D2Mit164	TCTCTGCTAATTAAGTTGAAGAGTGC	ACCAGTGTGTGTTTGTATGATGTG	FAM	148	120
D2Mit208	CAAAAAGCCACAGCCACC	GTTTATAATCAAGAGGCTATCTTGGG	HEX	115	195
D2Mit498	GCAGCCTTTCCTTCCTTTCT	CAGATAGAGCACTCAGACATACATACA	TET	129	121
D2Mit285	TCAATCCCTGTCTGTGGTAGG	TATGACACTTACAAGGTTTTTGGTG	FAM	145	155
D2Mit263	ACTGAATCATCTCTCTCCTCAGC	AGTTCAGTTCTTAGAACCCACAGC	HEX	147	141
D2Mit226	TTTTTGCAACTTTGTTAAGAATTCC	AAAACACCCTCCCACCCTT	TET	109	131
D2Mit147	CATCCCTAAGACAAGCAACTCC	GTCACAATGTCCTTCTCCATCA	FAM	124	128
D2Mit457	GACTTTCACATGAAAGTTGTTAGACC	TAGTGATTGCACTTAATTGTATGCC	HEX	127	121
D3Mit130	AACACATGAAACGTGTGCGT	TGATAGGCATGCTTAAGCCC	TET	156	128
D3Mit92	CCTCTGTTAGGATATCCAATCCC	CTTGTGTCCCTCCACTTGGT	FAM	249	245
D3Mit203	CTGAATCCTTATGTCCACTGAGG	GGGCACCTGCATTCATGT	HEX	161	145
D3Mit179	TTTCCACAGGGAACCATACTT	AACACACTACCTATGTTTTCTTTCTCT	TET	157	134
D3Mit354	ACTCGAGATTCCTCTCAA	CTAGGAAAGAAAGCTATTGCAT	FAM	367	312
D3Mit333	CTCCCTCCCTTCCTCCTTC	ACAAAAGCAGAAGACTGATCCC	HEX	133	151
D3Mit67	AGCATACATCATAGCCTAAAATGG	GTAACTAGGGAGACAGCCACTTG	TET	153	151
D3Mit199	CCAGACCTCAGAAAGTGAGTCC	ACCATGACATTCATGCTATTGTG	FAM	148	121
D3Mit339	TCTATATTTGGGGGGAAGGG	GATTTAGTGTCAAAGGCTATGCA	HEX	155	125
D3Mit74	TCATCGTAGCAATAGAAATCCTG	CACCGTTTCCTGACCTCTGT	TET	159	155
D3Mit101	CCTCTAGATGCATACATGTGCC	GGTCAAGTTAAGTGTATTTTTTTCCC	FAM	115	133
D3Mit57	TCCAGTTACTTGGTGAACTCCA	ATATGTGTACATGTTCATGGTGTG	HEX	165	163
D3Mit216	AGGACTGAAGAAACATACACATGC	AGAAACATCTTGATTTTCAACAAGG	TET	129	149
D3Mit14	ATTGCGGTTAAAGTTTGCTT	TCCTGCAAATTGTCCTCTGA	FAM	177	205
D3Mit257	CCTAGCGCAGGAATAGTTAACC	ACAAACAGAACAAAACAAAAAGTCC	HEX	135	227

(*Continued on the next page*)

Supplement table S1. **Continuation.**

				Expected fragment lengths	
Marker name	**Forward primer**	**Reverse primer**	**5' - Label**	**C57BL/6J**	**C3H/HeJ**
D3Mit18	GAACAGTTCCCAGGTCCTCA	CTGCCTTTAAATTCTGTCACCC	TET	238	219
D3Mit262	TTGTGTTTTTTATTGTTTGTTTTGG	GAGGTAGAGAAATCTGACAGAGCC	FAM	142	146
D3Mit116	TCACTGCCCATCTTTGTAACC	CCCAGAGACCCGGAATAGAA	HEX	270	282
D3Mit19	CAGCCAGAGAGGAGCTGTCT	GAACATTGGGGTGTTTGCTT	TET	167	183
D4Mit149	TGAATTCAGAAGGATGTGTGTATG	ATGTGAGAATCAACACCTGAGG	FAM	121	137
D4Mit50	AAGGCAGAAACACAAAAATACATG	TGATGGTGTTCCATATGTCTTATG	HEX	103	109
D4Mit39	TCTTTTCTGCCCTCACAGCT	GTCTATCTTGCCAATTTCAGGG	TET	179	175
D4Mit286	ATGGGGTCTAGGAAAACATGG	AAATTATGAGTATTTCACCTGAGTGTG	FAM	103	83
D4Mit89	GTGGGCATTTTTTTTGTGGA	TTCCCAGATCCTCTCCCTCT	HEX	139	125
D4Mit139	CAGCCGTAGAAGAGAAGTAATTTT	ATCAAACTGGGAAGAGCCAA	TET	159	151
D4Mit87	ACAGGTAGGAATGGAGCCCT	TCATCCCTTTGCCAAAGC	FAM	123	127
D4Mit178	GCCCTGAAGGTAAATCAGTAACT	GCTCAGGAGGTACATTGCCT	HEX	153	177
D4Mit27	GCACGGTAGTTTTTCCAGGA	TGGTGGGCAGGCAATAGT	TET	157	125
D4Mit153	ATATGGAGTCTGTGTGTGTGTGC	CACTGAATTTCTATTGTTGGAATAGG	FAM	129	119
D4Mit31	ACGAGTTGTCCTCTGATCAACA	AGCCAGAGCAAACACCAACT	HEX	129	119
D4Mit76	TGAAGGAACCTGAAGCAAGG	ACCTCCCAGGAGTGTCCAG	TET	189	179
D4Mit203	GAATTCTTCCTGGGCCTTTC	CAAGAGCCCAGGTGTGGTAT	FAM	151	131
D4Mit251	AAAAATCGTTCTTTGACTTCTACATG	TTTAAAAGGGTTTCTTTATCCTGTG	HEX	123	135
D4Mit32	CCCTGGATAAACGTCATTTAATTC	ATGGTTGGGTGTTACCAGGA	TET	155	191
D4Mit233	TGGTCATGTGTGTCCATGC	ACTTCATGTAGCCAGGTGGG	FAM	184	174
D4Mit42	CATGTTTGCCACCCTGAAAC	CCTCACTTAGGCAGGTGACTC	HEX	109	101
D5Mit146	TTAAATCTGAAGGTGTGGCTATAGC	GAGATTGCAAGTAAAGTGAGAGAGG	TET	133	129
D5Mit348	CTGACCAGAACACAGCATAGTACA	TTTAAAATAGGAAAAGCATTCTTTCC	FAM	130	140
D5Mit387	CCCCATGTATCTCTAGATTAACAATG	GCACTCGTGTACATAACCAAATAC	HEX	181	189
D5Mit352	CCCAGAGCCCACATCAAG	TAGGTGGGTGTGTCTCTCCC	TET	120	127
D5Mit106	GTCAGGCATGGTGATTCCAT	ATGGATGACTGTGAACATACAACT	FAM	170	160
D5Mit183	TATAAAGATAATCAGGGCTTAAACTCG	ACCTCCACAACATGAGCACA	HEX	120	122
D5Mit254	GTGCAGGCCTGAATTGAAAT	CAAAGTGCCTGTGCATGTG	TET	143	131
D5Mit201	GAGGACTCCTTCGATTTCCC	TTCCTAAGCAGGAACTGACCA	FAM	117	111
D5Mit18	CTGTAGTGGGTGGTTTTAAAATTG	ATGCCACTGGTGCTCTCTG	HEX	245	227
D5Mit91	TCTGCAGGTGTCTTCTGCC	CTTAGCTTTTACCTGTCTGCACC	TET	211	225
D5Mit277	GTGTGTTTGTGCATGGGTATG	ACCATCGGGAAAAAATGTAGC	FAM	131	147
D5Mit210	GATGGGTGCATTCATCCTG	TGAAAGTGATTCCTCAGGGG	HEX	201	213
D5Mit95	TGTTCTTGTCCATGTCTGATCC	AACCAAAGCATGAAACAGCC	TET	123	141
D5Mit163	AATTAGATTAGAAGTGCTGGGTCG	GCTCAAAGAGTTCCAATTCCC	FAM	174	152
D5Mit99	CAGAAAAGAGAAAACGGAGGG	TTCCTGCTGCCTGAAGTTTT	HEX	105	211
D5Mit409	GACACAGTTTGGTCACTTGCA	ACACACTCTCTCTATTCCACTTTCTG	TET	205	221
D6Mit138	GCTCTTATTAATGAAGAAGAAGGAGG	CAAAGAAAGCATTTCAAGACTGC	FAM	118	142
D6Mit83	TTCTGTAAATTGCTAATCTGTCCA	TTGTATGCATTTAACAACTCAGGA	HEX	157	137
D6Mit159	CATATTCAAGACGGAGACTAGTTCC	CACATGAAACACATGCACACA	TET	123	147
D6Mit268	AGTCAGAATATGGCAAGTCAGTG	TTTCAGAGTCTTTCTTTCAGTATCTCC	FAM	130	117
D6Mit274	GCAATGCCAAAATGTTCAAA	TCCTTCTCCATTTACACTTACAACA	HEX	122	102
D6Mit384	AATGCTTTATATGCAAACTACTCTCTC	GAATATAGCAAGACAAGGGAGACA	TET	133	155
D6Mit209	CTCCCCCTCTGTGTGATTGT	TTATTACACCAGACCCATGTGG	FAM	141	145
D6Mit284	GGCTGCTGAGAAACAACCTC	TGAGTATTGAGCCAAATCCTCC	HEX	152	150
D6Mit132	TTGTTGTTTTTTACCTCTCATTGG	GATCACGAGACTACGGAGGC	TET	227	207
D6Mit36	ACCATCTGCATGGACTCACA	GTTGAAGAGGACGACCAAGTG	FAM	203	185
D6Mit62	CTCACCCACACTCCTGTTAGC	TTGTGTGTGATAGACTTACTGGGG	HEX	297	173
D6Mit335	CTATGATATGTGCGCGCG	AGTAATTCAGACACCAATTAAAATTTT	TET	131	125
D6Mit219	AAATGTTGACTTTAATGAGGTAATTG	TTCACATATCCCTCAGACATGC	FAM	195	185
D6Mit59	GCCATCCTTTGTAATAACAAACA	CGTCTGGGAAAACCTCAAAA	HEX	175	185
D6Mit15	CACTGACCCTAGCACAGCAG	TCCTGGCTTCCACAGGTACT	TET	267	202
D6Mit374	TTCTGGCTCTTAACAGTCTGTCC	TACATATGCCAATGATATTCTCCC	FAM	185	153
D7Mit21	GGGTTGAACCTTACAGGGGT	ATCAAACCAGCCCAAGTGAC	HEX	135	139
D7Mit191	TTGGGTTTGTACTACCTAGATACCTC	CCTCTAGGGCTCTTGCACAC	TET	158	190
D7Mit266	TCAGGGATGTCTTAAAACTGGG	CGCTGTAAAGCGTATTCGTG	FAM	129	123
D7Mit224	CCATGCAGAGGTTTGGAAGT	CCCAATGTTCTTGATTCCCA	HEX	138	150
D7Mit228	ATTCTTGGCCTTTTCTTGTAACA	AAACCTCCACACTGACTTCCA	TET	155	147
D7Mit229	GGTTCTCTTTCCTTGTTTGCC	TACTGGTTACATCTGGTGGGTG	FAM	130	150
D7Mit248	AATCAGGCAACTCAGGCACT	TCCTTAGGTCTCCAGTGAAAGC	HEX	115	119
D7Mit346	CTCCTTTTTGGTACATATATACACACA	ACACTGGAGAGCCAGGAGAA	TET	103	109
D7Mit163	GGACAGACACCCTCACCG	CGGCTGTGAGAGCATAGTGA	FAM	200	150
D7Mit31	TTCAAACCATCCAGTAAGTCCA	TTGGTGAACTGCTTCAATGC	HEX	253	233
D7Mit323	TTTCACCTTCTAATCCTACTTCCTG	TGTCCAGAACAGGAAATAGAGTACC	TET	123	149
D7Mit281	TTCCTCTACCTCCTGAGCCA	GCCACAAGGAAGACACCATT	FAM	120	210
D7Mit66	TTCACTCCCAGCCAGTCTCT	TAACCAGGAAACACACGAACC	HEX	171	155
D7Mit105	AGCAAAGTAAGGCAGACTTTGG	AGGAGAGGCAGAACATGGAA	TET	267	249
D7Mit109	TCAACACCAGGAAGTCTCTTCA	CCTCCATCTCCCATCCAATA	FAM	119	115
D7Mit259	CCCCTCCTCCTGACCTCTT	GTCTCCATGGGAACCACACT	HEX	155	159

(*Continued on the next page*)

Supplement table S1. **Continuation.**

				Expected fragment lengths	
Marker name	**Forward primer**	**Reverse primer**	**5' - Label**	**C57BL/6J**	**C3H/HeJ**
D8Mit155	TTGGACAGGGAAAATTCTGC	TGAGGACTTGCTTTAAGAGTACTCC	TET	158	122
D8Mit124	CAACTGTGTATCATAAACTGGGAA	GAAGAATCACTCAGCAGTGTATGG	FAM	136	142
D8Mit289	AAAAAGAAAAGAAGGCTTAGTAATGTG	CTTGCTATTCATTGCAAAATTCC	HEX	159	125
D8Mit63	TCTGGAACACAGTCCAATTCC	ATATGTGTGAGGGTTTTACCGG	TET	215	229
D8Mit190	CTTTGTTGCTGTTTCATTCTGG	AGTCATATACAAGGTCAACCTGAGC	FAM	145	123
D8Mit339	ACCTATGGTACACACACATCGC	CAAACATTTTTAGGCATTTAGATCC	HEX	129	113
D8Mit231	TGCAAAGAAAAAAGTATCAAAATTG	TGTGTCCTATTTGCAATGTAACTG	TET	149	167
D8Mit135	TTGTTTTAAAAGGAAGGTCTATTTTAG	CAGAGCCCACATGACAGAGA	FAM	207	181
D8Mit80	TGCATTTGTCAGGGCTCTC	ATGACACATGAGCCTCCACA	HEX	115	141
D8Mit242	TGTGCAACCAATTTCTTCCA	CCCATGATTTATTCAGACTGAGG	TET	173	203
D8Mit113	GGTCACATAATAAGAAAGCCCG	AACCCGTTAGGAGGACCG	FAM	145	167
D8Mit271	GGCAGAACCACAGGTTGATT	GGAATGAGGTTTGGGTCAAA	HEX	103	127
D8Mit89	TGTTTTGAATCTGTTATTAGGTGTG	GAGAGAAAGGAACAAATTTATCAAGG	TET	139	129
D8Mit49	TCTGTGCATGGCTGTGTATG	TGGTGTGCTGCTGATGCT	FAM	159	161
D8Mit13	CCTCTCTCCAGCCCTGTAAG	AACGTTTGTGCTAAGTGGCC	HEX	105	98
D8Mit280	CATGCAATTCCAATGTCAGTG	TAGCACTCAATCAAACCCCC	TET	115	167
D9Mit126	GCTGCCAATCAGGTAAAGGA	GGGGTAGGTATCTGAGCAAGG	FAM	134	136
D9Mit1001	ATATCAGGCATGCATTATGATCC	TCTCTCTAGTGGGATTATCAACACA	HEX	129	139
D9Mit2	GTGGTCTGCCCTCTTCACAT	CAAAGCCAGTCCAACTCCAA	TET	182	189
D9Mit328	CATTTACTGTCTCTCTTTCATTCTCTG	CTTACATCTGGTCCACAAGAAGG	FAM	191	237
D9Mit129	TTGTCTTTTAACCTCCTGGAGC	TCCCATCTTTCTCCTTGTGG	HEX	139	157
D9Mit162	ACCACCAAATACAACCACTTCC	GACTGAACAATCAGGAGTATGGC	TET	147	129
D9Mit208	GCCTCTCTTTCTTTAAACACTTTAAG	CCTCCACACACCTGTTTGTG	FAM	119	93
D9Mit75	AGACTGTGACTTACTACGGCTTCA	CACACATCTCCCTGTCCCTT	HEX	203	151
D9Mit269	TTTTTGGACTAATAGTCAACTGTGTAA	AGGAAGACTGAAAACTTGTGGG	TET	183	155
D9Mit110	CCAGAAGGGGTGTGTTTTGC	CTACCCTCCTTTCTAGTTTTTGTCC	FAM	82	157
D9Mit35	CCAGCGCACTGTTCTGATAA	AGGTGCCTTCTGCTTTGAAA	HEX	131	119
D9Mit53	ATTCATGTGTCTCCAAAATCCC	CAAACTCTTGCTGGGTGTGA	TET	219	211
D9Mit214	AGCACAGGAAAAGGACGCTA	AACCTGTCTCTGTAAAACTATCTCCA	FAM	147	123
D9Mit279	CTCCAGAAACTTGTCCGCTC	AATTGAAACTGTATCTAAGGCATGG	HEX	153	143
D9Mit18	TCACTGTAGCCCAGAGCAGT	CCTGTTGTCAACACCTGATG	TET	187	217
D10Mit49	GGAATTTACACTGGAATACAACCC	GTGGGCATTTGCACTGTG	FAM	113	115
D10Mit189	TGTGTAGGTATGTGTGTGCATAGG	ATCAGACAGCACCTGGGAAC	HEX	113	137
D10Mit213	CTCCTCCTACTGATTGTCCCC	GGGACAAACTTTTAAAAATTGCA	TET	157	143
D10Mit86	TTTGCCTGTAACAAGCCAGA	TTGAGGCTATCAGTTTAAAATCCC	FAM	163	157
D10Mit126	ACATTCACAAAATGTGTATGTATGTG	TGTTTTTCATTAATCTCTTGAGATGG	HEX	136	132
D10Mit38	CGATGAGCCCTAACACCAAT	CCTGTTACAAACTAAACCAAACCC	TET	173	203
D10Mit130	TGCCACACAAACACCACC	ATTCATCAGTGTGAAATATGGCC	FAM	157	165
D10Mit31	CATAAGGAGCACAGGCATGA	CCCTCTACGTGCATGCTGTA	HEX	159	161
D10Mit139	AAGTAAATGAGCAGGATGAAAACC	GGGTATGCTGACAGCAAGGT	TET	158	154
D10Mit42	GCATTCAGAAGCTGGAAAGG	TGCCCAGCATATGTTTAAAGG	FAM	191	203
D10Mit95	CCAGTCTCAAAACAACAACAAAC	TTGCACCTAGATTGCCTGA	HEX	208	174
D10Mit96	ATGTCCAAAACACCAGCCAG	GGAAGTGATGGAGCTCTGTT	TET	160	134
D10Mit233	GTGCTTTATATTGGAGATCATCACA	GTCCCGAATTTCACATACATAGC	FAM	137	115
D10Mit180	GACCTTCCTTTATACACAAGTCATAGC	GTGGTACAGAACTTAGGTGTTTAATTG	HEX	141	213
D10Mit103	TATGCCGACAATATTTCATTGC	GCCTCTGCATACATACCAATACC	TET	149	151
D11Mit71	GCCATACCTGGTAGCGTGTT	AATTTTCAGATGTAGCCATAAGCC	FAM	221	245
D11Mit2	TCCCAGAGGTCTCCAAGACA	CCACAGTGTGTGATGTCTTC	HEX	129	144
D11Mit295	GTTCTAAAATGCAAGTCCCTGG	CTCTTTGATACCCCCACCCT	TET	119	123
D11Mit163	AACCCTGCTATTGTGCTGCT	CTAGAACACACATGCATGCTCA	FAM	144	162
D11Mit296	TAGGGCATATTAAAATATAAAGGCTG	CTGCACCAATGGTTTATATTTCC	HEX	127	103
D11Mit86	TTGACATTGTGACAAAGACTTTCA	AAGGCATCATGAGGTTTTTAGTG	TET	133	141
D11Mit242	GAAGCCAGCAAGAAAAATGC	CTGTCTGGTAGTGCAGCCAA	FAM	129	143
D11Mit351	GTATGTGAGGGAGAGTACTCACATG	TCTCAGTAACATGAGATATTCAGTGTG	HEX	117	111
D11Mit29	TTGAGGCATGAGGGGATTAG	TTTCCGTCATTGCTAAAGGG	TET	151	157
D11Mit245	ATGAGACCATGCTCCTCCAC	TTGTCCTCTGACCTTCACACC	FAM	159	147
D11Mit356	GGCAAGCAACTTCTTCCATC	TTCAGAAATTTGGGTATTAGAGTGG	HEX	103	123
D11Mit285	CATGAATCCATCACCAGCAG	TTTTTCAGTCATGCAGGCAG	TET	128	139
D11Mit289	CTTTGGTTGGTTTTAAATGTTTTAA	AAGGAGAAAGCAGATTCATACACA	FAM	133	126
D11Mit198	TGAAAATATGCAGCCTCCG	ATCTGCAAAGGGATCTGGTG	HEX	293	305
D11Mit258	AAACAGAGATAAACCACGGGG	TGTGGAACTAACTCTCAGAAGGC	TET	137	177
D11Mit214	CATACAGCCTTCAACAATGACA	ACTGCATACATGTGCACTCATG	FAM	156	142
D11Mit337	CCTGGCAACCTTCCACTTTA	ACCTTCTGACCTCCACATGTG	HEX	121	113
D11Mit104	CACATGATCATACACTGTTTCTCC	GCCACGTGTTCTAACCTTCC	TET	163	169
D12Mit103	ACTATGGTGAAATCATACCCACG	ATCAATGGATCTTTTTTGGTGG	FAM	140	142
D12Mit11	TCCCAAATGGAAGACAGGAA	CCCTCCCATTGCCTTTTAAT	HEX	179	173
D12Mit185	TGGAACTAGAAATCCATGTTAAAGG	ACTCAGGTATTTGTGCAATTGG	TET	159	137
D12Mit59	AGTGAAATTCAGAGCACAAAAGC	ACCCTATATCTCCATGGTACGTG	FAM	157	159
D12Mit222	TTTAAAAACAACAACAACAAAAAAGG	ATCTGGGTTTTGAAATAAGAGCC	HEX	121	127

(*Continued on the next page*)

Supplement table S1. **Continuation.**

				Expected fragment lengths	
Marker name	**Forward primer**	**Reverse primer**	**5' - Label**	**C57BL/6J**	**C3H/HeJ**
D12Mit112	CTTCAGGCCTCCCTGGTAC	TGCCTCCAAATATACTCACAAGC	TET	161	143
D12Mit210	CTGATGTGAAATTCACAAAGAACC	TGGGGCCCACTCTACATTAG	FAM	157	167
D12Mit52	CCATCTTCTGGCATTTTGCT	AGACAGGAGGGTCCCAAAGT	HEX	147	125
D12Mit158	CATTGGGCAATGGAATTTG	ATGAGAGAAAACCAGAAACAAAGG	TET	154	159
D12Mit239	AAAGCATTTCTTGTTTTATGTAATGTG	CATGCATCTGCAACTCGC	FAM	97	107
D12Mit101	GCTTTTCCTTATCAAGATATGCG	GCAGCAGAAAGAGAGGGAAA	HEX	177	125
D12Mit17	TCCGAGTGTTGCTTCTCCTT	CCTTAGATGCTCAAGGCTGG	TET	187	177
D12Mit144	CCACACATGTGCAGACACAG	CTGGCTCTAAACCTTAGCACTAGG	FAM	312	316
D13Mit303	AGTTCAAGTTTGAGACAGATTCAGG	TTCTCTCGCTTCATAAAGTCCC	HEX	131	123
D13Mit3	TCAGGCTCATCCCAGATACC	TTTTGCAGAGAACACACACC	TET	166	203
D13Mit133	TAGACACTTAATTCTGTGATGAAATGG	AGCAAAAGCCCCAGTTAGTG	FAM	124	118
D13Mit275	TTAGCAAGGGAACAGAGAGAGG	CAATCAAGGTATCCCTGTCTCC	HEX	115	123
D13Mit18	TGTATCCAGCTCATCCTGATAGG	ACTTCCTTTGAACTTCATGACTTC	TET	203	197
D13Mit19	GGTGAGTTGTGTAATGATGGACA	AGCAACAGGGCTACTAAACACA	FAM	157	159
D13Mit179	GACCAATGCCCTACAATTTCA	CAGAAGCAGTTTGTCTTTGTGG	HEX	103	89
D13Mit248	TAAAGTAGAAGGCAGCATGAGTG	ACCCAAATGTTTTGGATCCA	TET	121	101
D13Mit231	GCACGGAGGGAGAAATGTAA	GTACTTAGGGACTCTTCAGCGTG	FAM	123	151
D13Mit24	TGCATGACTGTGTAATGCTTTG	GAAGAACTGGGGAAACTGAGG	HEX	213	173
D13Mit159	CCCATTGTCCCTGTTCAGAT	AAACCCACCATGAATTAAATGC	TET	149	167
D13Mit147	CATCCAGGAAGGCAATAAGG	CAAATGCACAGTGCCGAG	FAM	115	100
D13Mit30	TTTTTGATGTGTATGCTTGTTGG	AAAGAGAAGACGGGGAGGAG	HEX	115	119
D13Mit213	GCCTGAAACTCTACATAAAATACATCC	AGTTTCATTGCTTTAGTTACATTTTCA	TET	155	167
D13Mit292	AAATGACATTTTTGTATGCACACA	GAGACAGAGTAATGACCGAATGG	FAM	159	145
D13Mit262	CTGCGGCTGTAGGTTAAGTATG	AGGCTGCTGCTAACAGATGG	HEX	133	123
D13Mit35	GATTTTCCAGGTAAGTGGCG	CACATTCACTGTGAGTGCACA	TET	197	189
D14Mit48	TTTCTAGCCCTGACCCCC	TCTGTTCACTCTGTGTAATTCTCC	FAM	125	93
D14Mit207	TCCAACTAGTCCCCCTCTACTT	CTGTGACTATCTGTACAAGACCTGC	HEX	135	111
D14Mit127	AAACTTTACCTACCAGTGTCAAGTTAG	GTGTTGAACAACTCTATGTCTGTCTG	TET	154	156
D14Mit60	TATTCCCCTATTTTACACTTTCCG	ACCTCAATGGATTTTTAAAAGTGG	FAM	143	119
D14Mit259	TGGTGTCTCCTTCGGAATTT	TAAATGTAAAAGGTAAAGGCAATGG	HEX	133	153
D14Mit5	CACATGAACAGAGGGGCAG	GTCATGAAGTGCCCACCTTT	TET	185	111
D14Mit157	GGTTGACCTCTGACCTCCAC	AATAGCACTGGAATTAAAAATGTGG	FAM	157	153
D14Mit85	TCCCACATATGCACATACACG	ATTCTGATTGCAGATTCCGG	HEX	167	163
D14Mit68	GTGGCATGCACAACCGTATA	CCCTTTTGAGGTGCTTGTTT	TET	161	163
D14Mit263	TGAGCACAGAGCCTATGTGG	ACAGAGAAATACCATGAAAACACC	FAM	129	125
D14Mit106	CATAGGCTCTAGCGCTGACC	ATTGCATTGATGTCATAATTTCA	HEX	316	314
D14Mit166	TGGGGTTAGAGTAACTAGAATATAGGG	GGGGGCATTGTATGCTTAAA	TET	151	135
D14Mit97	TCAGTCCAAACTCTGTTAATCTTCC	CAGCTCCACATTTTTGCTCA	FAM	162	168
D14Mit266	ATGCACAGGATTGATCTGCA	AGCATGACCTAAATAATGAGACCC	HEX	155	183
D15Mit102	TATGGAACACACACAAGCATACA	TGATCATTCATGAATAGGTTGAGG	TET	208	195
D15Mit256	CCACCCTTCCAAGTTCTTATACC	GGAATGGCTAATAAATAAAGACTCTTG	FAM	125	167
D15Mit138	TTCAATTCCCTTTTGTCAAATG	CAAGACCCTAGATTCAGTCTACCC	HEX	156	134
D15Mit152	AAATGTAGGACTTACACAGTTTGTGC	CAAAGTTTAGTGTCAGAACGAATACA	TET	113	89
D15Mit229	AGAGTGATTATTTACAAGAAACACACA	GATTAATGTTTAAAATCATGGCTGC	FAM	117	147
D15Mit88	TAGCAATCACAGGAGGAATTAGG	TTACTGAACTTAAGAACTGGAATCATT	HEX	216	208
D15Mit209	TTGTGCTTCACTAGATGTAGACCA	TTTTATAGTTGCACATAAGCAGCA	TET	134	113
D15Mit156	CCCACATTCATGCACATATAGG	AACAAATCAAGAACCAATTGGG	FAM	152	130
D15Mit188	TTCACTCCAAATCCTCCGAC	GAAGAGGAAATGCAAGCCAG	HEX	170	189
D15Mit107	CAACACTTATACACTTGTGTCAGGG	TCATGGTTGGAACAGCAGAC	TET	158	152
D15Mit43	GAGTTTGGTTCGGTTGTAGAGG	CTGGGTACCTCAGCTTTTGC	FAM	211	227
D15Mit44	ACCTGCATAGATGTTGAGTCACA	AGGCACAAAAGGAGCAGAGA	HEX	157	155
D15Mit79	CGAAACATTTGGGCACTTG	CCCCATTCCTGAGTCTCTTG	TET	283	289
D15Mit161	TCTGTTTTGTTTGTTCGTTTGC	TAAAATCTCCCTGTATACAAGTCTGTG	FAM	135	109
D16Mit107	ACCCCATGAGACTCAGCATC	GAAAGCCTGAACACATGGGT	HEX	197	199
D16Mit100	AGTCTTGTCCGCGTCAGAAT	AAAAGGATTGCAGGGACTACTG	TET	154	160
D16Mit144	AACTATCCAGGCCACAGTCTG	CAACCGCATTAGTCAGGGTT	FAM	153	157
D16Mit101	TTATGAAATGTTTTATCTTTTGGGG	CTCCAGATGTAGAAATTAAAATCTTGG	HEX	157	153
D16Mit134	ATGGGAAGCAATCAGTAATAACTG	ACCACATAGACATCATGGTATACACA	TET	157	165
D16Mit12	GAACTCAGTAAGCTCTCTATGCCC	GGAGGACTAGCAGGCTAGAGC	FAM	199	163
D16Mit42	TAACCATCACATTCTTTTTCATGT	TGTGGCATAAGGCAGGCT	HEX	135	159
D16Mit30	GTGCACATACATACCACAGCG	TCACTGCAGGGAGGTTCAG	TET	159	117
D16Mit140	ATAGTTGAAAAACTTGAACATGCG	GAAAAGGTTAATGCTGGTCACC	FAM	157	171
D16Mit139	GTATGTAAGGAATGGTCAAATTCTTG	TCATTGTGATTGTGAAAGAATGC	HEX	155	179
D16Mit27	AGAAAAGAATGAAAATCACGCA	TAGAGACCTTTTGTCTGAAATCCA	TET	91	81
D16Mit189	ACAGTGTTTGTTTGTTTGTTTGTG	CAGTACAGGAAGTCTTTGCATCC	FAM	206	192
D16Mit70	GGATCTATATGCTATAGAACCATTCA	GTCATCAATTCCATTTCCTAATATAGA	HEX	196	176
D16Mit86	TAATGTGGCAAGCAACCAAA	GCATGTTTCCATGTGTCTGG	TET	135	129
D17Mit164	AGGCCCTAACATGTAGCAGG	TATTATTGAGACTGTGGTTGTTGTTG	FAM	143	133
D17Mit133	TCTGCTGTGTTCACAGGTGA	GCCCCTGCTAGATCTGACAG	HEX	202	174

(*Continued on the next page*)

Supplement table S1. **Continuation.**

				Expected fragment lengths	
Marker name	**Forward primer**	**Reverse primer**	**5' - Label**	**C57BL/6J**	**C3H/HeJ**
D17Mit29	CATCTTTCCAGTCCAAATCTCC	CTTCTGGCTTCCTCAACCC	TET	157	153
D17Mit33	TGTTGGAGCTGAATACACGC	CCAAACACCAGGGTCTCTGT	FAM	201	179
D17Mit49	TCTTAGAACTCACATCAATGCCA	TCCAGGGACCTTTTGTCTTG	HEX	257	231
D17Mit180	AGACACTGTCTAAAAACACAAGATGG	TTGTGTTCATATGCATGTGTGC	TET	155	157
D17Mit20	AGAACAGGACACCGGACATC	TCATAAGTAGGCACACCAATGC	FAM	187	177
D17Mit119	CCTCCTGTTCTGAACTTCAGC	TCGATGCAACCCAGTATAAAA	HEX	153	145
D17Mit218	GGAGAAGATGGGAGAAAGGC	CAAAGCATTTCCAAGCATAGG	TET	123	103
D17Mit142	AATATATATATCCTGGAGCCAACACA	ACCTTTATGAAGTTATGCTGAGTATCA	FAM	154	128
D17Mit76	CTCCTCACCCAGATTCTTGTAA	TTTCGCAAGTTATTTTAACCCG	HEX	131	97
D18Mit64	TCAGATTCACTGCTAAGTCTTTTC	AGCAAGAAAAGCAGGTGAGG	TET	159	179
D18Mit116	CCTTAAAGGAGTGTGTATATTTTTGTG	TTGATGTTATCCTCTGGGCC	FAM	140	96
D18Mit34	CACTGGATGACACAGCCTGT	GATGTTTCCTTGGGTTTGTCA	HEX	141	149
D18Mit119	AGATGCTTGTGAAACATACATATGTG	GAGTGTATAGCGGACTTTTGGG	TET	162	136
D18Mit177	CTGTAGTTTATCAGTTCACCCTGTG	TGTGCTGTTAAACAAATATCTCTGG	FAM	179	175
D18Mit74	AGCCAGAGCTACAAAGTTTCAA	GCTCTTGTAGAGCCATCATTCC	HEX	227	191
D18Mit124	CCCAAATGGGGTGTCTTTTA	CTGCCACACATTTGTGTGTATG	TET	158	146
D18Mit40	GGTAGGAGTCACTTTCCGTCC	TTTTGTGAGCATTTTTATACCATT	FAM	149	139
D18Mit184	CACACATGTGTAGGTAGGTAGGTAGG	CGCACAAGGACTACTGAAACA	HEX	179	134
D18Mit186	AAGTGTTGGGCAAAGGCTAA	CTTTAGTATAGTGTGCATGAGTGTGA	TET	133	115
D18Mit48	TTGCACTCACAGGGCACAT	TCAGAGTTTCCAGAAGACACCA	FAM	173	165
D18Mit144	TAGGGTTTTTTTTTTCTTTTTCTCC	GATAAAAAAATATGTTCACAAAACGC	HEX	187	184
D19Mit59	CTCTAACTATCCTCTGACCTTCACA	TTTTAAGCAGAACATTGAGGACC	TET	206	146
D19Mit56	CTGAATGTGTATGTGTGCAAGTATG	ATTATGAATTCAAGACTAGCCTAGGA	FAM	145	137
D19Mit128	GGCAGGAGAATGTATTCAGAAA	TCCTCCAACCTGCTTCCTC	HEX	130	152
D19Mit96	CTTAACTGCAGTTTTAAAAGACATTTG	CATTTGAGAGAATGTTTGAACATACA	TET	128	120
D19Mit106	CCTTTTTTTTTTTAACCAGACAGG	ATCAATGAATGAAGAACAAATAGTTTC	FAM	131	123
D19Mit40	CAGGGTAGTATTGCAGATAATCAA	AAAGTTTCTTTGTGTGTGCACG	HEX	119	113
D19Mit119	CACCCACATACCTTGATT	CTCTCTTTATCTCTCCTCTCTCT	TET	271	283
D19Mit13	TCTGGCACAAAGAGTTCGTG	CTTTTGCAGGAGCAGGTAGG	FAM	253	269
D19Mit90	GTGGGAATCAATTTTAGTATGAACA	GGATGCTTGATATCATGTACATACA	HEX	139	133
D19Mit83	GACACATGCGGCATACAGTC	CTTGCTCTGAGTATTTTAATGACTGC	TET	134	112
D19Mit26	TTGTTACACAGCAAAATCCTGC	TTGAGGAGTAAGGCAAAAAAGG	FAM	139	141
D19Mit6	ATTAGTAAACTGACTCCCATGCG	CTCATGAGTCCCCTGGGTTA	HEX	119	123
DXMit54	ACATCTGATGTGGATGTTCAGC	CTCCGATGGAAACTGTTGGT	TET	199	151
DXMit81	GAGGAGCATCAACCTTCTCG	GAGGTGGGGAGAAACAGAGG	FAM	206	200
DXMit105	AAATTGGAGTGACCTCAGATTTG	CCATGTTTCTCACCATGAAGA	HEX	157	153
DXMit140	ACATGAAAGTTAGAAAGAGACCCG	GTGCACATTTGTGTGTGTATGC	TET	115	129
DXMit46	CTTTCCTGAGTGCCTCTTGG	TTCTGAATCTGTAATCTGTCTGGC	FAM	257	253
DXMit119	CTTTAACCATAATAATGGCCTTGC	GGGTTCTGTGATCGCAAGTT	HEX	161	175
DXMit93	TTGTCAGAATGATCGATTCTTATATC	CACCCAAAGTAGTTAGATCTTATCATT	TET	207	209
DXMit16	CTGCAATGCCTGCTGTTTTA	CCGGAGTACAAAGGGAGTCA	FAM	125	93
DXMit170	TGCAGGCACTAACAGTGAGG	TAGTTTCACTGTGCCATTGTATACA	HEX	123	129
DXMit64	GGATCAGTTAGCAGGGAAAGG	CACAGACTGAGAAGGCTGTCC	TET	141	121
DXMit173	ATTTGATGTCCTCGTCTGGTG	TAATTATACTGGGGACTAGAACTCAGG	FAM	132	136
DXMit130	TTCATATCGCCCCAACCTAC	TATTTTGAAACCTCTGCCATTT	HEX	175	153
DXMit10	GAATTACAGGCATGCGTCCT	TGTTTGACTGAGAGGATGCG	TET	247	245
DXMit186	ATCAATGCATAGTATTTGGGCC	AATTTGTCACTGCGGGTAGG	FAM	135	127
DXMit223	TTGGTTTGGGGTTTTTTTTG	ATTCCTGATAATGTCTTCTGGACA	HEX	112	116

8.2 Publications

DREWS, E., I. RÁCZ, A. DIAZ LACAVA, A. BARTH, A. BILKEI-GORZÓ, T.F. WIENKER, A. ZIMMER, 2009 Quantitative trait loci contributing to physiological and behavioral ethanol responses after acute and chronic treatment. Int J Neuropsychopharmacol, submitted.

8.3 Declaration

I hereby solely declare that I prepared this thesis entitled: “Identification of Quantitative Trait Loci in Alcoholism” entirely by myself except otherwise stated. All text passages that are literally or correspondingly taken from published or unpublished papers/writings are indicated as such. All materials or services provided by other persons are equally indicated.

Danksagung

Besonderer Dank gilt Herrn Prof. Dr. Andreas Zimmer für die Überlassung des Themas, für seinen fachlichen Rat und die fortdauernde Unterstützung während der gesamten Zeit meiner Doktorarbeit.

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Auf keinen Fall unerwähnt bleiben dürfen Britta Schürmann, Jennifer Rehnelt, Pamela Frisch, Julia Woelke, Angela Harmeth und Kerstin Michel: Ohne euch wäre ich oftmals verzweifelt!

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So eine Arbeit wird eigentlich nie fertig, man
muss sie für fertig erklären, wenn man nach
Zeit und Umständen das Möglichste getan hat.

Johann Wolfgang von Goethe (*1749, †1832)