Identification of genetic factors involved in the regulation of stress

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Abbreviations

A	Adenine / Adenosine
ACTH	Adrenocorticothropic hormone
ßgeo	ß-galactosidase / neomycin phosphotransferase
bp	Base pair
C	Cytosine / Cytidine
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
cM	Centimorgan
cm	Centimeter
CMS	Chronic mild stress
CO_2	Carbon dioxide
CRH	Corticotrophin releasing hormone
dB	Decibel
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
ENU	N-ethyl-N-nitrosourea
Enoph1	Enolase-phosphatase 1
ES cell	Embryonic stem cell
F1	First filial generation
F2	Second filial generation
FAM	6-carboxy-fluorescine
FST	Forced swim test
g	Gravity
G	Guanine / Guanosin
GABA	Gamma-aminobutyric acid
GAD	Generalized anxiety disorder
GMEM	Glasgow's minimal essential medium
HEX	Hexachloro-6-carboxy-fluorescine
HMM	Hidden Markov model
HPA	Hypothalamo-pituitary-adrenocortical
HPLC	High performance liquid chromatography

Hsd17b11	17ß-hydroxysteroid dehydrogenase 11
Hsd17b13	17ß-hydroxysteroid dehydrogenase 13
kb	Kilobase pair
kHz	Kilohertz
LOD	Logarithm of the odds
LOG	Common logarithm
m	Meter
mA	Milliampere
Mb	Mega base
MDD	Major depressive disorder
mg	Milligram
MgCl2	Magnesium chloride
min	Minutes
mm	Millimeter
mM	Millimol
mRNA	Messenger RNA
ms	Milliseconds
n	Sample size
nm	Nanometer
ng	Nanogram
OMIM	Online Mendelian Inheritance in Man
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Panic disorder
Pde6b	Phosphodiesterase 6B
PVN	Paraventricular nucleus
QTL	Quantitative trait locus / loci
rd1	Retinal degeneration 1
RFLP	Restriction fragment length polymorphism
RI	Recombinant inbred
RNA	Ribonucleic acid
ROX	6-carboxy-X-rhodamine
RT	Room temperature
SA	Splice acceptor

SAM	S-adenosylmethionine
SD	Splice donor
sec	Seconds
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
T	Thymine / Thymidine
TAE	Tris-acetate-EDTA
TE	Tris EDTA
TET	Tetrachloro-6-carboxy-fluorescine
TPH2	Tryptophan hydroxylase 2
Tris	Tris (hydroxymethyl) aminomethane
TST	Tail suspension test
U	(Enzyme) Unit
UV	Ultraviolet
V	Volt
vol.	Volume
W	Watt
wt	Wild type
µl	Microlitre

Index

VIII

1 Introduction

In modern societies, the appearance of many diseases can be traced back to an increased stress exposure. The response to stress is highly dependent on the distinct reaction of each individual and ranges from highly stress-sensitive people to those who can tolerate intensive stress without any outcome, especially in health (Herman and Cullinan 1997). Responses to stress are shaped by a combination of several components, which may be vegetative, cognitive, emotional or behavioral in nature. In mammals, this response to stress is evolutionarily conserved and facilitates appropriate behavioral reactions to anxious or fearful situations. Behavioral responses like avoidance, flight or estimation of the potential risk are defensive and induced by intermediate anxiety states. In cases where flight is impossible or the situation is more fearful, the defensive behavior is replaced by an offensive fight response. When very strong threats induce panic or extreme anxiety, a freezing response is elicited as a worst-case reaction in order to escape this situation by mimicking a dead individual (Blanchard et al. 2003). The main system of the brain, which regulates the response to stress, is the limbic system. It is established by the interconnections of other brain regions, mainly the hippocampus, the amygdala, the hypothalamus and the prefrontal cortex. All these regions fulfill specific functions during stress response, like the amygdala, which processes the reaction to environmental input (Gordon and Hen 2004, McEwen and Gianaros 2010).

If an individual is in an anxious situation, which will interrupt the homeostasis, the brain will induce corresponding behaviors and elicit changes in the hormone system in order to adapt homeostasis to the situation. Behavioral stress responses like fear and anxiety also include emotional aspects, which demonstrate a high variability among individuals. This seems to be a strategy of survival for the individuals, since the variation ensures the survival of the population (Levine and Ursin 1991). Because of these circumstances, the response to stress does not harm the individual's health; rather it enhances it. Normally the response occurs only for a short period of time, when threatening stimuli are present, and relapses afterwards. If an individual experiences stress for a long time, the homeostasis can be changed substantially and can adversely affect health (Fuchs and Flügge 2003).

Anxiety-related behavior is not solely affected by stress, since stress can also strongly influence depression. It is known that depression is strongly connected with

stressful life events. Stress in early life seems to be a major risk factor for the later development of depression disorders. The possibility of developing stress-related disorders is determined by the individual's genetic predisposition and whether they had previously suffered stress in early life and for long periods (Charney and Manji 2004).

When focusing on psychiatric disorders which are related to stress, anxiety and depression disorders are the most common ones. Worldwide, 10-15 % of the human population are affected by these disorders (Reul and Holsboer 2002). In Germany, a survey revealed in 1998 that over 14 % of the 18 to 65 year old people interviewed suffered from clinically relevant anxiety disorders. Female patients are affected twice more when compared to male patients. The survey indicats furthermore that 15 % of the women and 8.1 % of the men questioned had suffered a depressive phase in the last twelve months. There are also twice more women than men affected by depression. The study also shows that 40-70 % of suicides can be traced back to depression. In 2004, a survey revealed that 11,000 suicides were registered in Germany that year (Robert Koch-Institut 2006). This demonstrates the importance of stress as well as the stress response to mood constitution and the homeostasis in humans.

1.1 Anxiety and depression

Several emotions exist in animals and humans, anxiety being a fundamental one. Fearful situations or other anxious stimuli like novel environments, loud noise, sudden movements or odors of enemies can induce such emotions as responses (Blanchard and Blanchard 1972, Endler 1986, King 1999).

Anxiety disorders can be categorized in six different types. The first is panic disorder, which is marked by sudden bursts of anxiety. Generalized anxiety disorder (GAD) forms the second type, which is expressed by strong anxiety in various situations without any distinct stimuli. The third type is social phobia, which is marked by the avoidance of social situations. Specific phobias form the fourth type of anxiety disorders. Phobias induce strong fear as a response to naturally fearful stimuli like spiders or small spaces. The fifth type is the post-traumatic stress disorder, which is characterized by traumatic memories eliciting anxiety episodes. Obsessive-compulsive disorder is the last type, marked by mania and compulsive behaviors, which are both induced by anxiety (Gordon and Hen 2004).

Besides the different brain regions, there are also different neurotransmitter systems, which regulate the stress response and thus anxiety. Such neurotransmitters are the serotonergic, the noradrenergic and the GABAergic systems. Furthermore, the hormonal systems, especially the hypothalamo-pituitary-adrenocortical (HPA) axis, are involved in the regulation of stress response and also influence anxiety as a modulatory system (Hohoff 2009, Reul and Holsboer 2002). In the HPA axis, the corticotrophin-releasing hormone (CRH) and vasopressin are synthesized and secreted from the paraventricular nucleus (PVN) of the hypothalamus. These two peptides react on the anterior lobe of the pituitary gland where they stimulate the secretion of the adrenocorticotropic hormone (ACTH). The ACTH itself stimulates the production and release of glucocorticoid hormones (mainly corticosterone in rodents) in the adrenal cortex. The glucocorticoids build a negative feedback on the hypothalamus and pituitary gland in order to suppress the CRH and ACTH production (Figure 1) (Holsboer and Ising 2008).

In recent years, many studies have identified the importance of CRH, as well as its receptors, in the regulation of anxiety and depression (Arborelius et al. 1999, Hauger et al. 2009, Reul and Holsboer 2002). Several clinical studies also revealed increased CRH levels in patients with anxiety or depression disorders (Bremner et al. 1997, Erhardt et al. 2006, Landgraf 2006, Raadsheer et al. 1994). The glucocorticoid hormone cortisol, respectively corticosterone, synthesis is increased by high CRH levels via risen ACTH levels. The glucocorticoid hormones regulate the expression of several other genes in the brain, like tyrosine aminotransferase, phosphoenolpyruvate carboxykinase, insulin-like growth factor 1, pro-opiomelanocortin, prolactin, and the neuronal serotonin receptor (Revollo and Cidlowski 2009). Therefore, long-term exposure to this stress hormone can induce stable changes in gene expression patterns in the brain, leading to emotional changes like depressed mood (Holsboer and Ising 2008). Since the HPA axis plays a major role in the regulation of stress response, it is also involved in the outcome of stress-dependent disorders like anxiety and major depressive disorder (MDD).

Naturally, anxiety and depression are two separated disorders, but they often present comorbidity. In the United States, a survey revealed that 58 % of the people who suffered from a major depressive disorder also came down with an anxiety disorder. Equal results could be identified for the reverse, as 67 % of patients with generalized anxiety disorder also exhibited a unipolar depressive disorder (Judd et al. 1998,

Kessler et al. 2005, Pollack 2005, Simon 2009). The diagnosis of comorbid anxiety and depression disorders tends to be difficult, since anxiety disorders normally develop in early life and before depressive disorders. Thus, a GAD will be diagnosed prior to an MDD in patients with comorbidity. Furthermore, patients present a higher possibility for the onset of MDD when they have developed a GAD the year before (Kessler et al. 1996). The biological basis of comorbidity in anxiety and depression disorders is completely unknown, various possible factors have been identified which seem to play a role in this context. The HPA axis is one of these factors, while overlapping genetic sources in both diseases and equal environmental factors are also probably involved (Simon 2009).
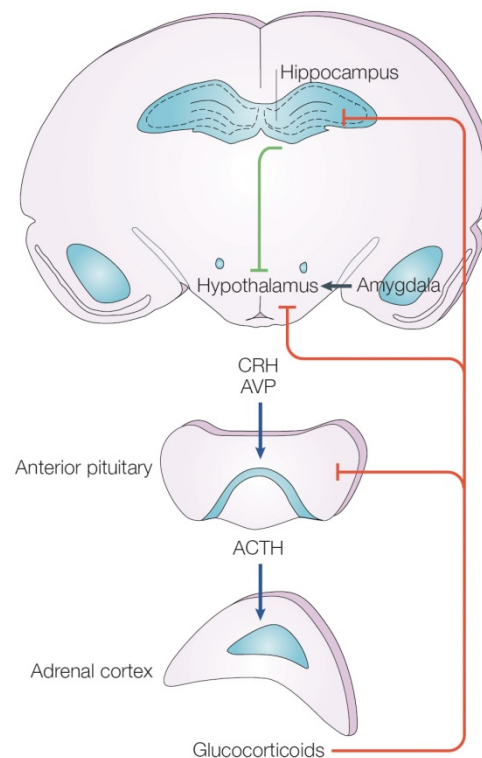
Figure 1. Schematic of the HPA axis. Reduction in glucocorticoid levels leads to an increase in corticotropin-releasing hormone (CRH) and vasopressin (AVP) production in the hypothalamus. Both hormones stimulate the production of adrenocorticotropic hormone (ACTH) in the anterior pituitary gland. This hormone enhances the secretion of glucocorticoids from the adrenal cortex in kidney. Then, the secreted glucocorticoids inhibit the secretion from the anterior pituitary and the hypothalamus by a negative-feedback loop. Additionally, glucocorticoids can bind to nuclear corticosteroid receptors in these brain regions in order to regulate gene expression. (According to Sandi et al. 2004)

1.2 Animal models in anxiety and depression

Various approaches are applied for studying the pathogenesis of human anxiety and depression disorders as well as their underlying complex biological mechanisms. Since human anxiety and depression diseases include complex emotions and cognition, unique experiences and different genetic backgrounds, simplified approaches are utilized to study these diseases. Mice serve as a useful tool for this purpose (Blanchard et al. 2001, Gordon and Hen 2004). These approaches should meet three criteria before they are validated as adequate animal models. The first criterion is construct validity, which implies that the model is comparable with the human cause of the disease. The next criterion is face validity, which claims an analogy between the phenotype of the animal model and the outcome of the human disease. Predictive validity marks the third criterion and requests equal responses of the model to treatments, which proved effective in humans. It is mostly verified by the application of drugs known to be effective in humans and generates results comparable to autonomic and behavioral reactions in humans (Chadman et al. 2009).

Besides the brain areas, which are connected to anxiety and depression, the neurotransmitters and neuromodulators are also conserved between mice and humans (Leonardo and Hen 2006, Urani et al. 2005). For this reason, different paradigms were developed in order to measure anxiety- and depression-related behaviors in mice (Dalvi and Lucki 1999, Hohoff 2009). The response of humans to stressful or threatening stimuli can be simulated in aspects by these paradigms. The behavioral paradigms are sorted into five groups for the measure of anxiety-related behavior: ethological conflict tests, conditioned fear tests, punishment-induced conflict tests, aversive tests and drug discrimination tests (Shekhar et al. 2001).

All these paradigms can furthermore be grouped into two classes, i.e. the conditioned and the unconditioned models. The conditioned models are comparable with human fear conditioning, since they are strongly influenced by learning and memory, as well as the motivation of mice. The unconditioned models mimic human panic disorder (PD) or GAD. These models induce a conflict between the natural exploration behavior of the mice and their natural aversion towards novel, high, open or bright environments. This conflict leads to avoidance, hiding, freezing or panic behavior in the mice (Hohoff 2009).

The type of anxiety measured by behavioral paradigms can be separated into 'state' and 'trait' anxiety. State anxiety is experienced exactly at the moment of the test and it is not stable, since there are temporary fluctuations mostly induced by external stimuli. In contrast, 'trait' anxiety describes the reaction to different situations by generally elevated anxiety levels for a longer time. External stimuli do not influence this type of anxiety and it is more constant over time compared to state anxiety (Andreatini and Bacellar 2000, Belzung and Griebel 2001). The most common tests to achieve 'state' anxiety behavior are the zero maze, the elevated plus maze, the light dark and the open field test. All these paradigms induce a conflict between the mice's interest in a novel environment and avoidance of an aversive, mainly open surrounding. For the measurement of 'trait' anxiety, the acoustic startle response is widely used, which measures the intensity of a startle reflex after the presentation of a sudden loud noise (Andreatini and Bacellar 2000, Belzung and Griebel 2001, Hohoff 2009).

Depression is a very heterogeneous disorder, as it turned out difficult to copy, even in parts, in the laboratory (Cryan and Mombereau 2004). Although several drugs or stressful situations can induce anxiety, which can be handled quite easy, the initiation of depression in animals or humans is more difficult (Blanchard et al. 2003, Shekhar et al. 2001, Sullivan et al. 2003). Most of the depression-related paradigms for mice demonstrated only predictive validity, confirmed with clinically effective antidepressant drugs. There exist four major models for depression, which are widely used in depression research (Dalvi and Lucki 1999), i.e. the forced swim test (FST) (Porsolt et al. 1978), the tail suspension test (TST) (Steru et al. 1985), the olfactory bulbectomy (OB) (Kelly et al. 1997) and the chronic mild stress (CMS) model (Willner et al. 1997). Among these, the FST and TST are the most commonly applied paradigms, since they can also be easily conducted in high-throughput testing (Cryan and Mombereau 2004).

The numerous inbred mouse strains presented substantially different behaviors in anxiety- as well as depression-related paradigms (Crawley et al. 1997, Lucki et al. 2001). Thus, the background strain should be selected carefully for use in genome-wide studies. In order to retrieve enough power for genome-wide studies, there should be a robust behavioral difference in anxiety- and depression-related behavior between the background strains.

1.3 Genetics of anxiety and depression

There exists a broad individual variability in the risk to develop an anxiety disorder, with 30–50 % of the variability depending on genetic factors and interactions between genes. The remaining variability is mainly influenced by gene-environment interactions (Hettema et al. 2001, Kendler et al. 2001, Roy-Byrne et al. 2002). Similar data were identified in human twin studies for depression disorders (Sullivan et al. 2000). The main focus in the neuroscientific area lies in revealing the numerous genetic factors of psychiatric diseases that are responsible for the genetic variability of such diseases. Genetic linkage studies have emerged as a powerful tool to identify the respective genes. Regarding this aspect, a number of genetic studies focused mainly on genetic associations in the past decade. The genetic linkage approach is based on tracing chromosomal segments in families, in order to search for specific haplotypes that segregate in individuals carrying the disease. In the classical analysis, a likelihood-based method is mostly applied, which evaluates the likelihood that the disease can be correlated with a particular marker and both segregated within the family. The likelihood-based research for linkage is an excellent approach to identify rare DNA variants that lead to large effects in individuals. This method was intensively applied in genetic studies of psychiatric disorders, since its first successes in other medical disorders. However, major results were still missing, and remain so even nowadays. The unsuccessful outcome of these linkage studies for psychiatric disorders is a result of the large number of genes, which all contribute to the risk of these complex disorders and present low influence on the liability of the diseases. This changed comprehensively with the rise of genetic association approaches that emerged as a new tool for the identification complex traits. Like linkage, the genetic association method is based on the assumption that the human population can be handled as a large pedigree. In this pedigree, large DNA segments were cropped by recombination to very small chromosomal regions, where a genetic variant can be detected, which is shared by many unrelated people with the same disorder. During the past decade, thousands of genetic studies were carried out with this approach for several psychiatric disorders (Hamilton 2009). These studies are mainly based on two types of variation. Single nucleotide polymorphisms (SNPs) are the first type of variation, which occur in single DNA bases. SNPs are highly common in the genome and can be found at 1 out of 1,000 bases on average. The second type of variation is based on short repetitive sequences, which consist of two to six nucleotides and can

be detected with variable length, mainly around genes. This variation type is also known as DNA microsatellites and they are completely independent of diseases in contrast to SNPs (Smoller et al. 2009).

Linkage studies have revealed several suggestive linkages for panic anxiety phenotypes on a broad range of chromosomal regions (Table 1). Phobic disorders were linked with this method to 3 chromosomes (Table 1), whereby obsessive-compulsive disorders were linked to 4 loci (Table 1) (Smoller et al. 2009). With bipolar depression linkage studies, as many as 10 chromosomes were associated with this disorder and 11 loci were mapped (Table 1). For MDD, 7 genetic regions were identified (Table 1) (Hamet and Tremblay 2005).

Table 1. Linkage of human genetic regions to psychiatric diseases (Modified according to Hamet and Tremblay 2005, Smoller et al. 2009)

Disorders	Genetic loci
Panic anxiety phenotypes	1q, 2q, 7p, 9q, 12q, 15q, 22q
Phobic disorders	3q, 14q, 16q
Obsessive-compulsive disorder	3q, 9p, 10p, 14q
Bipolar depression	1q, 4p, 10p, 11, 12q, 13q, 18pq, 20q, 21q, 22q, Xq
Major depressive disorder	1q, 4q, 7q, 8p, 11q, 12q, 13q

Despite the fact that many loci are already linked to psychiatric disorders, there is still a relative lack of success for this method. This is mostly due to the large number of genes contributing to these complex disorders and furthermore to the limited effect of each gene for the whole phenotype (Hamilton 2009).

Based on new technical developments like high-throughput microsatellite or SNP screenings, association studies evolved as a follow-up approach to linkage studies. They focused on candidate genes, which were identified in earlier studies or were located within the genomic regions indicated in linkage studies so far. The candidate genes, which were associated with psychiatric disorders, encode for receptors, transporters or they play a role in neurotransmitter systems. Other candidate genes encode for neuropeptides, which were identified in earlier animal studies (Smoller et al. 2009). The association studies have discovered 76 discrete genes in anxiety disorders. However, there are huge differences in the phenotypic assessment in these studies, leading to controversial findings. Despite the high number of individually assessed candidate genes, only a handful were identified in two or more studies as risk genes for anxiety disorders, like the catechol-O-methyltransferase or

serotonin transporter (COMT, SLC6A4) (Hamilton 2009, Smoller et al. 2009). A very similar situation can be seen for depression. Until now, not only several loci were identified in linkage studies but also many genetic variants were detected in association studies (Levinson 2006, Schulze 2010). However, the meta-analysis of genetic studies on major depressive disorders only revealed six susceptibility genes with statistical significance. The major problem of the predominant number of studies was the insufficient statistical power due to small sample sets (Lopez-Leon et al. 2005, Lopez-Leon et al. 2008).

Besides the linkage and association approaches on a genome-wide range, the classical candidate gene identification and evaluation in genetically modified mice is still an important source of information. For the functional analysis of genes, generation of transgenic mice and gene knockouts by homologous recombination were the methods of choice in recent decades. This method, however, reaches its limit in the detection of phenotypic variances caused by a gene, which had only minor effects on these variances like in the complex traits of anxiety- and depression-related behaviors (Gordon and Hen 2004, Hamet and Tremblay 2005). Collaborations like the International Mouse Knockout Consortium have been working to knock out every protein-coding gene until the end of 2012. This enthusiastic goal shall be reached using the gene trap technique, a high-throughput knockout strategy with random integration of viral vectors in mouse embryonic stem (ES) cells (The International Mouse Knockout Consortium 2007). The completion of this project raises the possibility of detecting behavioral phenotypes of new gene knockouts and identifying many new candidate genes. However, the problem of small effect sizes of many genes still remains. It seems that only a combination of genome-wide studies with candidate gene analyses and growing bioinformatic analyses will be able to reveal significant candidate genes for complex traits.

1.4 Quantitative trait loci analysis

Anxiety- and depression-related behaviors are complex traits, which means that these behaviors are influenced by many genetic loci, each contributing only to a limited extent to the phenotypic variance. This variance is described by a quantitative trait and the genetic locus, that contributes to this quantitative trait is, determined by a quantitative trait locus (QTL). Thus, a QTL analysis associates the genetic variation with trait variation and identifies the QTL.

Researchers have mapped several thousand QTL for human and mouse traits. The genes of mice and humans are arranged syntenically, thus enabling the comparison and validation of the according QTL for equal traits (Pennacchio 2003). This concept was confirmed, for instance, in atherosclerosis (Wang et al. 2005) or kidney disease (Korstanje and DiPetrillo 2004), demonstrating the assignability of QTL studies in humans and mice. Using mice for a QTL approach offers a number of advantages compared to human studies. The numerous inbred mouse strains differ in their physical and behavioral phenotypes, which are passed on and stable among each strain. Additionally, the perturbing problem of genetic variability can be excluded, since the genomes of animals within the same inbred strain are identical and the influence of environmental factors is controlled by the experimental procedures. Consequently, the variance in the behavior of mouse strains are a result of the genetic differences, which can be detected and analyzed by QTL mapping (Hovatta and Barlow 2008). For this purpose, the strains should exhibit strong variance in behavioral phenotypes. The level of difference between the two strains in the phenotype of choice reflects the level of probability to identify related QTL loci (Hovatta and Barlow 2008, Moore and Nagle 2000, Peters et al. 2007). Following the selection of appropriate inbred strains, mice are bred in order to receive a second (F2) generation, in which the phenotype-causing alleles segregate. There are two options to receive this F2 generation, either by intercrossing offspring of the first (F1) generation, or by backcrossing the F1 generation with the parental strains. A third method of QTL analysis is based on the generation of recombinant inbred (RI) strains. For this method, the F2 generation mice are continuously bred for 20 generations by brother-sister mating in order to get inbred animals that are homozygous for recombinant chromosomes. The RI strains are very popular in QTL studies, despite the fact that the number of strains in the classical RI strains is relatively small and therefore resolution of the QTL analysis is limited (Flint 2003, Moore and Nagle 2000, Peters et al. 2007). The most commonly used strategy is to produce a second-generation cohort by intercrossing. This strategy is most suitable when on the one hand both parental strains exhibit different phenotypes within the same behavioral test and on the other hand an intermediate phenotype can be detected in the F1 generation (Moore and Nagle 2000).

For the assessment of genetic variance in a QTL study, microsatellite markers are widely used. These markers are short DNA sequences in non-coding genomic

regions, which consist of short tandem repeats of one to six nucleotides. The lengths of the repeats exhibit a wide variance between human individuals and in different inbred mouse strains (Bennett 2000, Schlötterer 2000). Microsatellite markers are randomly distributed throughout the genome and their high level of polymorphism strongly facilitates their use for the construction of genetic maps. Microsatellites can be easily amplified by polymerase chain reaction (PCR) and can be subsequently sized for their length in high-throughput capillary sequencers, which make them highly versatile markers (Bennett 2000, Dietrich et al. 1994).

Figure 2. Illustration of microsatellites. The upper part shows three different alleles of a microsatellite marker with a CA di-nucleotide (C: Cytidine; A: Adenosine). The first allele has 15 repeats, the second 17 repeats and the third 18 repeats. The arrows indicate the flanking primers for the amplification of the microsatellite by PCR. In the bottom part, a schematic gel is illustrated with probes of individuals, which are either homozygous for each allele or heterozygous for all possible combinations. The different lengths of the respective alleles can be clearly identified in the gel. (According to Silver et al. 1995)

One can conclude that even though thousands of QTL have been identified so far, elucidation of their underlying genes is mainly missing and only a handful of genes could be verified as candidate genes (Flint et al. 2005). It is therefore an important, though challenging issue to narrow a QTL interval in order to get a small number of candidate genes that can then be validated by follow-up experiments. A promising strategy is the implementation of a comparative genomics approach, if this is feasible. Such an approach is based on the structural conservation among the mammalian genomes. The comparison of human and mouse genomes revealed approximately 340 conserved syntenic segments (Pennacchio 2003). This offers the

opportunity to compare human and mouse QTL linked to equal traits to narrow the QTL, supposing that the underlying gene lies in the common region (DiPetrillo et al. 2005). The feasibility of this approach was elegantly demonstrated by the identification of 66 candidate genes for kidney disease by comparison of rat and human QTL (Vitt et al. 2004). In order to select possible candidate genes, an innovative method arose within the last year, which is based on semantic similarity in biomedical ontologies. These ontologies grew rapidly in coverage, formality and integration in recent years, making them suitable for similarity searches in gene ontologies (Pesquita et al. 2009). In this context, Schlicker and Albrecht developed a software tool for the comparisons of gene ontologies and the identification of prominent candidate genes. Gene ontologies of candidate genes are compared to those of a known disease gene The software generates a list of genes, prioritized in their functional similarity to the known disease gene (Schlicker and Albrecht 2010). This approach will dramatically influence the detection of candidate genes in the coming years.

Moreover, the identification of QTL will be also enhanced in the near future, since the mapping of genetic variants is starting to be carried out with high-throughput SNP screenings instead of microsatellites. This will improve the detection of QTL with small effect sizes, even in crosses of closely related inbred strains (Eisener-Dorman et al. 2010). Likewise, the Collaborative Cross will be available soon, which consists of about 1,000 recombinant inbred strains derived from eight classical inbred strains. Although the Collaborative Cross will not have a resolution on gene basis, it will still result in shorter QTL intervals, which subsequently improve the detection of QTL with lower effect sizes (Flint and Mott 2008).

1.5 Gene trap knockout

The gene knockout technique in mice is an invaluable tool to get deeper insights into the functions of genes. This technique can be performed by either homologous recombination, by random mutagenesis in embryonic stem (ES) cells with mutagens like N-ethyl-N-nitrosourea (ENU), or by gene trapping with viral vectors. Even though the homologous recombination generates a well-defined knockout, it is a very time-consuming method. The ENU mutagenesis is an inexpensive, though completely undirected method of generating knockout mice. Moreover, the screening for the affected genes is time-intensive (Skarnes 2005). Vectors of retroviruses, on the other

hand, show a high affinity to insert in the 5' region of a gene, mainly in the 5' untranslated region and the first intron. This enhances the efficiency of the insertion and thus results in a higher percentage of null mutations. The use of viral vectors further ensures that there occurs only a single integration of the vector in the genome (Stanford et al. 2001). Therefore, gene trap vectors based on retroviruses are a convincing method for high-throughput mutagenesis projects.

The gene trap vector contains a gene-trapping cassette, which is made up of a promoterless reporter gene combined with a marker gene for selection. A widely used reporter gene is ß-galactosidase. The neomycin-resistence gene is commonly chosen to select clones with integration. A 3' splice acceptor site is positioned upstream of the gene-trapping cassette and a polyadenylation site terminates the cassette downstream. Long terminal repeats surround the whole cassette and mediate the viral integration in the genome. When the whole gene trap cassette is successfully inserted into an intron of a gene, it is expressed under the control of the endogenous promoter. Due to the new splice site, a fusion transcript is transcribed consisting of exons upstream of the insertion site and the reporter respectively the selection genes. The new polyadenylation site leads to the termination of transcription. The final fusion transcript results now in a truncated and therefore nonfunctional protein (Stanford et al. 2001).

Using gene trap vectors, it is also feasible to generate a conditional gene knockout mouse. Herefore, several recombination sites for Cre- and FLPe recombinases flank the gene trap cassette. The gene trap cassette has a classical design composed of a splice acceptor, a reporter gene and a polyadenylation site (Figure 3A). Due to this, the whole cassette can be inversed with FLPe recombinase in ES cells, leading to the gene trap being inactivated. At a later point in time, this process can be reversed with Cre-recombinase, i.e. the gene trap is activated (Figure 3B). For this second step, mice expressing Cre-recombinase under tissue-specific promoters will be quite useful, since tissue and cell-type-specific gene traps are possible (Schnütgen et al. 2005, Xin et al. 2005).

Mouse ES cells with gene trap insertions are catalogued and available through the International Gene Trap Consortium or the subgroup German Gene Trap Consortium, which have generated thousands of gene knockouts in recent years. Considering this background, the generation of knockout mice based on existing ES cell clones can be regarded as highly promising.
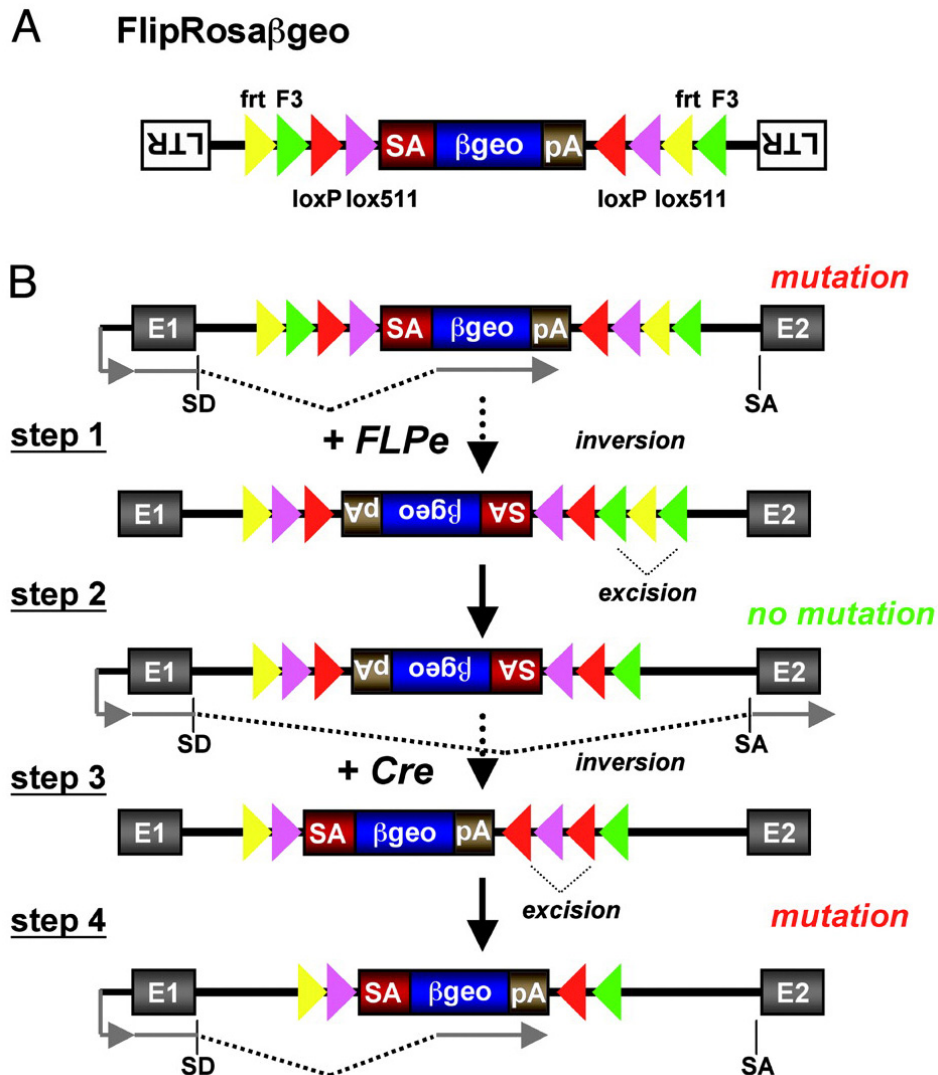
Figure 3. Scheme of a conditional gene trap. A: Illustration of the retroviral gene trap cassette. Abbreviations: LTR, long terminal repeat; frt (yellow triangles) and F3 (green triangles) are target sites for the FLPe recombinase; loxP (red triangles) and lox511 (purple triangles) are target sites for the Cre-recombinase; SA, splice acceptor; ßgeo, ß-galactosidase/neomycin phosphotransferase fusion gene; pA, polyadenylation signal B: Conditional gene knockout by a retroviral gene trap cassette. The integration of the gene trap cassette in an intron of a gene is presented and expressed transcripts (gray arrows) are spliced between the splice donor (SD) of exon 1 (E1) and the SA of the gene trap cassette. In this case, the expression of the ßgeo gene is activated and the early termination of the endogenous transcript leads to a knockout. In step 1, the FLPe recombinase inverts the gene trap cassette at the designated frt recombination sites. Additionally, the FLPe recombinase simultaneously excises the frt recombination sites (step 2), which locks the cassette for a reinversion. This induces normal splicing with the endogenous splice sites and rescuing the knockout. It is reversed in steps 3 and 4 by Cre-recombinase, which inverts the gene trap cassette back to the active position and leads to gene knockout. This stable recombination results in a product, which cannot be reversed. (Modified according to Schnütgen et al. 2005)

1.6 Aim of the study

Several studies have identified genetic regions linked to anxiety- or depression-related behavior in mice or to the respective anxiety and depression disorders in humans like GAD or MDD. Despite the enormous amount of data retrieved by all of these linkage or association approaches, only a handful of genes that are involved in anxiety or depression are identified as having statistical significance. Thus, the genetic background of these disorders remains unclear in numerous parts and many genes are still unknown due to their limited effect size on complex disorders or behaviors. It is an important issue to firstly reveal the genetic basis of anxiety and depression before the even more complex gene-environment interaction can be examined in detail.

For this reason, the aim of the present study was the detection of new genetic loci and the identification of new candidate genes contributing to anxiety- and depression-related behavior. A QTL study was carried out in the F2 generation of an intercross between C57BL/6J and C3H/HeJ mice. At first, over 500 animals of the F2 generation were phenotyped in different behavioral paradigms evaluating their anxiety- and depression-like behaviors. In the next phase, all animals were genotyped by microsatellite markers. The establishment of a high-throughput microsatellite mapping with a dense marker map of 269 microsatellites for the parental mouse strains was also an aim of this study. Subsequently, the phenotypic and genotypic data were conducted in a QTL analysis in order to reveal the respective genetic loci linked to anxiety- or depression-like behavior. Prominent candidate genes were selected, after narrowing of some QTL. Moreover, new bioinformatic methods were applied in order to reach this goal. The verification of selected candidate genes was finally accomplished by an evaluation with biomolecular as well as biochemical analyses.

2 Material

2.1 Equipment

Activity-tracking System	Actimot, TSE Systems
Centrifuges	Biofuge fresco, Heraeus Instruments
	Biofuge stratos, Heraeus Instruments
Digital gel documentation	Chemi Doc Syst CCIR, Bio-Rad Laboratories
Electrophoresis chamber	Sub-Cell GT, Bio-Rad Laboratories
Genetic Analyzer	ABI 3130xl, Applied Biosystems
Homogenizator	Precellys 24, Bertin Technologies
HPLC system	Autosampler 3900; Pump 1000; Manager 5000; UV Detektor 2900; 250 x 2 mm Eurospher 100-3 C18 column: all from Knauer
Light-Dark test chamber	In-house workshop
Liquid handling platform	Multiprobe II, PerkinElmer
Magnetic stirrer	MR 3001 K, Heidolph, Fisher
Microplate Reader	MRX TC II Microplate Reader, Dynex Technologies
PCR cycler	iCycler, Bio-Rad Laboratories
Pipetts	Research (variable), Eppendorf
	Research® pro (multichannel), Eppendorf
	Multipette plus, Eppendorf
pH meter	inoLab, WTW
Pump	Chemistry-Hybrid-Pump RC5, Vacuubrand
Real-time PCR system	7900HT Fast Real-Time PCR System, Applied Biosystems
Spectral photometer	ND-1000, Thermo Fisher Scientific
Startle response system	Startle Response System, TSE Systems
Sterilising oven	Varioklav 25T, H+P Labortechnik
Vacuum dryer	Speed Vac, Savant Instruments
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, Ver. 3.0
GeneMapper	Applied Biosystems, Ver. 3.7
Microsoft Office 2008	Microsoft, Ver. 12.2.3
NanoDrop 1000	Thermo Fisher Scientific, Ver. 3.7.1
PASW Statistics 17	SPSS, Ver. 17.0.2.90
Revelation	Dynex Technologies, Ver. 4.2.2.1
R software language	R, Ver. 2.8.1
R/qtl	R/qtl, Ver. 1.11-12
SDS 2	Applied Biosystems, Ver. 2.2.0.1
Vector NTI Advance	Invitrogen, Ver. 11.0
WinPrep	PerkinElmer, Ver. 1.0.0.1

2.3 Databases and computer programs

The ensembl browser (http://www.ensembl.org) and the National Center for Biology and Information (NCBI; http://www.ncbi.nlm.nih.gov) were the sources of murine as well as human genome sequences.

Ensembl (http://www.ensembl.org):
Ensembl also offers a human homology search function to identify syntenic genomic regions in mice and vice versa. This database was used for homology queries in this study.

FunSimMat (http://funsimmat.bioinf.mpi-inf.mpg.de):
FunSimMat is a comprehensive resource of semantic and functional similarity values retrieved from comparisons of candidate genes with a reference gene. It offers the possibility of disease gene prioritization and was used to prioritize candidate genes.

Mouse genome informatics (MGI; http://www.informatics.jax.org):
The MGI database offers information in genetics, proteomics, phenotypes and other biological data for the laboratory mouse strains. It was used to access data for microsatellite markers, genes, proteins and SNPs, as well as information of the used laboratory mouse strains.

Online Mendelian Inheritance in Man (OMIM; http://www.ncbi.nlm.nih.gov/Omim):
The OMIM database provides information about genes associated or linked with human disorders. It was utilized to identify the reference gene used for the FunSimMat software.

2.4 Chemicals and reagents

All chemicals and reagents used in this work were purchased from Invitrogen, Carl Roth, Merck or Sigma-Aldrich. Otherwise it is indicated in the specific method.

2.5 Enzymes

The HotStarTaq DNA polymerase from Qiagen and the Taq DNA polymerase from New England Biolabs were used for PCR reactions. Restriction enzymes were purchased from New England Biolabs. Superscript™ II from Invitrogen was used for cDNA synthesis and the Platinum$^®$ Taq from Invitrogen for SNP analyses.

2.6 Kits

The following Kits were used:

DNeasy Blood & Tissue Kit	Qiagen
DNeasy 96 Blood & Tissue Kit	Qiagen
BCA Protein Assay Kit	Perbio
peqGOLD Gel Extraction Kit	Peqlab
QIAshredder	Qiagen
RNeasy Mini Kit	Qiagen

2.7 Oligonucleotides

The oligonucleotides used in the experiments of this work were purchased by Metabion International AG. All unlabeled PCR primers are given in Table 1. The primer pairs used to amplify microsatellite loci were ordered as follows:

A fluorescent dye (FAM, HEX or TET) was attached at the 5' end of each forward primer and all reverse primers were labeled at their 5' ends with the sequence GTGTCTT (5'–3'). This sequence promotes the template unspecific addition of nucleotides (+ A) in the PCR reaction. All detailed information about each primer is provided in the appendix (Supplement table S1).

The TaqMan® gene expression assays were purchased from Applied Biosystems. For Enoph1 gene, the Mm01207771_m1 assay was utilized and for the Hsd17b11, the Mm00504406_m1 assay was applied, as well as the Mm01203271_m1 assay for the Hsd17b13 gene. The TATA binding protein (TBP) gene was chosen as the reference household gene with the assay Mm00446973_m1.

Table 1. Unlabeled PCR primers

Name	Sequence 5' - 3'	Comment
SRY 2	TCTTAAACTCTGAAGAAGAGAC	forward primer for mouse sexing; 404 bp; Y chromosome
SRY 4	GTCTTGCCTGTATGTGATGG	reverse primer for mouse sexing; 404 bp; Y chromosome
ZFY 3	AAGATAAGCTTACATAATCACATGGA	forward primer for mouse sexing; 617 bp; Y chromosome
ZFY 4	CCTATGAAATCCTTTGCTGCACATGT	reverse primer for mouse sexing ;617 bp; Y chromosome
NDS 3	GAGTGCCTCATCTATACTTACAG	forward primer for mouse sexing; 244 bp; X chromosome
NDS 4	TCTAGTTCATTGTTGATTAGTTGC	reverse primer for mouse sexing; 244 bp; X chromosome
W149	CATCCCACCTGAGCTCACAGAAAG	forward primer for mutation of Pde6b gene; 298 bp
W150	GCCTACAACAGAGGAGCTTCTAGC	reverse primer for mutation of Pde6b gene; 298 bp
Enoph RT 1 F	GTGTTGCCCTCCTTAACCAA	forward primer for RT PCR of Enoph1 or genetrap; 207 or 256 bp
Enoph RT 1 R	ACACTCCTCCTCCTCCCAGT	reverse primer for RT PCR of Enoph1; 207 bp
1st Race Primer	CAGGGTTTTCCCAGTCACGAC	reverse primer for RT PCR of genetrap; 256 bp
PCR 1 5' SPLK R	CGACCAGCTGTGCGCATAGTG	reverse primer for sequencing of gene trap clones E122 & E237
PCR 1 3' SPLK R	AGTCATAGACACTAGACAATCGG	forward primer for sequencing of gene trap clones E122
PCR 2 5' SPLK R	TTTGGCAAGCTAGCACAACC	reverse primer for sequencing of gene trap clones E261
PCR 2 3' SPLK R	CAGTCAATCGGAGGACTGGCG	forward primer for sequencing of gene trap clones E237 & E261
E122G092 F	TGTCCAGACAAAGCCAGACA	forward primer for sequencing of gene trap clones E122
E122G092 R	AATAAGGCACTCGCCCACTA	reverse primer for sequencing of gene trap clones E122
E237D042 F	CTAGAAGGCAGGAGCAGGTG	forward primer for sequencing of gene trap clones E237
E237D041 R	CAGTCCACGCTAACCACAGA	reverse primer for sequencing of gene trap clones E237
E261G041 F	TGCTGACTAGCAGGGAGATG	forward primer for sequencing of gene trap clones E261
E261G041 R	CTCAAAGGACATGGGAAAGG	reverse primer for sequencing of gene trap clones E261

2.8 Molecular weight standards

As length standards in agarose gel- and capillary electrophoresis molecular weight standards were utilized. For agarose gel electrophoresis, 100 bp and 1 kb DNA ladder from New England Biolabs were used. For capillary electrophoresis, GeneScan - 500 ROX Size Standard from Applied Biosystems were used.

2.9 Solutions

6x loading dye: Glycerol 60 % (v/v)

Tris, pH 7.4 150 mM 40 % (v/v)

Bromophenol blue 0.0025 % (w/v)

Xylene-Cyanol 0.0025 % (w/v)

1x TAE buffer: EDTA 0.5 mM

Tris-Acetat 40 mM

1x TE buffer: Tris, pH 7.4 10 mM

EDTA 1 mM

2.10 Mouse strains

For QTL analysis it is essential to have phenotypic differences in the parental strains increasing the chance to detect a QTL within this phenotype. Additionally, a high degree of DNA sequence variation facilitates the choice of polymorphic markers (Liu 1997). The C57BL/6J and C3H/HeJ mouse strains fulfilled these requirements, so that these mice were used to create a mapping population in the present study. This selection was also supported by the positions of the parental strains on the mouse family tree. They are located on distant branches, thus ensuring high genetic difference (Witmer et al. 2003). The phenotypic variance for anxiety- and depression-related behavior of the parental strains was verified in several studies published recently (Crawley et al. 1997, Crowley et al. 2005, Griebel et al. 2000, Lucki et al. 2001, Milner and Crabbe 2008).

The C3H/HeJ mice comprise a potential disturbing factor for the behavioral analysis, since they are all homozygous for a mutation in the rod photoreceptor cGMP phosphodiesterase 6 β-subunit (Pde6b) gene, which causes loss of rods by weaning age (Hart et al. 2005, Pittler and Baehr 1991, Sidman and Green 1965). An additional caveat of C3H/HeJ mice is an inversion on Chromosome 6 (Akeson et al. 2006), which covers 20% of the Chromosome, but does not cause a phenotype.

2.11 Cell culture media

ES cell medium: 1x GMEM medium (Sigma)

2 mM glutamine (Gibco)

1 mM sodium pyruvate (Gibco)

1x nonessential amino acids

10 % (v/v) fetal bovine serum (Hyclone)

1:1000 dilution of beta-mercaptoethanol stock solution

1 ml of leukocyte inhibitory factor solution (625 ng/ml) (Sigma)

1x trypsin solution: 100 mg of EDTA tetrasodium salt (Sigma)

500 ml of PBS (Gibco)

10 ml of 2.5 % trypsin solution (Gibco)

5 ml of chicken serum (Gibco)

Stored in 20 ml aliquots at -20 °C

Geneticin (Gibco): 125 mg/ml stock solution in PBS

filter sterilized and stored at -20 °C

1:1000 dilutions for work solutions

3 Methods

3.1 Methods involving Animals

All experiments comprising animals like breeding, behavioral analysis and tail biopsies were carried out by Dr. Andras Bilkei-Gorzo and Kerstin Michel (Institute of Molecular Psychiatry, Bonn, Germany)

3.1.1 Animal housing

C57BL/6J and C3H/HeJ mice were obtained from Janvier Laboratory, all with an age of eight to ten weeks. These animals were crossed to the F1 generation and subsequently intercrossed within this generation in order to obtain the F2 generation. For the studies, F2 generation mice of both sexes with an age of eight to ten weeks were used for the studies. The animals were housed in groups of three to five per cage in the animal facility of the university's medical center. They were kept under constant temperature (23 ± 1°C) and in a 12 hour reversed light-dark cycle (lights on at 19:00 and lights off at 9:00). Each animal had free access to standard food pellets and water *ad libitum*. Animal care and experiments were carried out according to national regulations (Tierschutzgesetz) and were approved by legal authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW). All animals were acclimatized to the animal facility for at least two weeks prior to experiments.

3.1.2 Behavioral experiments

All behavioral experiments were conducted with animals from the F2 generation, as well as with parental mice. Each animal was analyzed once in all behavioral tests and was left undisturbed for 7 days between two experiments. The behavioral examination was achieved in the following order: 1. zero maze, 2. light-dark test, 3. startle response test, 4. forced swim test.

The elevated zero maze was utilized to measure trait anxiety levels of mice. It consisted of an elevated annular and white platform (outer diameter 47 cm, 5.6 cm width, 40 cm above ground), which was separated into four equal compartments, two opposing ones were enclosed by walls (11 cm high).

The complete apparatus was brightly illuminated (550-600 lux) and each mouse was placed on one open part of the platform. The behavior was recorded for 5 min and

the time spent as well as motor activity in the open area (Shepherd et al. 1994) was evaluated.

The light-dark test is another behavioral paradigm to assess trait anxiety levels of the animals. It consisted of a two-compartment test chamber with a dark box (15 x 45 x 22 cm) and a bigger box (30 x 45 x 22 cm), which were connected by a 6 x 6 cm hole. The test chamber was illuminated with a 20 W white neon lamp fitted 30 cm above the box. Each mouse was placed in the center of the lighted area of the big box and observed for 5 min. The time spent and horizontal activity in the open area was detected with the Actimot system (Costall et al. 1989).

The state anxiety rates of mice were measured with a startle response apparatus, which consisted of an 11 x 5.5 x 6 cm Plexiglas cage with a floor of metal bars. The cage was surrounded by two speakers and was mounted on a vibration-sensitive platform in a sound-attenuated chamber (35 x 32 x 35 cm). For habituation (5 min) and during the whole test a background white noise (65 dB) was given and the startle reactivity was measured after an acoustic signal (12 kHz, 110 dB, 40 ms). This measurement was repeated seven times (40-80 sec between trials) and the amplitude of the startle response was evaluated (Davis 1990).

The forced swim test was used to detect depression-related behavior. For this purpose a Plexiglas cylinder (10 cm diameter, 50 cm high) was filled with 23 ± 2 °C water (20 cm height) and the animals were placed directly on the water. The test takes 6 min and behavioral measurement started after the second minute. The immobility time was assessed, which was the time animals made only movements necessary to keep its head above the water (Porsolt et al. 1978).

3.1.3 Tissue preparation

Tail biopsies (< 1 cm) were obtained from living animals and stored at -20 °C. For preparation of brain and liver tissue, mice were sacrificed by cervical dislocation and the liver was directly shock-frozen in dry ice-cooled isopentan. Brains were cut in coronal sections of one mm thickness, whereby selected brain regions were punched out. The punched tissue was also immediately shock-frozen in dry ice-cooled isopentan. For biochemical analysis, brains were sagittally sliced into two equally sized halves and frozen in dry ice-cooled isopentan. For SNP analysis, whole brains were immediately shock-frozen in dry ice-cooled isopentan. All tissues were stored at -80 °C.

3.2 Methods involving DNA

3.2.1 DNA isolation

Genomic DNA was isolated from tail biopsies utilizing the DNeasy Blood & Tissue Kit in single tubes or 96-well plates. Preparation was performed according to the manufacturer's manual. Isolated DNA was stored at 4 °C. Genomic DNA of mice from the F2 generation was isolated by Kerstin Michel (Institute of Molecular Psychiatry, Bonn, Germany).

3.2.2 Measurement of DNA concentration

Concentration of DNA was evaluated using the NanoDrop photometer in 2 µl sample volume. Concentration was calculated by the NanoDrop Software utilizing the absorption values at 260 nm and the Beer-Lambert equation.

3.2.3 Normalization of DNA

DNA was normalized to a concentration of 5 ng/µl and stored at 4 °C until further use in the microsatellite PCR. Normalization was carried out by means of the liquid-handling platform Multiprobe II in a high-throughput manner. Normalization of DNA was conducted in order to ensure equal performance of the PCR and equal signal strengths in the fragment length analysis.

3.2.4 Polymerase chain reactions

The specific amplification of DNA fragments was performed by polymerase chain reaction (PCR). The reaction mix and PCR program was adjusted according to the thermodynamic properties of primers used and expected product.

Microsatellite PCR

For the microsatellite PCR, either the HotStar Taq or the NEB Taq DNA polymerase was used, depending on the primer pair. The used polymerase for each primer pair is listed in the appendix (Supplement table S1).

Reaction mix and program using HotStar Taq DNA polymerase (10 µl)

1 µl	DNA (5 ng/µl)
6.075 µl	Aqua bidest.
1 µl	10x HotStar Taq buffer
0.9 µl	25 mM MgCl$_2$
0.4 µl	forward primer
0.4 µl	reverse primer
0.2 µl	dNTP (10 mM each)
0.025 µl	HotStar Taq polymerase

Step	Temperature	Time	
Activation	95°C	15 min	
Denaturation	94°C	45 sec	
Annealing	60°C	45 sec	35 cycles
Elongation	72°C	45 sec	
Final elongation	72°C	10 min	
Storage	4°C	∞	

For some primer pairs the annealing temperature was changed to 65°C or 50°C in order to achieve a proper amplification in the PCR. The affected primer pairs are listed in the appendix (Supplement table S1).

Reaction mix and program for NEB Taq DNA polymerase (10 µl)

1 µl	DNA (5 ng/µl)
6.975 µl	Aqua bidest.
1 µl	10x Thermo Pol buffer
0.4 µl	forward primer
0.4 µl	reverse primer
0.2 µl	dNTP (10 mM each)
0.025 µl	Taq DNA polymerase

Step	Temperature	Time	
Activation	95°C	3 min	
Denaturation	95°C	45 sec	
Annealing	60°C	45 sec	35 cycles
Elongation	72°C	45 sec	
Final elongation	72°C	7 min	
Storage	4°C	∞	

All microsatellite PCR reactions were diluted with water in a 1:1 ratio. The PCR products were separated by capillary electrophoresis and detected due to the specific dye of each primer pair.

Mouse sexing PCR

PCR was used for sexing of mice depending on one X chromosome marker and two Y chromosome-specific genes. The genes SRY and ZFY are both Y-chromosome specific and the microsatellite marker DXNds3 (NDS) can be found on the X chromosome. The PCR products exhibit different lengths: 617 bp for ZFY gene, 404 bp for the SRY gene and 244 bp for the DXNds3 marker. Primers for all genes (Sry 2; Sry 4; Zfy 3; Zfy 4; Nds 3; Nds 4) were mixed in a multiplex reaction generating three different products for males (244, 404 and 617 bp) and one product (244 bp) for females (Greenlee et al. 1998, Kunieda et al. 1992).

Reaction mix and program for HotStar Taq DNA polymerase (20 µl)

5 µl	DNA (5 ng/µl)
11.4 µl	Aqua bidest.
2 µl	10x HotStar Taq buffer
0.5 µl	forward primer
0.5 µl	reverse primer
0.5 µl	dNTP (10 mM each)
0.1 µl	HotStar Taq polymerase

Step	Temperature	Time	
Activation	95°C	15 min	
Denaturation	94°C	15 sec	
Annealing	60°C	15 sec	35 cycles
Elongation	72°C	30 sec	
Final elongation	72°C	10 min	
Storage	4°C	∞	

The amplified PCR products were separated on a 2 % agarose gel.

PCR for screening Pde6b mutations

The primer W150 and W149 served for the amplification of a DNA fragment (603 bp), which harbored a base exchange mutation in the Pde6b gene. This mutation introduces a new restriction site for the enzyme Dde I (Kuenzi et al. 2003, Pittler and Baehr 1991).

Reaction mix and program for HotStar Taq DNA polymerase (20 µl)

5 µl	DNA (5 ng/µl)
7.2 µl	Aqua bidest.
2 µl	10x HotStar Taq buffer
1.2 µl	25 mM $MgCl_2$
0.5 µl	forward primer
0.5 µl	reverse primer
0.5 µl	dNTP (10 mM each)
0.1 µl	HotStar Taq polymerase

Step	Temperature	Time	
Activation	95°C	15 min	
Denaturation	94°C	60 sec	
Annealing	55°C	60 sec	35 cycles
Elongation	72°C	90 sec	
Final elongation	72°C	10 min	
Storage	4°C	∞	

After amplification, the PCR mix was supplemented with 0.5 µl restriction endonuclease Dde I and then incubated at 37 °C for 3 hours. The restriction led to a 603 bp fragment for the wiltype allele or a 511 bp fragment for the mutated allele, which were separated on a 2 % agarose gel.

PCR for sequencing ES-cell clones

PCR was used for amplification of genomic sequences flanking the insertion site of the gene trap vectors. Primers were generated on the basis of sequencing data provided by the German Gene Trap Consortium. Since the integration was specific for every ES-cell clone, specific primers were constructed for every clone (Table 2).

Table 2. Sequencing primers for three ES-cell clones:

ES-cell clone	Forward Primer	Reverse Primer	Location	Annealing Temperature
E122G09	E122G09 2 F	PCR 1 5' SPLK R	5' of gene trap	60°C
	PCR 1 3' SPLK R	E122G09 2 R	3' of gene trap	60°C
E237D04	E237D04 2 F	PCR 1 5' SPLK R	5' of gene trap	55°C
	PCR 2 3' SPLK R	E237D04 1 R	3' of gene trap	55°C
E261G04	E261G04 1 F	PCR 2 5' SPLK R	5' of gene trap	55°C
	PCR 2 3' SPLK R	E261G04 1 R	3' of gene trap	55°C

Reaction mix and program for HotStar Taq DNA polymerase (20 µl)

2 µl	DNA (25 ng/µl)
14.4 µl	Aqua bidest.
2 µl	10x HotStar Taq buffer
0.5 µl	forward primer
0.5 µl	reverse primer
0.5 µl	dNTP (10 mM each)
0.1 µl	HotStar Taq polymerase

Step	Temperature	Time	
Activation	95°C	15 min	
Denaturation	94°C	30 sec	
Annealing	60°C or 55°C	30 sec	40 cycles
Elongation	72°C	90 sec	
Final elongation	72°C	10 min	
Storage	4°C	∞	

After amplification, PCR products were cleaned up with the peqGOLD Gel Extraction Kit according to the manufacturer's manual and sequenced by Macrogen Inc.

PCR for detection of gene trap transcripts

PCR was used for the detection of wildtype and gene trap transcripts in cDNA from mouse tail biopsies. Two different primer pairs were designed, the first one (Enoph RT 1 F / Enoph RT 1 R) detects the wildtype allele with a boundary of exon one and two being hallmarked by a product length of 207 bp. The second primer pair (Enoph RT 1 F / 1st Race Primer) amplifies a 256 bp product identifying the allele with gene trap insertion.

Reaction mix and program for HotStar Taq DNA polymerase (20 µl)

2 µl	cDNA (~ 50 ng)
14.4 µl	Aqua bidest.
2 µl	10x HotStar Taq buffer
0.5 µl	forward primer
0.5 µl	reverse primer
0.5 µl	dNTP (10 mM each)
0.1 µl	HotStar Taq polymerase

Step	Temperature	Time	
Activation	95°C	15 min	
Denaturation	94°C	15 sec	
Annealing	60°C - 1°C every cycle	15 sec	5 cycles
Elongation	72°C	60 sec	
Denaturation	94°C	15 sec	
Annealing	55°C	15 sec	30 cycles
Elongation	72°C	60 sec	
Final elongation	72°C	10 min	
Storage	4°C	∞	

The amplified PCR products were separated on a 2 % agarose gel.

3.2.5 Fragment lengths analysis

Fragment lengths of the microsatellite PCR products were determined on a ABI 3130xl Genetic Analyzer with the ABI GeneMapper® software. This Genetic Analyzer operated with 16 capillaries of 36 cm length. The GeneScan™ 500 ROX™ Size Standard was used as the internal DNA size standard for sizing of DNA fragments.

The PCR products were diluted with Aqua bidest. before mixing with Hi-Di™ Formamid and ROX™ Size Standard. Up to four PCR reactions were multiplexed for the fragment length analysis. All steps of the fragment length analysis were conducted according to the manufacturer's manual.

Reaction setup for the Genetic Analyzer:

9.75 µl	Hi-Di™ Formamid
0.25 µl	GeneScan™ 500 ROX™ Size Standard
1 µl	each PCR product

3.2.6 Electrophoresis in agarose gels

Separation of DNA fragments was carried out on agarose gels containing 2 % agarose. For this purpose, agarose was boiled up in 1x TAE buffer and the gel was casted in Bio-Rad casting chambers. Fragments were separated in 1x TAE buffer with 10 V/cm and afterwards stained with ethidium bromide. Visualization and documentation of stained DNA fragments was conducted with a Bio-Rad Chemi Doc System and related software. For the estimation of fragment sizes, appropriate size standards were used.

3.2.7 Sequencing

DNA sequencing was performed by Macrogen Inc. (South Korea) or Medigenomix GmbH (Germany).

3.3 Methods involving RNA

3.3.1 RNA isolation

Frozen tissue samples were homogenized in MagNA Lyser tubes with the Precellys homogenizator. Each sample (< 50 mg) was homogenized in 800 µl TRIzol reagent using program five of the homogenizator (twice). Samples were centrifuged at 10,000 g for 10 min at 4 °C, the supernatant was transferred in a new tube and incubated for 5 min at RT. 160 µl chloroform was added and the samples were mixed on a vortex machine for 30 sec before they were incubated for 3 min at RT. After a centrifugation at 10,000 g for 5 min at 4 °C, the aqueous phase was transferred to a new tube and mixed with 400 µl 100 % isopropyl alcohol on a vortex machine. The samples were incubated for 10 min at RT and centrifuged at 10,000 g for 10 min at 4 °C until the supernatant could be removed. Subsequently the RNA pellet was washed with 1 ml 75 % ethanol on a vortex machine, centrifuged at 10,000 g for 5 min at 4 °C and the supernatant was removed. This washing step was repeated twice, and then the RNA was dried for 5 to 10 min at 50 °C. RNA was resolved in 20 µl ultraPURE™ water and incubated for 10 min at RT before 15 min incubation at 60 °C. The solved RNA was stored at -80 °C.

3.3.2 Measurement of RNA concentration

Concentration of RNA was evaluated using the NanoDrop photometer in 2 µl sample volume. Concentration was calculated by the NanoDrop Software utilizing absorption values at 260 nm and the Beer-Lambert equation.

3.3.3 cDNA synthesis

PCR tubes were filled with 500 ng RNA of each sample and filled up to a volume of 11 µl with RNase-free water. The samples were supplemented with 1 µl Oligo dT12-18 (0.5 µg /µl) and 1 µl 10 mM dNTP Mix before an incubation of 5 min at 65 °C followed by 2 min at 4 °C. 4 µl 5x First-Strand Buffer and 2 µl 0.1 M DTT was added to each sample prior to an incubation of 2 min at 42 °C. After cooling for 2 min at 4 °C, 1 µl SuperScript $^{®}$ II RT (200 U / µl) was added and mixed by pipetting up and down. The samples were incubated for 50 min at 42 °C, next for 15 min at 70 °C and at last for 2 min at 4 °C. The cDNA was filled up to 160 µl with ultraPURE™ water and stored at -20 °C. The final concentration was 3.125 ng / µl.

3.3.4 Real-time PCR

For TaqMan® gene expression assays, 25 ng cDNA was used per sample and prepared as triplicates for each assay and sample. 20 µl reaction mixture as well as PCR program for the 7900HT PCR System was applied according to the manufacture's manual.

3.3.5 SNP analysis

The SNP analysis was performed by Dr. Jeeva Varadarajulu (Max Planck Institute of Psychiatry, Munich, Germany) according to the protocol recently published (Ditzen et al. 2010). In short:

Mouse brains were homogenized by grinding in liquid nitrogen and subsequent utilization of QIAshredder spin columns. The extraction of RNA was carried out with a RNeasy Kit and cDNA was synthesized with the SuperScript™ II One-Step RT-PCR. A DNA fragment carrying both SNPs were amplified by PCR and confirmed by DNA sequencing.

3.4 Biochemistry

3.4.1 SAM isolation

Sagittally divided brains were weighed and homogenized in 10x vol. 5% trichloroacetic acid by sonication for 90 s at 40 W on ice. 200 µl of 100 µM N6-methyladenosine was added as an internal standard and homogenates were centrifuged at 10,000 g for 30 min at 4 °C. The supernatant was transferred in a new tube, washed with 10x vol. water-saturated diethyl ether and centrifuged at 10,000 g for 10 min at 4 °C. This step was repeated twice until the pellet was vacuum dried to remove diethyl ether residues from the aqueous phase. Finally, the aqueous phase was analyzed by HPLC.

3.4.2 HPLC analysis of SAM

SAM was detected on the Knauer HPLC system equipped with a Knauer 250 x 2 mm Eurospher 100-3 C18 column. UV detection was carried out at 254 nm. The mobile phase consisted of phase A (40 mM $NH_4H_2PO_4$, 8 mM 1-heptanesulfonic acid sodium salt, pH 4.5, 0.45 µm membrane filtered) and phase B (100 % methanol). The following gradient was applied: 0 min. - 5 % phase B / 10 min - 18 % phase B / 20

min - 18 % phase B / 25 min - 5 % phase B, then 10 min 5 % phase B for equilibration (Gospe et al. 1995). Flow rate was set to 0.2 ml / min. at RT. Results were corrected according to the internal standard N6-methyladenosine. HPLC analysis was performed by Dr David Otte (Institute of Molecular Psychiatry, Bonn, Germany).

3.4.3 Polyamine isolation

Sagittally halved brains were weighed and homogenized in 3x vol. 4 % perchloric acid by sonication for 90 s at 40 W on ice. The homogenates were incubated over night at 4 °C. After centrifugation at 10,000 g for 20 min., 100 µl of the supernatant were mixed with 300 µl 2N sodium hydroxide and 3 µl benzoyl chlorid on a vortex machine for 30 s. The samples were incubated for 20 min. at RT and the reaction was stopped by addition of 500 µl saturated sodium chloride solution. After mixing with 500 µl chloroform on a vortex machine and a centrifugation at 10,000 g for 10 min, the chloroform phase was transferred in a new tube, followed by vacuum drying. The sediment was redissolved in 100 µl 55 % methanol and analyzed by HPLC (Ditzen at al. 2010).

3.4.4 HPLC analysis of polyamines

SAM was detected on the Knauer HPLC sytem equipped with a Knauer 250 x 2 mm Eurospher 100-3 C18 column. UV detection was carried out at 254 nm. The mobile phase consisted of phase A (water) and phase B (100 % methanol) with a gradient of 55 % - 84 % methanol over 23 minutes followed by isocratic elution at 84 % methanol for seven minutes (Ditzen et al. 2010). Flow rate was set to 0.2 ml / min. at RT. HPLC analysis was performed by Dr David Otte (Institute of Molecular Psychiatry, Bonn, Germany).

3.5 Cell biology

All experiments comprising ES-cell culture like thawing, freezing, culturing and passaging were carried out by Anne Zimmer (Institute of Molecular Psychiatry, Bonn, Germany)

3.5.1 Thawing of ES-cells

One vial containing 2×10^6 cells was thawed immediately in a water bath at 37°C and supplemented with 4 ml pre-warmed medium. The cells were plated in a well of a 6-well plate coated with 0.1 % gelatin. After 2 hours incubation at 37 °C in a humidified 7 % CO_2 incubator, the medium was replaced with 5 ml fresh medium.

3.5.2 Freezing and storage of ES-cells

The medium of a confluent well of a 6-well plate was aspirated off and the cells were washed twice with 5 ml pre-warmed PBS. Then cells were covered with 0.5 ml of 1x trypsin solution and incubated for 2 min at 37 °C. After addition of 5 ml medium, the cells were centrifuged at 1200 g for 3 min. The medium was removed and the cells were resuspended in 2 ml fresh freezing medium. 1 ml cell suspension was frozen in a cryotube at -80 °C over night and transferred to liquid nitrogen for long-term storage.

3.5.3 Culturing of ES-cells

ES-cells were cultured in ES-cell medium at 37 °C in a humidified incubator with 7 % CO_2. Due to the rapid growth of the ES-cells, medium was replaced every 24 hours and cells were passaged after two days. They were kept under selection with Geneticin® the whole time. The cell culture dishes were coated with 0.5 % gelatin before cells were plated.

3.5.4 Passaging of ES-cells

The medium of a confluent well of a 6-well plate was aspirated off and the cells were washed twice with 5 ml pre-warmed PBS. Then cells were covered with 0.5 ml of 1x trypsin solution and incubated for 2 min at 37 °C. Finally, the cells were resuspended gently in 4 ml medium. 1 ml of resuspended cells was plated onto a new well of a 6-well plate.

3.5.5 Microinjection of ES-cells

Cells were trypsinized as described in 3.5.4 and resuspended in 4 ml medium. Then they were injected into blastocysts harvested from super-ovulated 4 - 6 week old C57BL/6J mice. Blastocysts were then transferred to pseudo-pregnant foster animals. Tanja Tropartz performed the microinjections at the transgene service of the University Hospital Aachen. Chimeric male animals were transferred to the animal facility of the University Hospital Bonn and first tested for germline transmission by backcrossing with C57BL/6J mice.

3.6 Gene knockout in ES-cells

The gene Enoph1 was knocked out with a gene trap vector integrated in the first intron. This vector introduced a new splice acceptor site together with a polyadenylation signal resulting in a break of the mRNA after the first exon. Thus, no functional protein could be translated. The integration of the gene trap vector was carried out by the German Gene Trap Consortium, which uses a high-throughput approach to knock out every gene in mouse ES-cells. They used the ES-cell line E14TG2a.4, also known as the line 129P2. The consortium offered the ES-cells with integrated gene trap vector approved by PCR and sequencing. Altogether, six ES-cell clones (E261G04, E252B10, E237D04, E148E08, E123F08, and E122G09) were received from the German Gene Trap Consortium; all were carrying the gene trap in the first intron.

3.7 QTL detection

The mapping of QTL was carried out with the software R/qtl that was especially developed to map QTL in experimental crosses (Broman et al. 2003). It is written in the R language, a widely used language and environment for statistical computing that is freely available (R Development Core Team 2010). R/qtl calculates the probability of linkage between genotype and phenotype on the basis of the interval mapping method (Broman et al. 2003). The outputs of R/qtl are the logarithm of the odds (LOD) scores, which states the probability of a linkage between a trait and a marker. It measures the possibility for the presence of a QTL at a specific location, compared to the hypothesis that there is no QTL present. Therefore, larger LOD scores represent higher possibility for the presence of a QTL (Broman 2001).

Besides its mapping functions, R/qtl also offers the option for a quality control of the

input data. Additionally, the functions used in this context are also utilized to validate the reliability of results. Quality control measures the degree of missing genotypes for each individual and each marker. Additionally, it detects the recombination fractions (amount of non-parental recombinations) and LOD scores for linkage between all possible pairs of markers. Furthermore, the quality control functions estimates a marker map based on the observed data and compares it with the marker map based on database information. If there is a strong difference between the marker positions of the two maps detectable, this suggests a higher probability for genotyping errors. With this information, it is possible to assess the reliability of the collected genotype information.

QTL mapping was done by a single-QTL genome scan and the function scanone of R/qtl was used for this purpose. This function implements the commonly used interval mapping method by comparing the genotypes of each marker individually with the phenotypic data. The test for linkage was carried out with the analysis of variance. A big challenge in QTL analysis is the handling of missing genotype data between two markers as well as not genotyped marker. Therefore, the Haley-Knott regression was used (Broman and Sen 2009, Haley & Knott 1992) due to its faster calculation and robustness with data containing low error rates. This algorithm calculates an approximation of the standard interval mapping method by applying a single regression at each position, in this study every 1 cM (Broman and Sen 2009). For not normally distributed behavioral data the non-parametrical method was used in the scanone function, which was designed for mapping of binary traits (Broman et al. 2003). For the detection of significance thresholds, the permutation method was used. This algorithm shuffles the phenotypes and leave the genotype data unchanged in order obtain genome-scan-adjusted significant thresholds. In each replicate generated under the hypothesis of no genetic effect on the trait throughout the genome, peaks exceeding a pre-defined LOD threshold are counted. These counts – divided by the total number of replicates – determine the genome-wide significance level of the corresponding LOD threshold. The significance thresholds are based on 10,000 permutations. The function bayesint of R/qtl calculates the Bayesian credible intervals (confidence intervals) for a single-QTL and it returns an interval, which contains this QTL with a probability of 95%.

3.8 Prioritization of candidate genes

The FunSimMat software was used to identify prominent candidates in a list of genes. Therefore, it compares gene ontologies of phenotype-correlated reference genes from the database with respective ones from the candidate gene list. The results of this comparison are then used to prioritize candidate genes depending on their similarity to the reference gene. These comparisons are carried out on all three subtypes of gene ontologies, in detail the biological process, the molecular function and the cellular compartment; furthermore values are calculated for each subtype and comparison representing the amount of similarity based on four different algorithms for similarity measures (simRel, Lin, Resnik, and Jiang & Conrath). In cooperation with Dr. Andreas Schlicker (Max Planck Institute of Informatics, Saarbrücken, Germany), the developer of the software, the biological process similarity with simRel measures (BP simRel) and with Lin's measures (BP Lin) as well as the calculation of biological process and molecular function scores (rfunSim) were chosen as the prominent values for the identification of candidate genes. A value above 0.8 represents strong similarity, a value from 0.8 to 0.5 means mild similarity and a value under 0.5 implies no or negligible similarity. A gene was assigned as a candidate gene when at least two of the three values were above 0.5. As reference gene tryptophan hydroxylase 2 (TPH2) was chosen, which is related to the "susceptibility to unipolar depression" phenotype in the OMIM database (no. 607478). This phenotype was chosen due to its similarity to depression-related behavioral measurements in the forced swim test, where the strongest QTL was detected. UniProt codes were used as input format for the software.

3.9 Statistical methods

The descriptive statistics were performed on the basis of average values and their standard errors of mean (S.E.M.). All raw data were listed and evaluated with the spreadsheet program Excel®. Average value comparisons in pairs were examined with Students t-test and differences were termed as significant if the probability (p) was lower 0.05.

4 Results

A QTL approach was chosen to reveal new genetic regions, which contribute to anxiety- and depression-like phenotypes in mice. The goal of possible follow up studies is the identification and verification of the underlying genes. In order to reach this goal, mice of the parental and the F2 generation were tested for their anxiety- and depression-related behavior in four different paradigms. Phenotypes of the parental strains were evaluated to verify differences in anxiety- and depression-related behavior between these strains. Afterwards, QTL loci were assessed using phenotyping and genotyping data of the F2 mice. Finally, candidate genes were selected from QTL regions and validated by analysis of gene expression, SNP analysis and biochemical analysis.

4.1 Behavioral analysis of mice

The C57BL/6J and C3H/HeJ mouse strains exhibit high genetic diversity based on their distinct positions on branches of the family tree of laboratory mice (Witmer et al. 2003). This diversity facilitates the selection of suitable microsatellite markers for QTL mapping. Additionally, recent publications reported a substantial difference regarding their anxiety- and depression-related behavior (Crawley et al. 1997, Crowley et al. 2005, Griebel et al. 2000, Lucki et al. 2001, Milner and Crabbe 2008). The anxiety and depression levels were evaluated firstly in parental strains and subsequently in the respective F2 generation mice.

4.1.1 Analysis of parental mouse strains

The C57BL/6J and C3H/HeJ strains showed significant differences in their trait anxiety levels evaluated by light-dark and zero maze tests (Figure 4A-D). Mice of the C3H/HeJ strain spent significantly more time in the open area of the light-dark test ($p < 0.001$) (Figure 4A) and in the open area of the zero maze, compared with C57BL/6J mice ($p < 0.001$) (Figure 4C). Additionally, these mice covered substantially longer distances in the open areas in the light-dark test ($p < 0.05$) (Figure 4B), as well as in the zero maze ($p < 0.01$) (Figure 4D).

In order to investigate the state anxiety levels, both strains were analyzed in the startle response test, whereby C3H/HeJ mice exhibited significantly higher startle amplitude in contrast to C57BL/6J ($p < 0.01$) (Figure 4E).

Depression-related behavior was measured using the forced swim test. C3H/HeJ mice spent significant less time in an immobility phase compared to C57BL/6J mice (p < 0.001) (Figure 4F). Dr. Andras Bilkei-Gorzo and Kerstin Michel (Institute of Molecular Psychiatry, Bonn, Germany) performed the behavioral analysis of parental mice.
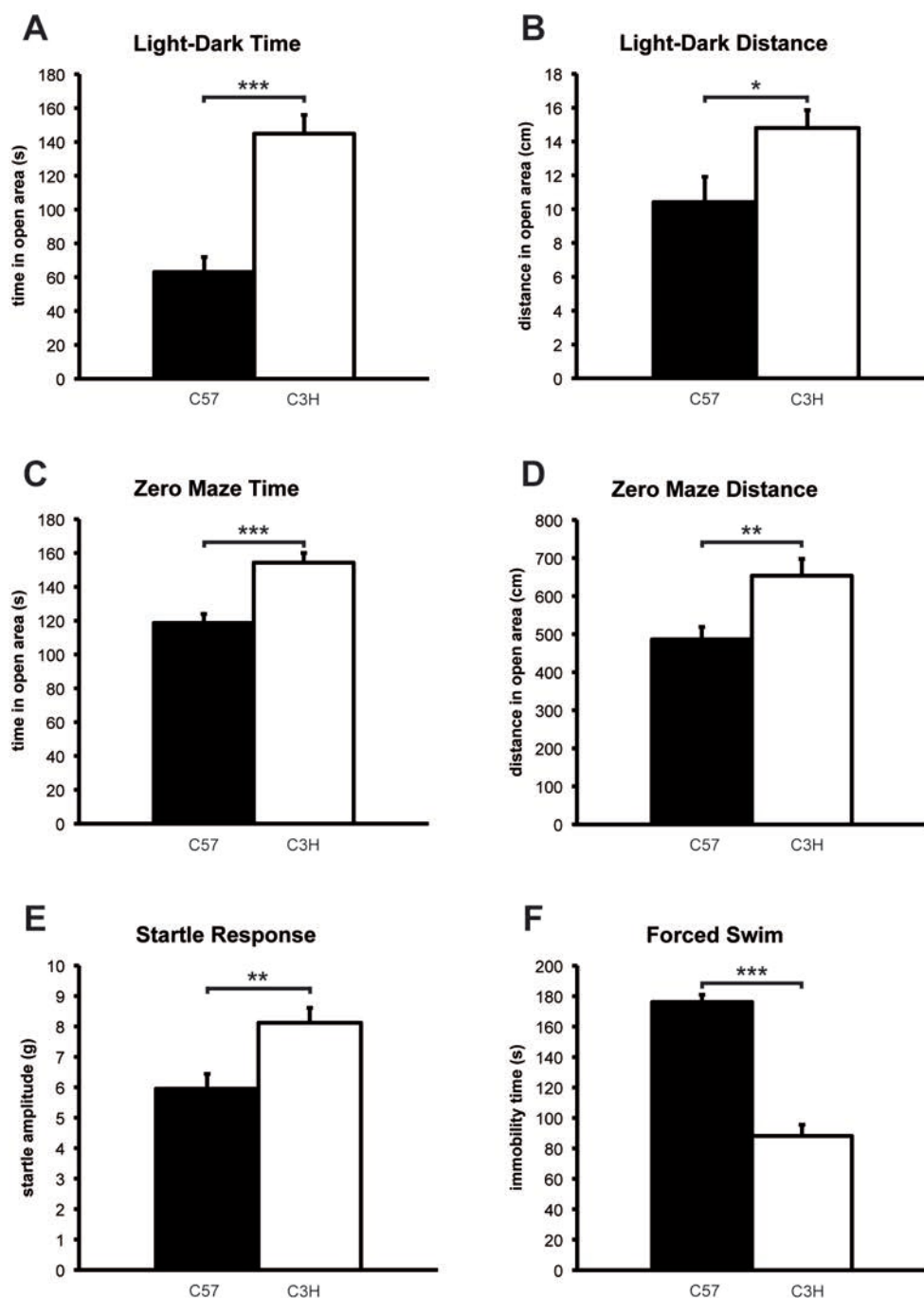

Figure 4. Anxiety and depression levels of parental mice. Mice of C57BL/6J and C3H/HeJ strains showed significant behavioral differences in state anxiety levels assessed by the time spent (A) and distance traveled (B) in the open areas in the light-dark test. Moreover, noteworthy differences were

observed for the respective values in the zero maze test (C & D). Furthermore, measurements of trait anxiety levels within the startle response test (E) revealed considerable differences between the strains. C57BL/6J and C3H/HeJ exhibited also largely different immobility times (F) in the forced swim test, which assessed depression-related behavior. Values represent mean ± SEM; n = 20; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.1.2 Analysis of second filial generation

A total of 543 mice from the F2 generation were examined in the same behavioral paradigms as the parental strains. Since a normal distribution of the data is regarded as the best source for further experiments, square root transformations were applied for distance traveled in the open area measure of the light-dark test (Figure 5B), as well as for time spent (Figure 5C) and distance traveled (Figure 5D) in the open part values of the zero maze to receive a normal distribution. In order to achieve this normal distribution, startle amplitude measurements were logarithmical transformed (Figure 5E). A high number of animals spent a prolonged time in the open area in the light-dark test (Figure 5A) and additionally the immobility time values of the forced swim test (Figure 5F) showed right shift. Due to these distributions, achieving a normal distribution by transformation of these data was not possible, thus raw data were used for further analysis. The black curve in each histogram represents the expected normal distribution for each value (Figure 5A-F). This calculation could not be applied for the time measurement of the light-dark test due to its non-parametric distribution. All behavioral data of the F2 Generation were assessed by Dr. Andras Bilkei-Gorzo and Kerstin Michel (Institute of Molecular Psychiatry, Bonn, Germany).
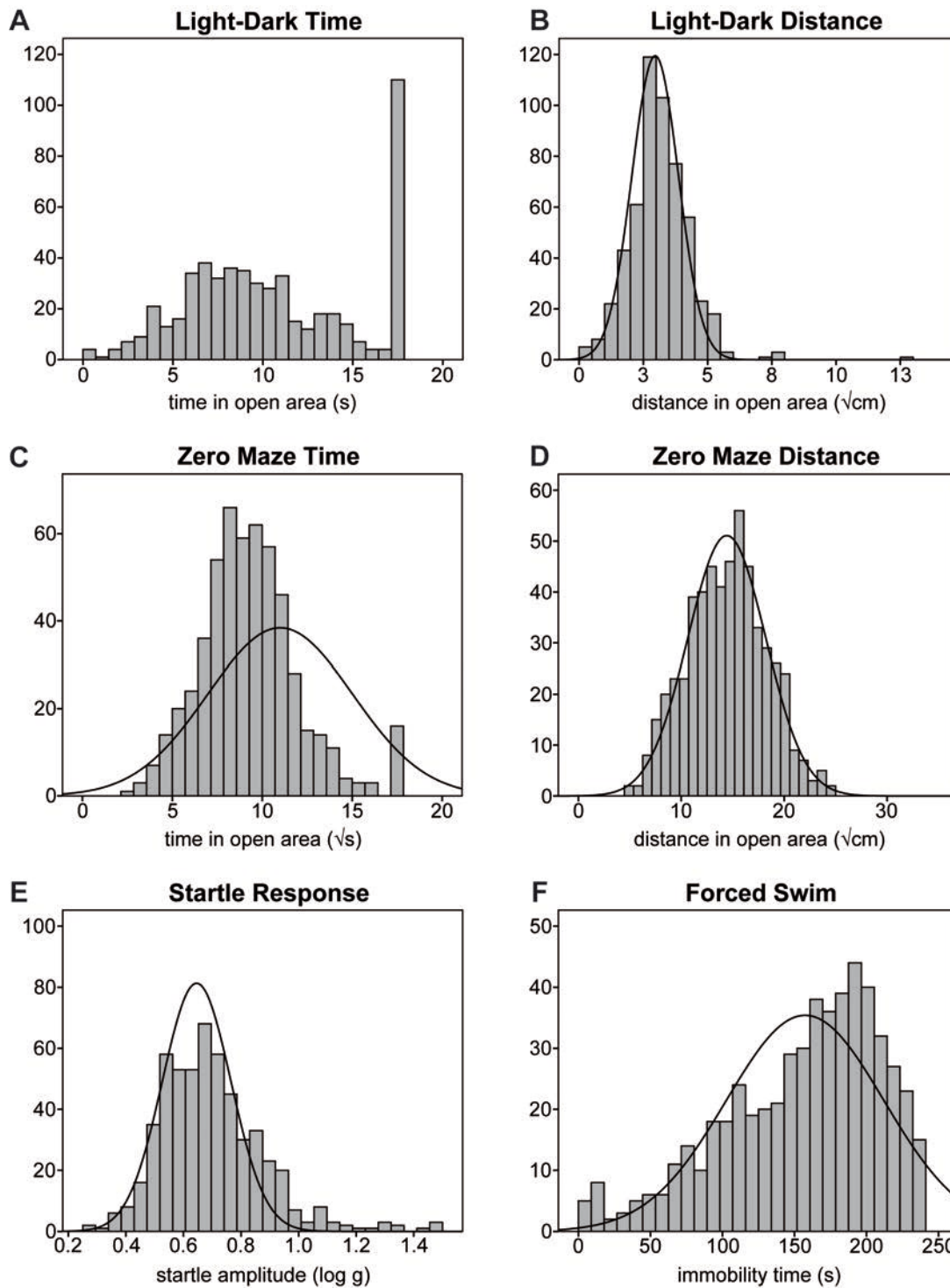
Figure 5. Distribution of animal behavior in F2 mice. The F2 progeny was tested in four paradigms for anxiety and depression. The histograms plotted the number of individuals against each value of the behavioral experiments; some of them were transformed to receive normal distribution. Raw data of time spend (A) and square root transformed distance traveled in the open area (B) in the light-dark test are shown. For the zero maze test, the values of time spend and distance traveled in open areas were square root transformed (C & D). The startle amplitude measurements were transformed by logarithm (E) and raw data of immobility time of the forced swim test were plotted. The curves illustrate the expected normal distributions, if they were able to be calculated.

4.1.3 Control analysis in second filial generation

Some animals of the F2 generation were assigned a false sex. Therefore all mice were screened with a sex determining PCR. Additionally, the influence of a possible homozygous retinal degeneration 1 (rd1) mutation in the gene phosphodiesterase 6 β-subunit (Pde6b) on the animals' behavior was elucidated. This mutation is known to be responsible for retinal degeneration and loss of rod cells leading to a reduced visual ability.

4.1.3.1 Sexing of the second filial generation

In order to exclude a false sex determination of mice, a multiplex PCR was chosen prior to a QTL analysis detecting three different markers within one reaction. As an example, figure 6 presents the PCR fragments of three male and three female wildetype C57BL/6J mice. The results of the sex determination for all mice from the second filial generation are displayed in the appendix (Supplement table S2).
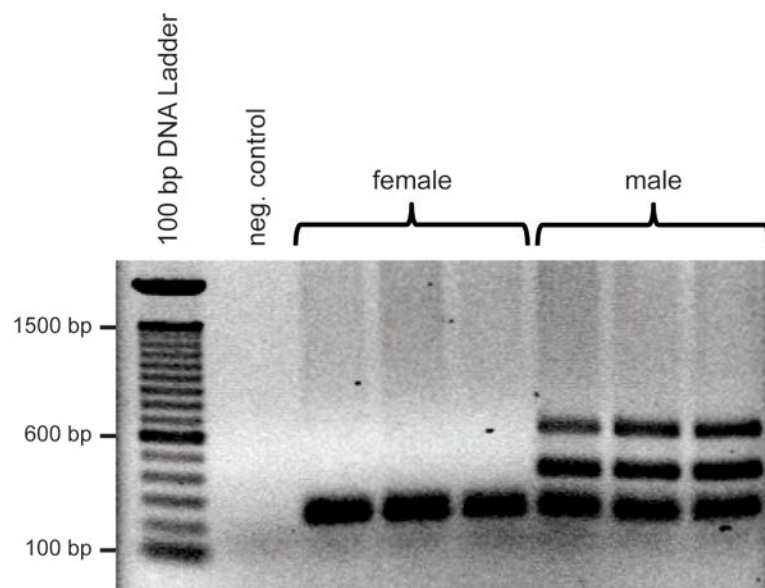
Figure 6. Example of sex determining PCR. DNA of three male and three female wildtype C57BL6/J mice was examined by multiplex PCR with three primer pairs. The primer pair SRY 2 / SRY 4 amplified a product of 404 bp and the primer pair ZFY 3/ ZFY 4 a product of 617 bp. Both products are Y-chromosome specific. Primer pair NDS 3 / NDS 4 amplified a X-chromosomal product of 244 bp. Negative control (neg. control) was carried out with aqua bidest. instead of DNA as template.

4.1.3.2 Analysis for the rd1 mutation in Pde6b gene

In order to investigate the influence of homozygous mutation rd1 in the Pde6b gene on the QTL analysis, a subset of 248 randomly chosen animals were screened for this mutation by PCR analysis. Afterwards, a restriction fragment length polymorphism (RFLP) analysis of the PCR product with the restriction endonuclease Dde I was carried out. The wildtype PCR product has a length of 511 bp, whereas the mutated allele is cut by Dde I into two fragments of 298 bp and 213 bp. As an example, figure 7 displays the results of wildtype C57BL6/J, C3H/HeJ mice, as well as of the first filial generation. Results of 248 mice from the second filial generation are presented in the appendix (Supplement table S2).
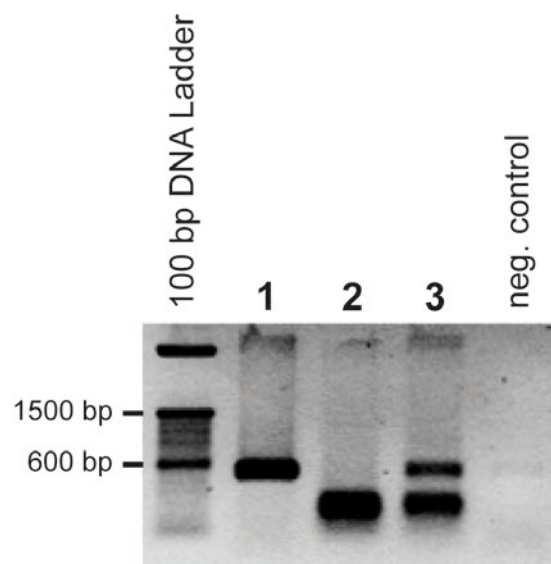
Figure 7. Example for analysis of Pde6b mutation. The Dde I restricted PCR products were separated in 2 % agarose gel. In C57BL6/J mice the primer pair W149 / W150 amplified a product of 511 bp length (1), which contained no mutation and was consequently not restricted by Dde I. The product amplified from C3H/HeJ was cut by Dde I resulting in two fragments of 298 bp and 213 bp length (2). For the F 1 generation of these strains, both results could be observed (3). Negative control (neg. control) contained aqua bidest. instead of DNA.

All animals, which were homozygous for the mutated allele in the Pde6b gene, were assigned as blind. The other mice carrying either homozygous wildtype or heterozygous alleles were defined as healthy, since these animals were hallmarked by normally developed retina. In the subset of 248 mice from the second filial generation, 58 animals were identified with the homozygous rd1 mutation in the Pde6b gene and 187 animals were assigned as healthy. The behavioral data of

these two groups were compared for activity measurement of the light-dark and zero maze tests. Activity is presented as the percentage traveled in the open area from overall movement. In the light-dark test, blind mice exhibited significant higher activity ($p < 0.05$) compared with wildtype mice (Figure 8). For the zero maze test, no difference was detected between both groups (Figure 8).
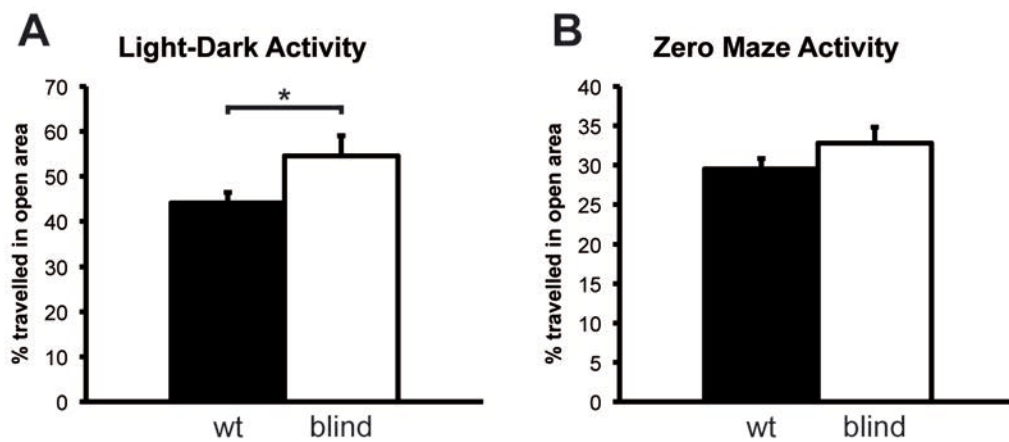
Figure 8. Behavioral comparison of blind and wildtype (wt) mice. (A) Blind mice travelled a significant higher percentage in open area of light-dark test compared to wildtype mice. (B) For the zero maze, no significant difference could be observed between blind and wildtype mice for the percentage traveled in open area. Bars represent mean with n = 58 for blind and n = 187 normal mice; ± SEM; * $p < 0.05$.

4.2 QTL analysis

The genomes of F2 generation mice from the C57BL/6J & C3H/HeJ intercross were genotyped by a microsatellite screening. QTL intervals were assessed by a linkage analysis of phenotypic and genotypic data utilizing parametrical, as well as non-parametrical approaches. Finally, the most prominent QTL were narrowed by comparative genomics in order to reduce the amount of candidate genes.

4.2.1 Data quality control

The software R/qtl contains several functions to control the quality of input data and consequently to ensure a solid QTL mapping. These include a graphical representation of marker distribution on the genome, distribution of missing genotypes and comparison of estimated genetic maps. In the present study, 264 microsatellite markers with an average distance of 5.56 cM were used, which can graphically represented in a high-density genetic map (Figure 9A). Additionally,

missing genotypes from the input data were presented by plotting all markers against all individuals (Figure 9B). Since a negligible amount of genotypes were missing, the data could be analyzed directly. In Figure 9C, a genetic map based on database information is compared with a genetic map estimated from the input data. By this comparison, small shifts could be observed between the marker positions of these maps resulting in extended chromosome lengths. However, these shifts are quite short and acceptable, since microsatellite marker positions are influenced by several experimental factors as well as differences in database information. The final quality control function is based on the estimated recombination fractions, which could identify potentially incorrect placement of markers on the genetic map. The estimated recombination fractions between markers and the LOD scores are quoted in the upper left triangle and the lower right triangle, respectively (Figure 9D). Red indicates pairs of markers that appear to be linked, non-linked pairs are highlighted in blue. Since no linkage between two independent markers can be seen, the presence of incorrectly placed markers can be excluded. These markers were consequently chosen for further QTL mapping.
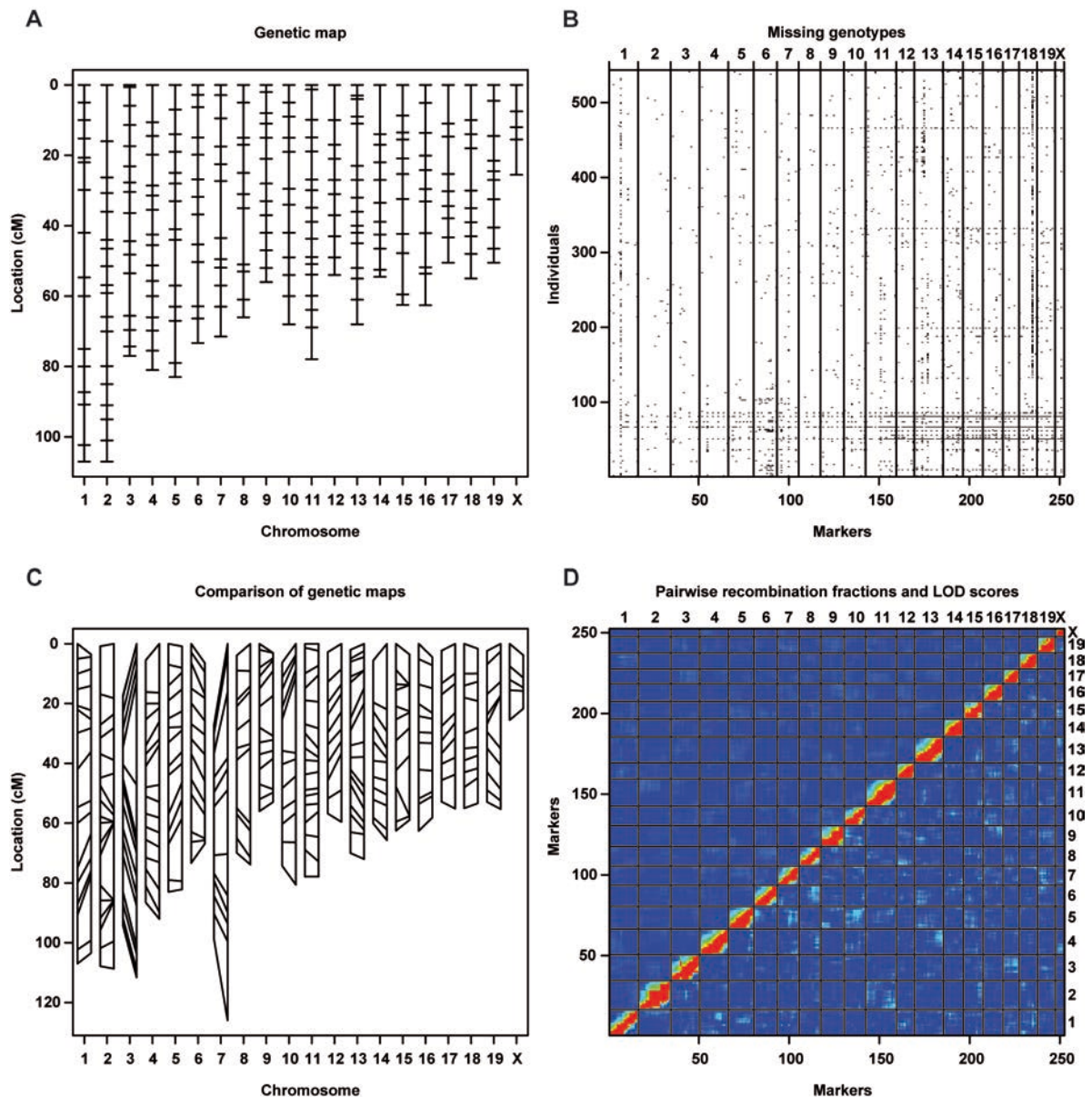
Figure 9. Data quality control. (A) Genetic map with marker positions throughout the genome. (B) Missing genotyping data in the F2 generation. Black dots indicate missing marker information for each individual. (C) Comparison of a genetic map based on MGI database information (left side of bars) with a genetic map calculated of the input data (right side of bars). (D) Estimated recombination fractions (upper left triangle) and LOD scores for all pairs of markers (lower right triangle).

4.2.2 QTL mapping

Mapping of QTL was carried out with the scanone function of R/qtl, which maps individually acting QTL. This single-QTL genome scan on all 543 F2 generation mice revealed one QTL on chromosome 5 at 58 cM for the light-dark test time parameter with a LOD score of 5.74 (Figure 10A) and a 95 % confidence interval from 49 to 60 cM, which accounts for 5.09 % of the phenotypic variance (Table 3). For the distance

parameter of the same behavioral test, two QTL could be identified. One is located on chromosome 1 at 57 cM with a LOD score of 4.69 (Figure 10B), representing 3.52 % of the phenotypic variance and with a 95 % confidence interval covering the 46 to 69 cM region (Table 3). The other one lies at 58 cM on chromosome 5 with a LOD score of 4.72 (Figure 10B) and a 95 % confidence interval spanning from 49 to 62 cM that covers 3.54 % of the phenotypic variance (Table 3).

The zero maze test revealed two further QTL, one for the time value on chromosome 15 at 22.2 cM and one for the distance value on chromosome 5 at 61 cM (Figure 10C-D). The first QTL for the time value had a LOD score of 4.59, representing 3.82 % of the phenotypic variance and a 95 % confidence interval from 8.7 to 25.7 cM (Table 3). The other detected QTL for the distance value revealed a LOD score of 4.84 and a 95 % confidence interval covering a region from 49 to 65 cM, which describes 4.02 % of the phenotypic variance (Table 3).

For the startle response test, no QTL could be discovered (Figure 10E). The forced swim test unveiled one QTL on chromosome 5 at 53 cM with a LOD score of 14.41 (Figure 10F), accounting for 12.4 % of the phenotypic variance and with a 95 % confidence interval from 41 to 58 cM (Table 3).
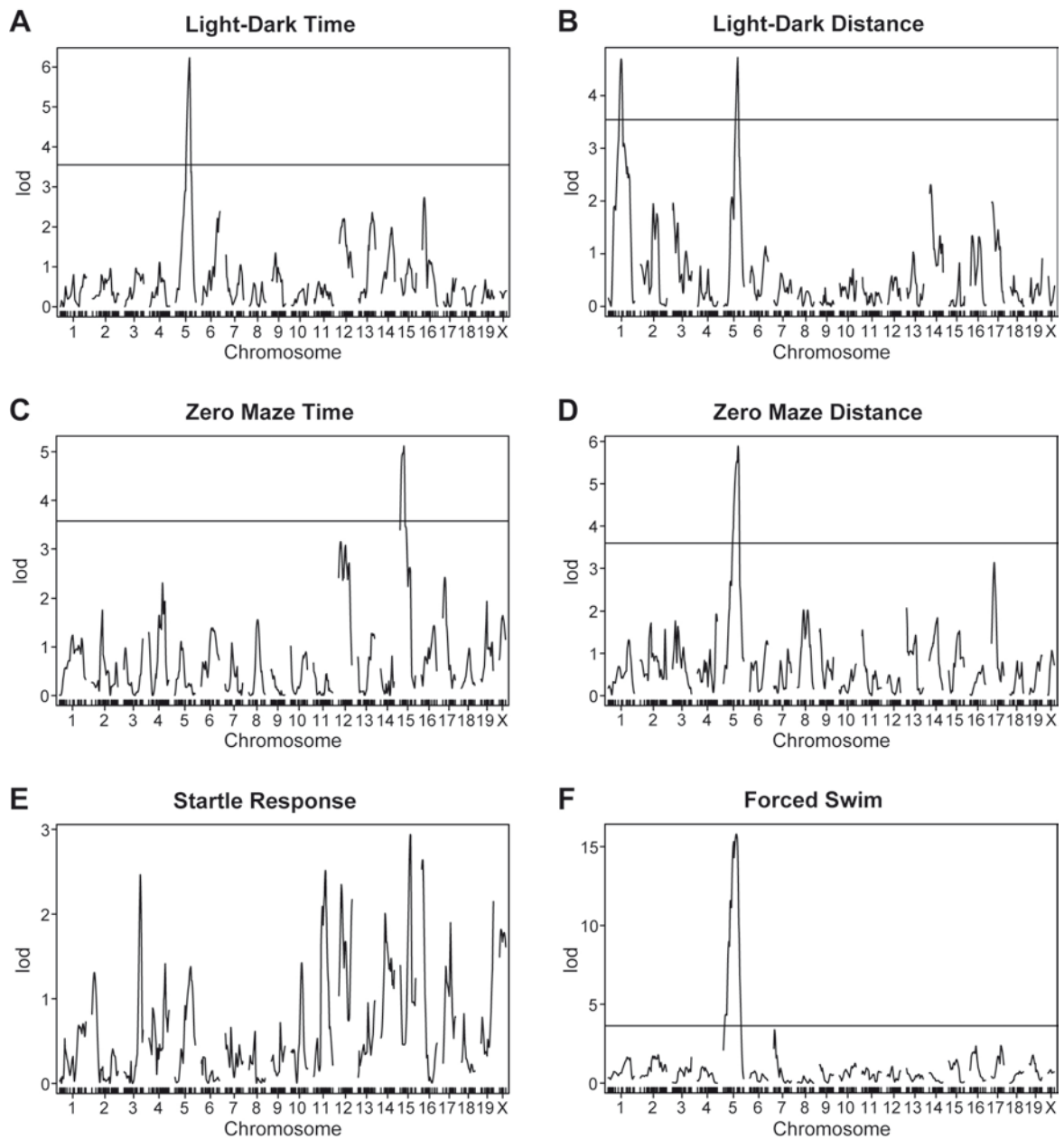
Figure 10. Genome-wide QTL analysis of the F2 generation. Graphs present LOD plots for (A) time in the open area and (B) distance in the open area in the light-dark test, for (C) time in the open area and (D) distance in the open area in the zero maze test, for (E) the startle amplitude in the startle response test and for (F) the immobility time in the forced swim test. The microsatellite markers are shown on the x-axis, sorted by their position in the genome and the LOD-scores are indicated on the y-axis. The horizontal line in each plot indicates the significance threshold with p < 0.05, indicating significant LOD-scores above this line.

Additional to the QTL mapping on the whole F2 generation, single-QTL analysis was also performed with the sample set separated into male and female individuals in order to identify sex-specific loci. Four male-specific QTL could be detected, two within the time parameter of the light-dark test on chromosome 5 at 57 cM with a

LOD score of 3.69 and on chromosome 14 at 55.5 cM with a LOD score of 4.42 (Table 3). The third QTL was revealed for the distance value of the light-dark test on chromosome 1 at 58 cM with a LOD score of 4.79 and the fourth one was observed within the forced swim test on chromosome 7 at 3.4 cM with a LOD score of 6.53 (Table 3). Moreover, QTL for the forced swim test were detected on chromosome 5 for both the male and female group (Table 3). The QTL for the males lay at 52 cM with a LOD score of 6.53 and a 95 % confidence interval from 42 to 59 cM (Table 3). The QTL of the female group lay at 40 cM with a LOD score of 9.1 and a 95 % confidence interval covering the region from 36 to 60 cM (Table 3).

Table 3. QTL found for the whole F2 generation and separated into sexes.

Trait	Chr.	Locus (cM)	LOD score	p-value	% var.	95% CI (cM)
Light-Dark Time	5	58	5,74	<0.001	5,09	49 - 60
Light-Dark Distance	1	57	4,69	0,003	3,52	46 - 69
	5	58	4,72	0,003	3,54	49 - 62
O-Maze Time	15	22,2	4,59	0,006	3,82	8,7 - 25,7
O-Maze Distance	5	61	4,84	0,005	4,02	49 - 65
Startle Response	---	---	---	---	---	---
Forced Swim	5	53	14,41	<0.001	12,4	41 - 58
Male						
Light-Dark Time	5	57	3,69	0,034	6,39	44 - 62
	14	55,5	4,42	0,007	5,57	38.5 - 58.5
Light-Dark Distance	1	58	4,79	0,002	6,82	50 - 85
O-Maze Time	---	---	---	---	---	---
O-Maze Distance	---	---	---	---	---	---
Startle Response	---	---	---	---	---	---
Forced Swim	5	52	6,53	<0.001	9,82	42 - 59
	7	3,4	4,02	0,016	6,42	1.5 - 8.5
Female						
Light-Dark Time	---	---	---	---	---	---
Light-Dark Distance	---	---	---	---	---	---
O-Maze Time	---	---	---	---	---	---
O-Maze Distance	---	---	---	---	---	---
Startle Response	---	---	---	---	---	---
Forced Swim	5	40	9,1	<0.001	10,3	36 - 60

Chr.: chromosome number; cM: centimorgan; 95 % CI: the 95 % confidence interval for the QTL (in cM) calculated by R/qtl; % var.: % of phenotypic variance the QTL accounts for. p value < 0.05 represents significant effects.

4.2.3 Narrowing of QTL intervals

Most QTL intervals were found on chromosome 5 for different behavioral measurements and all 95 % confidence intervals showed a common core interval. Therefore, these intervals were superposed in order to reveal the common core region. This core spans from 49 to 58 cM (Table 3) or from 90 Mb to 109 Mb. Interestingly, a previous publication linked agoraphobia and panic disorder in humans to a region from q21.21 to q22.3 on human chromosome 4 (Kaabi et al. 2006), which is syntenic to an interval on chromosome 5 of the mouse spanning from 28 to 67 cM. The syntenic region in the mouse also revealed a common core region with the previously narrowed QTL on chromosome 5 (Figure 11). For this reason, comparative genomic approaches were also implemented in the narrowing of the core QTL region on chromosome 5 resulting in a core QTL interval on chromosome 5 with a length of 15 Mb spanning from 90 to 105 Mb (Figure 11).
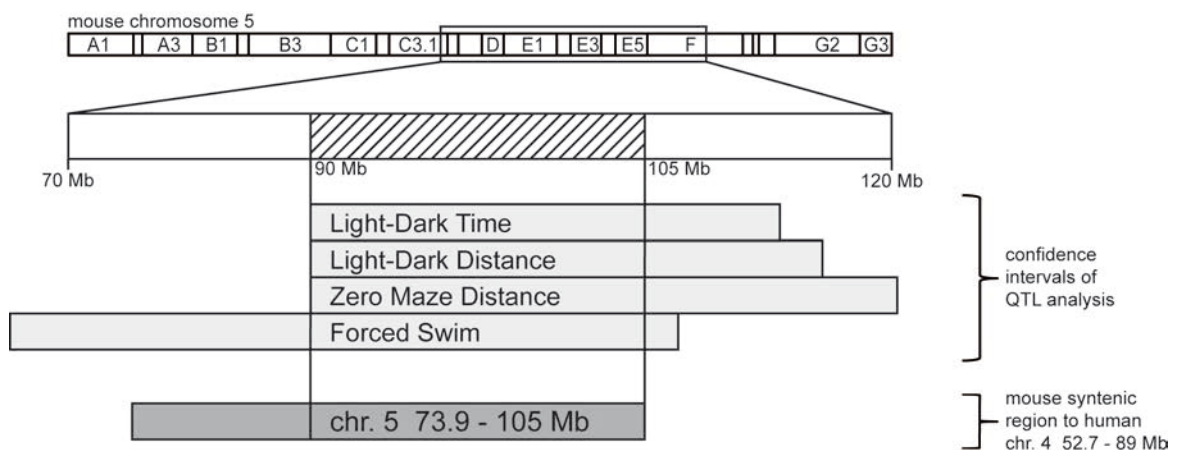
Figure 11. Narrowing of the QTL interval on chromosome 5. The thick white bar shows the magnification of the region on chromosome 5, where most of the QTL intervals were found. The light grey bars represent the identified QTL intervals on chromosome 5 with the corresponding trait. The dark grey bar indicates a syntenic region (73.9-105 Mb), homologue to a human region linked to agoraphobia and panic disorders (human chr. 4 52.7-89 Mb). The intervals were superposed to visualize their common intersection representing the narrowed QTL interval (shaded area).

4.3 Identification and validation of candidate genes

All genes lying in the narrowed QTL interval were prioritized, referring to the semantic similarities of their gene ontologies as compared to the respective ones of a reference gene. The most prominent candidate genes from this prioritization were validated by gene expression analysis in brain or liver tissue and by single nucleotide polymorphism analysis in the parental C57BL/6J and C3H/HeJ strains. Furthermore, biochemical analyses of interesting pathways were carried out within the parental mouse strains.

4.3.1 Identification of candidate genes

The narrowed QTL interval of 15 Mb length altogether contains 171 annotated genes. In order to identify prominent candidate genes in this region, a prioritization was carried out with the FunSimMat software. As the reference for the gene ontology comparison, the "susceptibility to unipolar depression" phenotype (no. 607478) from the "online Mendelian inheritance in man" database was used. 113 out of 171 annotated genes in the narrowed QTL interval possessed UniProt codes and could be used for the prioritization. The first eight genes of the prioritized list are presented in Table 5 and a complete gene list is presented in the appendix.

The first three genes, 17 β-hydroxysteroid dehydrogenase 13 (Hsd17b13), enolase-phosphatase 1 (Enoph1) and 17 β-hydroxysteroid dehydrogenase 11 (Hsd17b11) met the selection criteria that at least two of three values (BP simRel, BP Lin, rfunSim) were higher than 0.5 (Table 5). These genes were selected as possible candidate genes for validation experiments.

Table 4. Gene ontologies of the first eight genes on the prioritization list.

Genome Coordinates (strand)	Symbol	Gene Ontology		
		Biological Process	**Cellular Component**	**Molecular Function**
104384459-104406429 (-)	Hsd17b13	metabolic process oxidation reduction	extracellular region	binding catalytic activity oxidoreductase activity
100469063-100497779 (+)	Enoph1	cellular amino acid biosynthetic process metabolic process methionine biosynthetic process methionine salvage		acireductone synthase activity catalytic activity hydrolase activity magnesium ion binding metal ion binding phosphoglycolate phosphatase activity
104418781-104450938 (-)	Hsd17b11	metabolic process oxidation reduction lipid biosynthetic process steroid biosynthetic process	cytoplasm extracellular region	binding catalytic activity oxidoreductase activity estradiol 17-beta-dehydrogenase activity
97811243-97821349 (-)	Naa11	metabolic process	cytoplasm nucleus	transferase activity acyltransferase activity N-acetyltransferase activity peptide alpha-N-acetyltransferase activity
101227646-101233490 (+)	Mrps18c	translation	intracellular mitochondrion ribosome ribonucleoprotein complex	structural constituent of ribosome
101274732-101328121 (+)	Agpat9	metabolic process phospholipid biosynthetic process	membrane integral to membrane	transferase activity acyltransferase activity
93032125-93035605 (+)	Stbd1	carbohydrate metabolic process	membrane integral to membrane	catalytic activity carbohydrate binding
96638512-96695746 (+)	Mrpl1	translation RNA processing	intracellular ribosome ribonucleoprotein complex large ribosomal subunit mitochondrion mitochondrial large ribosomal subunit	RNA binding structural constituent of ribosome

Hsd17b13: hydroxysteroid (17-beta) dehydrogenase 13; Enoph1: enolase-phosphatase 1; Hsd17b11: hydroxysteroid (17-beta) dehydrogenase 11; Naa11: N(alpha)-acetyltransferase 11; Mrsp18c: mitochondrial ribosomal protein S18C; Agpat9: 1-acylglycerol-3-phosphate O-acyltransferase 9; Stbd1: starch binding domain 1; Mrpl1: mitochondrial ribosomal protein L1

Table 5. FunSimMat values of the first eight genes on the prioritization list.

Genome Coordinates (strand)	Symbol	SNPs		FunSimMat Values		
		SNPs	**SNP Type**	**BP simRel**	**BP Lin**	**rfunSim**
104384459-104406429 (-)	Hsd17b13	1	Coding-NonSynonymous	0.72	1.00	0.62
100469063-100497779 (+)	Enoph1	3	Coding-NonSynonymous (2) mRNA-UTR (1)	0.56	0.70	0.48
104418781-104450938 (-)	Hsd17b11			0.51	0.66	0.49
97811243-97821349 (-)	Naa11	1	mRNA-UTR (1)	0.45	1.00	0.33
101227646-101233490 (+)	Mrps18c			0.40	0.40	0.28
101274732-101328121 (+)	Agpat9	2	mRNA-UTR (2)	0.36	0.54	0.27
93032125-93035605 (+)	Stbd1			0.35	0.39	0.29
96638512-96695746 (+)	Mrpl1			0.35	0.37	0.26

The dotted line indicates the threshold for the candidate gene selection based on the FunSimMat values; SNP: single nucleotide polymorphism; BP simRel: biological process similarity with simRel measure; BP Lin: biological process similarity with Lin's measure; rfunSim: calculation of biological process and molecular function scores; Hsd17b13: hydroxysteroid (17-beta) dehydrogenase 13; Enoph1: enolase-phosphatase 1; Hsd17b11: hydroxysteroid (17-beta) dehydrogenase 11; Naa11: N(alpha)-acetyltransferase 11; Mrsp18c: mitochondrial ribosomal protein S18C; Agpat9: 1-acylglycerol-3-phosphate O-acyltransferase 9; Stbd1: starch binding domain 1; Mrpl1: mitochondrial ribosomal protein L1.

4.3.2 Expression analysis of candidate genes

In order to validate the previous identified candidate genes, expression differences in brain regions for Enoph1 gene were investigated in the parental C57BL/6J and C3H/HeJ strains. For the genes Hsd17b11 and Hsd17b13, expression differences in liver tissues of parental strains were analyzed since these genes showed high expression within this tissue (Horiguchi et al. 2008) and moreover due to their role in the hydroxysteroid hormone metabolism (Moeller and Adamski 2009). Enoph1 expression was detected in four different brain regions, the amygdala, cingulate cortex, hippocampus and hypothalamus, which play important roles in regulation of emotional behavior (Price and Drevets 2010; Shin and Liberzon 2010).

Quantitative PCR revealed significantly different expression levels of Enoph1 in cingulate cortex (p < 0.01), hippocampus (p < 0.01) and hypothalamus (p < 0.05) of C3H/HeJ mice compared to C57BL/6J mice (Figure 12). The expression of the Enoph1 gene was elevated in these brain regions in C3H/HeJ mice. No significant expression differences of Enoph1 were observable in the amygdala between these strains (Figure 12).
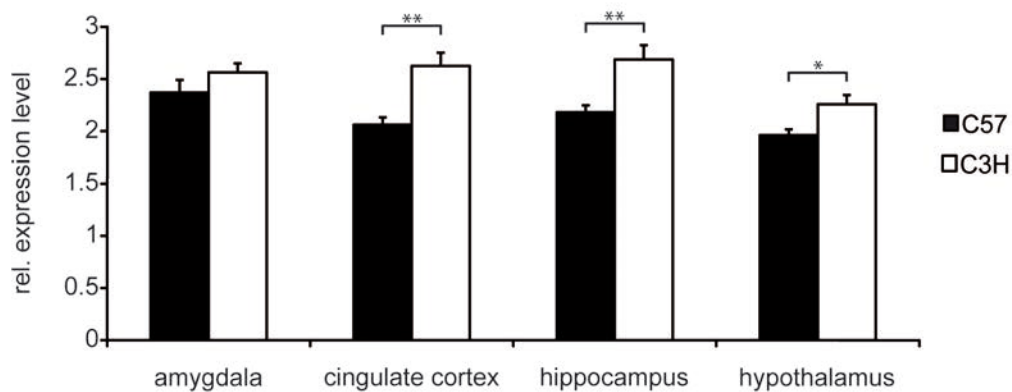
Figure 12. Expression of Enoph1 in different brain regions. The relative expression levels were calculated using the $2^{-\Delta C(t)}$ method with the TATA box binding protein as reference gene. The relative expression levels are shown on the y-axis and the different brain regions are presented on the x-axis. Bars indicate the mean of ten individuals ± SEM; n = 10; * p < 0.05; ** p < 0.01; C57 = C57BL/6J; C3H = C3H/HeJ.

Expression of Hsd17b11 was significantly higher in C3H/HeJ mice (nearly double) compared to C57BL/6J (Figure 13). The transcripts of Hsd17b13 demonstrate the

exact opposite, since expression was significantly higher in C57BL/6J mice (twofold higher as in C3H/HeJ mice) (Figure 13).
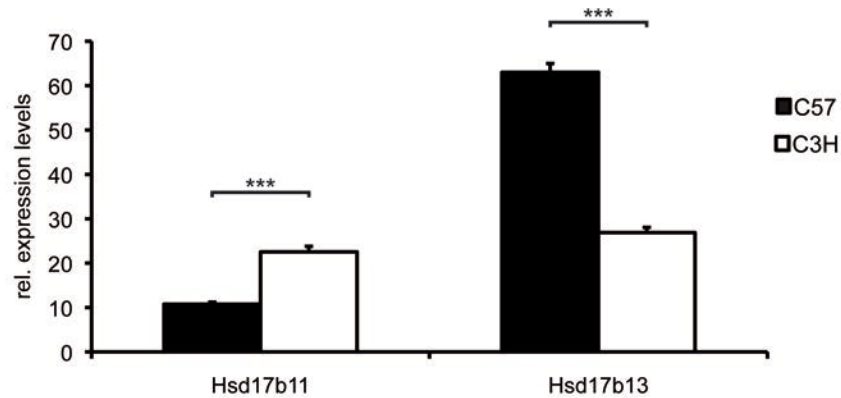
Figure 13. Expression of Hsd17b11 and Hsd17b13 in liver tissue. The relative expression levels were calculated using the $2^{-\Delta C(t)}$ method with the TATA box binding protein as reference gene. The relative expression levels are shown on the y-axis and the different genes were presented on the x-axis. Bars indicate the mean of ten individuals ± SEM; n = 10; *** p < 0.001; C57 = C57BL/6J; C3H = C3H/HeJ.

4.3.3 SNP analysis of Enoph1

Enoph1 gene was selected as the most interesting gene for further analysis, as not only expression differences could be detected, but also two publications correlated this gene with anxiety-related behavior in mice (Ditzen et al. 2006; Ditzen et al. 2010). A SNP analysis was conducted with brain tissue of the two parental strains for the Enoph1 gene. This gene contains two non-synonymous SNPs in its coding region, whereby the first one is located at the end of exon 2 (rs13460000) and the second one at the beginning of exon 3 (rs13460001).

The co-segregation of these two SNPs within the parental strains is hallmarked by specific nucleotide polymorphisms detected in the animals. All C57BL/6J mice carried a guanine base in the first and a cytosine base in the second SNP for both alleles (Figure 14). The C3H/HeJ strain carried an adenine base in the first and a thymine base in the second SNP for both alleles (Figure 14). These SNPs consequently result in amino acid exchanges in the final protein. The first SNP leads to an amino acid exchange from valine in C57BL/6J mice to isoleucine in C3H/HeJ mice at position 56 in the protein. The second SNP induces the change from proline in C57/BL/6J strain to leucine in C3H/HeJ strain at position 74 in the Enoph1 protein.

The SNP analysis was performed by Dr. Jeeva Varadarajulu (Max Planck Institute of Psychiatry, Munich, Germany).
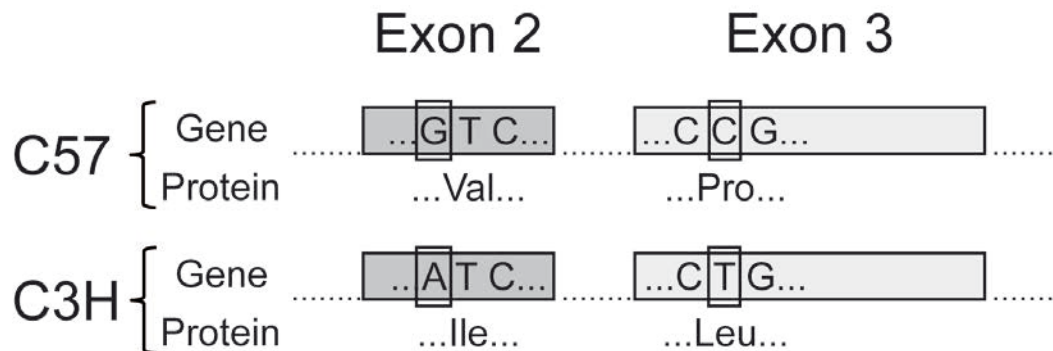
Figure 14. Single nucleotide polymorphisms (SNPs) of the Enoph1 gene. A comparison of the genomic sequences of C57BL/6J to C3H/HeJ mice is shown. The two SNPs are marked in the coding triplets at the end of exon 2 and beginning of exon 3. The resulting non-synonymous amino-acid substitutions in the Enoph1 protein are displayed beneath each triplet. SNP rs13460000 results in an amino acid change of isoleucine (Ile) to valine (Val) in position 56 of the protein; SNP rs13460001 causes an amino acid change of leucine (Leu) to proline (Pro) in position 74 of the protein. C57 = C57BL/6J; C3H = C3H/HeJ.

4.3.4 Biochemical analysis of methionine salvage pathway

The Enoph1 protein is part of the methionine salvage pathway, a ubiquitous pathway for the reconstitution of methionine. The well-known mood-enhancer S-adenosylmethionine (SAM) is also involved in this pathway, which is additionally connected to polyamines downstream of SAM (Pirkov et al. 2008). Recent studies revealed that polyamines are regulated in anxiety- and depression-related behavior (Fiori and Turecki 2008, Genedani et al. 2001, Hayashi et al. 2004, Lee et al. 2006). Therefore, the methionine salvage pathway could potentially regulate polyamines and might play a role in modulation of anxiety- and depression-related behavior. A HPLC analysis was conducted for SAM and the polyamines spermine and spermidine in brain tissue of the parental C57BL/6J and C3H/HeJ mice.

No different levels of SAM or spermine were detected in brain tissue of parental mice (Figure 15). In contrast to these findings, analyses of spermidine contents exhibited significantly different levels in these mice ($p < 0.05$), with elevated levels in the C3H/HeJ strain (Figure 15). The HPLC measurements were performed by Dr. David Otte (Institute of Molecular Psychiatry, Bonn, Germany).
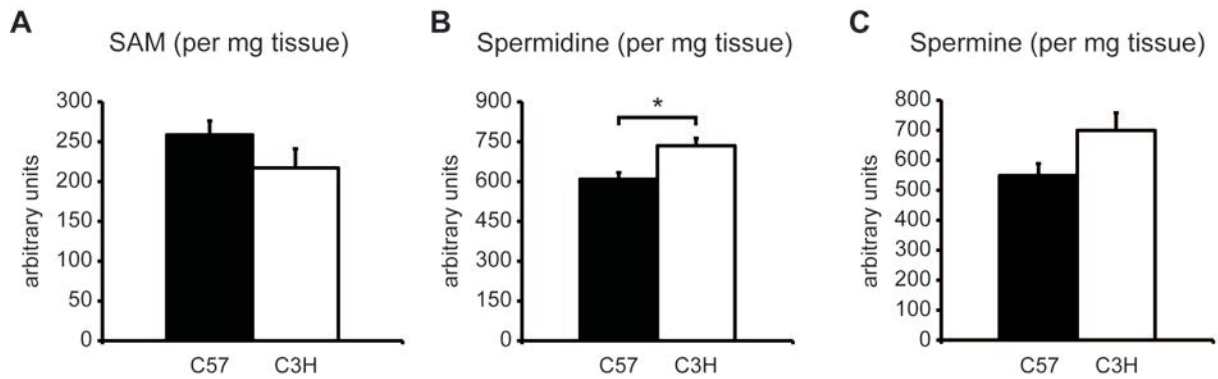
Figure 15. SAM and polyamine assays of C57BL/6J and C3H/HeJ brain tissues. SAM (A) was measured in brain homogenates by high performance liquid chromatography (HPLC) analysis, as well as spermidine (B) and spermine (C) levels after derivatization with benzoyl chloride. Measurements are presented in arbitrary units per mg tissue. Spermidine levels are significantly higher in C3H/HeJ mice compared to C57BL/6J mice. Bars represent mean ± SEM; n = 5; * $p < 0.05$; C57 = C57BL/6J; C3H = C3H/HeJ.

4.4 Generation of Enoph1 knockout mouse

Different expression levels as well as co-segregating SNPs in the parental strains indicate that Enoph1 is the most prominent candidate gene in the narrowed QTL interval. In order to gain deeper insights into its function, a knockout mouse was regarded as a suitable model. For this purpose, embryonic stem (ES) cells from the German Gene Trap Consortium were used, which contain a heterozygous Enoph1 gene knockout. A confirmation of the knockout by sequencing was performed prior to the injection of ES cells into blastocysts. Chimeric mice were screened for germline transmission and heterozygous mice are analyzed with RT-PCR.

4.4.1 Validation of ES cell clones

Six different ES cell clones were ordered from the German Gene Trap Consortium (E122G09; E237D04; E261G04; E252B10; E148E08; E123F08), all containing a gene trap insertion in the first intron of the Enoph1 gene. Initially, three clones (E122G09; E237D04; E261G04) were more closely analyzed for the exact insertion of the gene trap vector. For this purpose, the genomic 3' and 5' flanking sequences of the gene trap vector were amplified by PCR for each clone (Figure 16). All products exhibited the expected lengths and were subsequently sequenced and aligned with the respective reference sequence of the mouse in order to achieve the exact

position of the gene trap vector in the first intron of each clone. The results of sequence alignments are presented in the appendix (Supplement figure S1).
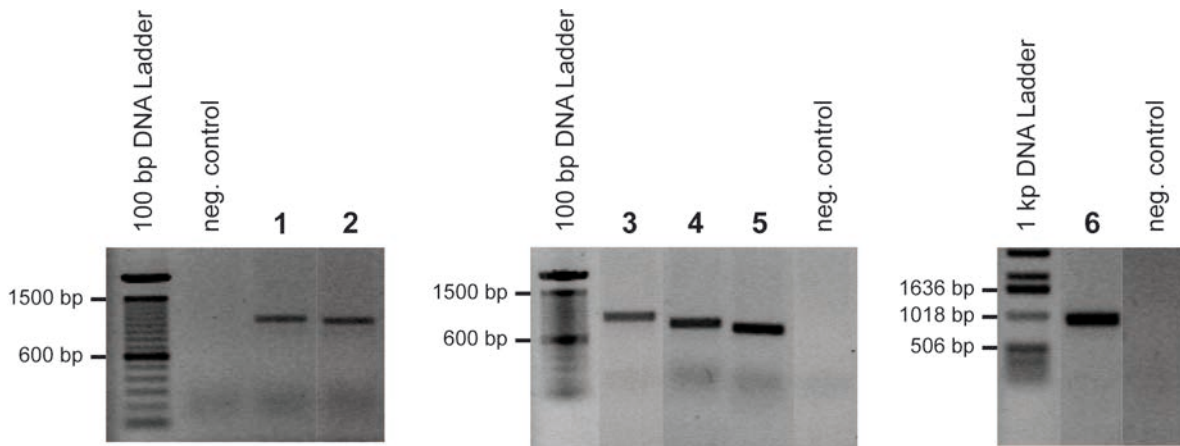
Figure 16. Amplification of gene trap flanking sequences. Products after amplification of the 3' and 5' gene trap flanking sequences of three different ES-cell clones (E122G09; E237D04; E261G04) were visualized in a 1 % agarose gel. The 3'-sequence (1) and the 5'-sequence (2) of clone E122G09 are highlighted on the gels. The 3'-sequence (3) and the 5'-sequence (6) of clone E237D04, as well as the 3'-sequence (4) and the 5'-sequence (5) of clone E261G04 (4). All bands exhibited the expected lengths and were subsequently sequenced. The negative controls (neg. control) contained aqua bidest. instead of DNA template.

Additionally, the three ES-cell clones were analyzed for the correct function of the gene trap insertion, which should result in a shorter transcript of the Enoph1 gene consisting of just the first intron. The transcript length was determined by PCR with cDNA samples prepared from tail biopsies.

All three ES-cell clones (lanes 2-4) presented the expected heterozygosis, one wildtype allele generating a 207 bp PCR product and the gene trap allele producing a 256 bp long product, confirming the correct function of the gene trap vector (Figure 17). As expected, the wildtype ES-cell (lane 1) contained only the wildtype allele, which produced the 207 bp PCR product exclusively (Figure 17). This PCR assay will also be applied for genotyping of knockout animals in later breeding.
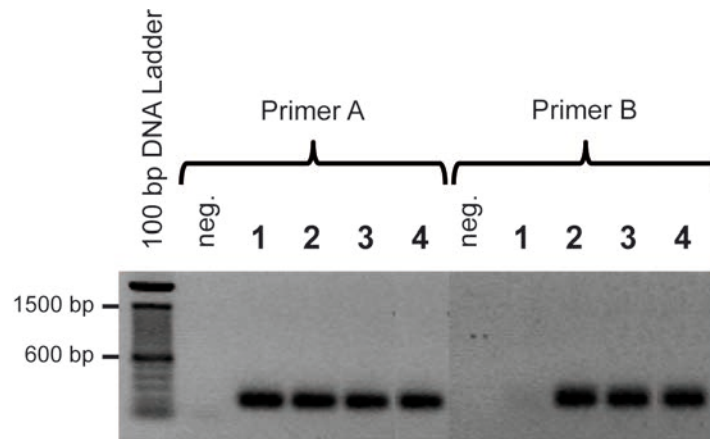
Figure 17. RT-PCR for gene trap insertion. The cDNA samples of wt ES-cells (1) or from the gene trap ES-cell clones E122G09 (2), E237D04 (3), E261G04 (4) were used as templates for the PCR. Negative control (neg.) contained aqua bidest. instead of cDNA. Two different primer pairs (**A** and **B**) were used. **A** contains primer pair Enoph RT 1 F / Enoph RT 1 R, which amplifies a wt allele in the Enoph1 cDNA with a product size of 207 bp. **B** contains primer pair Enoph RT 1 F / 1st Race Primer, which amplifies the gene trap allele in the Enoph1 cDNA with a product size of 256 bp.

4.4.2 Generation of chimeras and screening for germline transmission

The clone E261G04 of the three ES cell clones, confirmed by sequencing, was injected into blastocysts, which were subsequently transferred into super ovulated C57BL/6J female mice. Out of 16 injected and implanted blastocysts, one highly chimeric male mouse was obtained with approx. 70 % chimerity (Figure 18). Tanja Tropartz carried out the injections at the transgene service of the University Hospital Aachen and the chimeric mouse was then transferred to the animal facility in Bonn.

The chimeric male mouse was mated with wildtype C57BL/6J female mice in order to receive heterozygous knockout animals. However, within the time frame of this thesis no offspring was born. The uteri of three female mice were analyzed at day 7.5 after mating and no embryo was detected.
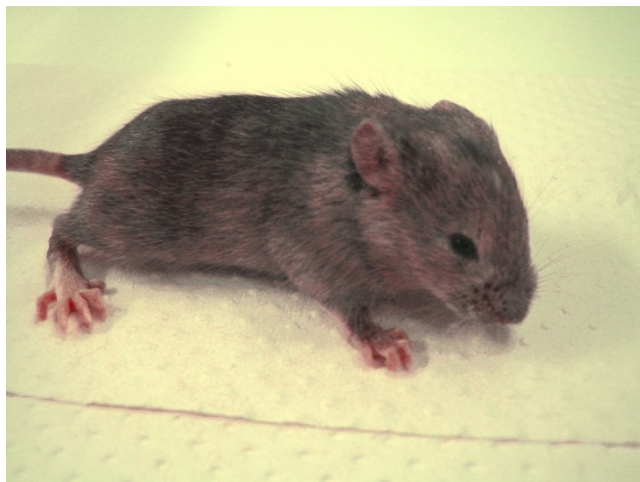
Figure 18. Highly chimaric male mouse. This mouse was obtained after injection of ES-cell clone E261G04 into blastocysts and implantation into superovulated C57BL/6J mice. It exhibited approx. 70 % chimerity.

5 Discussion

The identification of specific susceptibility genes in anxiety and depression is a challenging approach, since these behaviors have a complex genetic basis. In this study, differences in anxiety- and depression-related behavior, in combination with a QTL analysis, were used to gain a deeper insight into the complex genetic regulation of anxiety and depression in mice. A couple of genetic loci could be identified with a strong contribution to anxiety and depression. A few of them confirmed QTL that were detected in previous studies and consequently validated the chosen approach as being suitable for the aim. In addition, several new QTL could be mapped to anxiety- and depression-related behavior within a region on chromosome 5. This offered the ability to use a comparative genomics approach to reduce the length of the core QTL interval. The selected candidate gene Enoph1 was validated by expression, SNP, as well as biochemical analysis. The generation of a knockout mouse has recently started and will provide more information about the role of Enoph1 in anxiety- and depression-related behavior.

5.1 Behavioral screening of parental and F2 mice

The foundation of this QTL study was based on the genetic and behavioral differences of the parental mouse strains. The genetic difference of the strains was guaranteed by their phylogenetic distance on the mouse family tree (Witmer et al. 2003). Recent studies have published behavioral differences between the C57BL/6J and C3H/HeJ strains in anxiety- (Crawley et al. 1997, Griebel et al. 2000, Milner and Crabbe 2008) and depression-related behavior (Crowley et al. 2005, Lucki et al. 2001). These findings were further verified by the behavioral measurements in the two parental strains C57BL/6J and C3H/HeJ, presenting significant differences in all applied anxiety- and depression-related tests. In conclusion, these parental strains were an ideal basis for a QTL analysis due to their genetic and behavioral differences.

Behavioral analyses of the F2 generation mice in the same behavioral tests revealed a normal distribution of measured data for the majority of the parameters. For some parameters, the distribution was graphically hallmarked by a frequent shift to the left or a seldom shift to the right side. These data cannot be handled with standardized statistical tests, which mostly require a normal distribution. Deviation from normal

distribution resulted from test-specific limits or lay in the principles of the paradigm, meaning that it is not possible to retrieve normally distributed measures due to specific guidelines for some behavioral tests. Therefore, square root or logarithmic normalization was applied to receive a normal distribution, if it was necessary and possible. This normally distributed data could then be handled with the Haley-Knott algorithm of R/qtl, which is more stringent and robust compared to the algorithm used for non-normally distributed data. Most algorithms used for QTL mapping require normally distributed phenotypic data (Broman and Sen 2009). The algorithm for non-normally distributed data allows the input of non-parametric data for the QTL mapping – or, more precisely, for the phenotypic data of the time value of the light-dark test, as well as from the forced swim test.

However, the parental mouse strains posed a further challenge, because the C3H/HeJ are known to suffer from retinal degeneration due to inactivation of the rod photoreceptor cGMP phosphodiesterase 6 β-subunit (Pde6b) gene caused by a nonsense mutation (Hart et al. 2005, Pittler and Baehr 1991, Sidman and Green 1965). Mice homozygous for the Pde6b mutation develop pronounced visual impairment at weaning age due to the rapid loss of rod photoreceptor cells through apoptotic processes. PDE6b is considered to be a protein specifically expressed in the retina (Beavo 1995). The homozygous mutation in the Pde6b gene and therefore the retinal degeneration could have an influence on the behavior of the mice. Hence, identified QTL could be based on this gene due to its influence on the behavior. The Pde6b gene is located at the 3' site of the particular QTL, identified on chromosome 5, and maybe it could influence the detected QTL. In order to exclude this assumption, mice of the F2 generation were genotyped for the Pde6b mutation and grouped according to their genotype in wild-type and visually impaired mice. These groups were tested for significant differences in their activity in open areas of light-dark and zero maze tests. These tests were chosen since visual perception plays an important role in these paradigms and therefore also in QTL correlated to these traits. Concerning the light-dark test, the visually impaired mice exhibited a significantly higher activity in the open area compared to wild-type mice. There were not any differences detected in the zero maze test. These results lead to the assumption that the influence of mutation in the Pde6b gene on behavior could not be excluded completely, since differences were detected in one test. However, the other test did not revealed any behavioral differences and suggested that the Pde6b mutation may

only play a minor role in the behavioral response if there is one. Additionally, it is known from the literature that only rods are degraded by Pde6b mutation, whereas cones are still functional (Hart et al. 2005). This points to the fact that the examined mice were still able to distinguish between light and dark environments. Another interesting aspect is the influence of wall guidance on mouse behavior (Horev et al. 2007), which could have also influenced the behavioral parameters in the test. In the zero maze, for example, mice could recognize the closed areas through their tactile senses, such as whiskers or body hairs.

It can be concluded that the findings obtained from the genotyped and phenotyped mice of the F2 generation are suitable for the upcoming QTL analysis. The influences of the mutation in the Pde6b gene could not be excluded completely for the outcome of the behavioral analysis, but if there is one, it seems to be minor. Therefore, its influence on the QTL does not appear too strong and should not have an impact on the results of the QTL analysis.

5.2 QTL for anxiety and depression

Mapping of the QTL, which act on the behavioral measurements, was carried out with geno- and phenotypic data of the F2 generation mice and the R/qtl software. For the distance parameter in the light-dark test, a QTL on chromosome 1 at 57 cM was obtained. The association of this QTL to activity is supported by several studies, which similarly revealed a QTL for such a trait in this region on chromosome 1 (Gill and Boyle 2005, Koyner et al. 2000). It is most likely that this QTL is sex-dependent, since the analysis detected this locus only in male mice. Further studies also identified activity dependent QTL for the open-field test on chromosome 1 in a more distal position (Gershenfeld et al. 1997, Kelly et al. 2003, Singer et al. 2005, Turri et al. 2004), which points out that a minimum of two activity QTL are located on chromosome 1 (Bolivar et al. 2001, Turri et al. 1999).

A QTL on chromosome 15 at 22.2 cM was linked to the distance travelled in the zero maze with a very large 95 % confidence interval from 8.7 to 25.7 cM. This seems to be an anxiety-related QTL, because several studies likewise detected anxiety-related QTL in this region on chromosome 15. However, they did not provide any further positioning information (Laarakker et al. 2008, Singer et al. 2005, Turri et al. 2001a, Turri et al. 2001b, Turri et al. 2004).

Turri et al. linked this QTL to anxiety-related behavior measured in the open field, the elevated plus maze and the light-dark test, thus giving strong evidence for the importance of this QTL in relation to anxiety (Turri et al. 2001a). More detailed evidence came from three other studies, which also provided positional information of the identified QTL. Another study by Turri et al. mapped three QTL on chromosome 15, one at 20 cM for activity in the open field, the other at 22 cM for time in an open area in the elevated plus maze and the last at 24 cM for time in the enlightened area in the light-dark test (Turri et al. 2001b). The study of another group also found QTL in different measurements for open-field behaviors around 20 cM on chromosome 15; however, these are only suggestive ones due to their low P values (Eisener-Dorman et al. 2010). The most interesting work, which was performed by Henderson et al., identified a QTL from 22 to 28 cM on chromosome 15 linked to activity in aversive areas (Henderson et al. 2004). This is the only study in the available literature that provided information about confidence intervals, even though this QTL integrates completely in the interval found in this thesis and it revealed no options for narrowing the QTL on this chromosome. Despite the fact that their data suggested a relatively short interval, a database research revealed a large physical region of over 30 Mb.

In this study, a male-specific QTL for the time value in the light-dark test was detected at 55.5 cM on chromosome 14, which disappeared completely in the combined analysis for both sexes. Four studies have mapped an anxiety-related QTL to chromosome 14. Since this QTL was located in a position more upstream from the QTL detected in this study, it is possible that more than one QTL lies on this chromosome (Henderson et al. 2004, Turri et al. 2001a, Turri et al. 2001b, Turri et al. 2004). One recent approach discovered a QTL on chromosome 14 at 63 cM for an anxiety-related trait (Gill and Boyle 2005), which supports the finding in this study. There is not any information about the size of the interval. For this reason, a direct comparison with the QTL of this study consisting of a 20 cM interval was not possible.

All detected QTL are in accordance with the results of former QTL studies, which confirm the reliability of the present approach. Most of the intervals are very large and contain a high number of genes, which complicates an effective identification of possible candidate genes.

A completely new and exclusively male-specific QTL was detected on chromosome 7 at 3.4 cM for immobility time measured in the forced swim test, which is a depression-related paradigm. Recent studies identified non-gender-related QTL on this chromosome. These QTL are located in a region more upstream from 40 to 60 cM and solely for anxiety-related behavior measurements (Eisener-Dorman et al. 2010, Henderson et al. 2004, Turri et al. 2001a, Turri et al. 2001b, Turri et al. 2004). Therefore, it can be concluded that a new QTL for depression-related behavior in male mice was mapped. The selection of possible candidate genes turns out to be difficult due to the length of the QTL of 7 cM corresponding to a region of 27 Mb on the chromosome.

An outstanding finding of the present study was the identification of seven QTL on chromosome 5 in a region between 41 and 62 cM. These QTL were correlated with anxiety- as well as depression-related behavior and were mapped for both sexes. It was consequently assumed that these QTL and the according possible candidate genes contribute in getting further insights into the strong comorbidity of anxiety- and depression-related diseases (Simon 2009). This new discovery of a depression-related QTL position complements former studies, which only detected anxiety-related QTL more upstream on chromosome 5 (Gill and Boyle 2005, Turri et al. 2001a, Turri et al. 2001b). For depression-related traits, only one recent publication identified a QTL on chromosome 5 (Tomida et al. 2009). This one is located in an upstream region in relation to the QTL detected in this thesis. Another study mapped a QTL at 59 cM, an equal position on chromosome 5 to the findings shown here recently, which was correlated to ethanol-withdrawal-induced anxiety (Drews et al. 2010). Although it could be argued that anxiety-related behavior is the driving force for this QTL. Since the role of ethanol on this result cannot be excluded, this QTL was not considered for further comparisons or narrowing approaches.

All seven QTL on chromosome 5 were selected for an approach to narrow the length of the interval due to the fact that all QTL presented a common overlapping region. Therefore, the focus was set on the QTL within this common core region, where the underlying gene of these QTL should be located. Additionally, there was an opportunity to apply a comparative genomics procedure, which compares linked regions from other species with the identified QTL in order to reduce the length. Comparative genomics is commonly used to narrow QTL intervals (Burgess-Herbert et al. 2008, DiPetrillo et al. 2005). This technique also proved to be useful in several

studies (Jann et al. 2009, Rollins et al. 2006, Sheehan et al. 2007, Yamada et al. 2003). Kaabi and colleagues identified a region on the human chromosome 4, which is syntenic to a respective region on the mouse chromosome 5 and linked to anxiety disorders (Kaabi et al. 2006). This trait fits very well with the anxiety-related traits in mice used in the present thesis. Surprisingly, the syntenic region in mice partially overlapped with the narrowed core QTL on chromosome 5. This finding revealed that it was possible to reduce the length of the QTL interval even more to the final length of 15 Mb. The relevance of this locus is supported by recent studies linking this region to bipolar disorders in humans (Cassidy et al. 2007, McAuley et al. 2009). These bipolar disorders also present a high comorbidity with anxiety disorders (Baldessarini et al. 2010, Simon 2009, Young et al. 2009), suggesting that this locus plays an important role in stress-related psychiatric disorders.

In conclusion, the narrowing of QTL by comparative genomics was a sweeping success and the benefit of this method was proven by further identification of possible candidate genes.

5.3 Identification of candidate genes

The narrowed QTL on chromosome 5 contained 171 genes. An evaluation was carried out as to reveal which genes would be prominent candidates for the anxiety- and depression-related traits. To meet this challenge, the new FunSimMat software (Schlicker and Albrecht 2010) was utilized, which compared the gene ontologies of the candidate genes with the respective ontology of the reference gene TPH2. From the initial 171 genes, a few were excluded due to two major difficulties. Firstly, some genes had to be excluded due to missing information about a transcript and consequently a missing UniProt ID, which is required as input for the software. The second problem resulted from the missing gene ontology information for several genes, which leads to low values for genes with little or no information in the biological process and molecular function subgroups of the gene ontologies. These difficulties can be common when biological databases are used, since there is still a lack of content, despite the fact that information in these databases has grown immensely in recent years (Mooney et al. 2010). Additionally, biomedical research is mostly focused on specific topics and retrieves more information in the research fields of strong interest, which leads to more gene ontology annotations as well as biased gene ontologies in these topics (Done et al. 2010, Pesquita et al. 2009).

However, the information content of biological and especially genomic databases demonstrated a rapid growth in the past few years, recently reaching a point where their utility value strongly increased. For this reason, this approach was regarded as promising and highly beneficial for the candidate gene selection.

The comparison of the traits linked to the narrowed QTL with data from the Online Mendelian Inheritance in Man database suggested the entry 607478 containing the tryptophan hydroxylase 2 gene (Tph2) as a reference gene. SNPs in this gene were associated with depression disorders (Garriock et al. 2005; Zhang et al. 2005) and bipolar disorders in human patients (Cichon et al. 2008). Beaulieu et al. found abnormalities in depression- and anxiety-related behavior by expressing a variant of the human Tph2 in mice (Beaulieu 2008). Since these combined literature data fit quite well with the behavioral traits analyzed in this study, Tph2 was chosen as the reference gene for gene ontology comparison. This was the first approach to utilize the FunSimMat software for prioritization of candidate genes and no related publication data exist, because the software was only introduced in 2009.

The gene ontology comparison with this software revealed three candidate genes; the enolase phosphatase 1 and 17ß-hydroxysteroid dehydrogenases type 11 and 13, all confirmed by expression differences in brain or liver tissue in the parental strains. Furthermore, non-synonymous SNPs in coding regions – two for Enoph1 and one for Hsd17b13 – could be identified in a database research and SNP assay. The focus was set on the Enoph1 candidate gene in further experiments, since two prominent publications link this gene to anxiety-related behavior in mice (Ditzen et al. 2006, Ditzen et al. 2010). As Enoph1 is part of the methionine salvage pathway, it could potentially influence S-adenosylmethionine (SAM) levels via this pathway (Pirkov et al. 2008). Recent studies indicated that SAM is a mood enhancer (Baldessarini 1987; Benelli et al. 1999; Mischoulon and Fava 2002), suggesting that it is an interesting target. However, the present study revealed that the protein encoded by Enoph1 did not influence the S-adenosylmethionine (SAM) levels. SAM is also essential for the polyamine synthesis (Benelli et al. 1999), that synthesize spermidine as well as spermine and that is connected also to the methionine salvage pathway (Pirkov et al. 2008), downstream of SAM. Polyamines consist of small cationic molecules and play an important role in cellular proliferation. They can interact with a broad range of molecules like DNA, nucleotide triphosphates, proteins, and also RNA (Igarashi and Kashiwagi 2010). It is well known that polyamines themselves are regulated in

anxiety- and depression-related traits in rodents (Fiori and Turecki 2008, Genedani et al. 2001, Hayashi et al. 2004, Lee et al. 2006, Zomkowski et al. 2006) as well as in humans (Fiori and Turecki 2008, Sequeira et al. 2007). Differences in spermidine levels could be verified in the present study. This finding is consistent with literature data where decreased spermidine levels were detected in depression-related traits (Genedani et al. 2001). Other groups were not able to confirm these results, since they detected no differences in polyamine levels in mice with a restraint stress model (Hayashi et al. 2004). or even increased spermidine levels in the same stress model for mice (Lee et al. 2006). Furthermore, a human study with suicide victims suffering from depression revealed increased spermidine levels (Chen et al. 2010).

The regulation of polyamines in anxiety- and depression-related traits is not really understood and there is a controversial discussion with many unsolved questions. Taking this together with a lack of differences in SAM levels in the presented experiment, it can be concluded that Enoph1 did not influence SAM levels and also had no effect on polyamine levels via SAM.

This raises the question again of whether Enoph1 or one of the Hsd17b is the responsible gene behind the narrowed QTL. On one hand, Hsd17b genes are prominent candidates due to their expression differences, while on the other hand Enoph1 is also interesting if the focus is transferred to the function of SAM in epigenesis.

5.3.1 Enoph1 and epigenesis

The major function of SAM is the delivery of methyl groups to the *de novo* methyltransferase, and for this reason it plays an important role in DNA methylation (Detich et al. 2003, Hitchler and Domann 2007), which is one of the driving forces in epigenesis. Epigenesis is a major regulating process in the nervous system (Colvis et al. 2005, Feng et al. 2007, Feng and Fan 2009, Hsieh and Eisch 2010, Jiang et al. 2008) and it plays an important role in mood disorders shown in several recent studies (McGowan and Kato 2008, Renthal and Nestler 2009, Tsankova et al. 2006). Enoph1 could influence anxiety and depression through epigenetic regulation by changing SAM levels in an early postnatal phase, since in this phase the development of the central nervous system is in many aspects affected by epigenetic processes. It was shown that these processes have a strong influence on behavioral

development for dealing with stress (Branchi 2009, Holmes et al. 2005, Weaver 2009).

Studies have shown that the stress sensitivity is transferred from maternal behavior to offspring by epigenesis (Weaver et al. 2004; Weaver et al. 2005), demonstrating the crucial role of this process in behavioral development. It can be supposed that only in the early postnatal phase, Enoph1 has a regulating influence on SAM levels and thus on the epigenesis. The different activity of Enoph1 in C57BL/6J and C3H/HeJ mice could lead to different SAM levels in these strains only in the early postnatal phase, supporting the finding of equal SAM levels in adult animals. Differences in SAM levels between the parental mouse strains could then induce different genetic imprinting leading to changes in gene expression, which results in the different stress sensitivity of the mice. This might be the way that differences in Enoph1 activity, which are based on two SNPs, could be the cause of different stress sensitivities.

When the chromosomal imprinting is terminated, other controlling circuits will take over the control of SAM and Enoph1 will consequently lose its relevance, reaching the state observed in the adult animals. The polyamine system could then be regulated by the different genetic imprinting in epigenesis leading to the different levels in adult animals.

However, this hypothesis needs to be validated by further experiments observing SAM levels during postnatal development. Furthermore, the imprinting status of relevant genes should be mapped in this phase. However, the networks controlling DNA methylation and regulating the genetic imprinting remain unclear in many aspects. It would be a challenging approach to elucidate the influence of Enoph1 on epigenesis in this context.

5.3.2 17ß-hydroxysteroid dehydrogenases in anxiety and depression

The hydroxysteroid dehydrogenases are likewise prominent candidate genes as expression differences were found in liver tissue specimens from the parental strains. Hydroxysteroid dehydrogenases play an important role in steroid hormone metabolism (Moeller and Adamski 2006), which is involved in many processes like growth, differentiation, metabolism and reproduction (He et al. 2010). Steroid hormones are able to cross the blood brain barrier (Banks et al. 2009, Joëls 1997) and could influence the hypothalamic pituitary axis (Giussani et al. 2000), as well as

GABAergic transmission (Henderson 2007). Both are major pathways in the regulation of stress and play an important role in anxiety and depression.

Glucocorticoids are the most intensively studied steroid hormones due to their prominent role in the HPA axis and therefore in major stress response pathways (Chrousos and Kino 2009). However, other steroid hormones like androgens and estrogens are also capable of regulating stress response via the HPA axis (Bao et al. 2008, Young and Korszun 2010) and recent studies allocated their role in anxiety and depression (Amore et al. 2009, ter Horst 2010).

Androgens and estrogens are regulated by the dehydrogenase encoded by Hsd17b11 (Brereton et al. 2001, Li et al. 1998), whereas substrates for the dehydrogenase encoded by Hsd17b13 are not known so far. However, due to the similarity of 78 % (including 65 % identity in the amino acid sequences) of these two types of Hsd17b proteins in humans (Moeller and Adamski 2009), it can be assumed that they potentially share the same substrates. Hsd17b13 could be a tissue-specific variant of Hsd17b11, since it was exclusively detected in mouse liver, compared to the more ubiquitous expression of Hsd17b11 (Horiguchi et al. 2008). The Hsd17b proteins could influence the levels of steroid hormones due to their ability to catalyze the interconversion between active and inactive forms. Thus, the expression differences of the Hsd17b genes observed between the parental mouse strains could lead to changes in the levels of active androgens and estrogens, which might be responsible for a different regulation of the stress response in the C57BL/6J and C3H/HeJ mice. Raven et al. provided additional evidence when Hsd17b proteins were identified as biological markers of depression (Raven and Taylor 1998). These combined findings are strong evidence for a crucial role of androgens and estrogens in the regulation of anxiety and depression. Therefore, it can be assumed that Hsd17b11 and Hsd17b13 are likewise prominent candidate genes and it is worth investigating their role further in the regulation of steroid hormone levels. The influence of different steroid hormone levels on the major regulating pathways in anxiety and depression should also be elucidated.

5.4 Generation of Enoph1 knockout mouse

As the focus of the recent work was set on Enoph1 candidate gene, the intention arose to generate a knockout mouse in order to get a clearer view on the role of this gene according to anxiety- and depression-related behavior. Out of six different ES-

cell clones generated by the German Gene Trap Consortium, three of them were verified by RT-PCR and sequencing of genomic regions flanking the gene trap. So far, one clone has been successfully injected into blastocysts and one highly chimeric male mouse was received. However, further investigation points out that this mouse was infertile, since no embryo was detectable at day 7.5 after breeding with female mice. Since infertility is a well-known problem concerning the generation of knockout mice, it was decided to inject new clones to receive more chimeric males in the near future.

The generation of a knockout mouse is likewise interesting for the Hsd17b11 and Hsd17b13 genes to further examine their role in steroid hormone metabolism. However, a recent study demonstrated the possible difficulty of this approach, because the knockout of another Hsd17b gene encoding for type 12 17ß-hydroxysteroid-dehydrogenase is lethal at embryonic day 9.5 (Rantakari 2010). Furthermore, it was observed that this gene is highly expressed in neuronal tissue of the embryo. It can be assumed that the knockout of Hsd17b11 or Hsd17b13 could result in equal phenotypes. Difficulties due to a high mortality of the respective knockout mice could be avoided by using a conditional knockout system, for instance with a gene knockout in adult animals.

The gene trap technique also provides the option of a conditional knockout utilizing different recombinases. This would be the method of choice for generating Hsd17b11 or Hsd17b13 knockout mice.

6 Summary

In this QTL mapping study, several genetic regions were linked to anxiety- and depression-related behavior in the mouse. Furthermore, some previously identified QTL linked to these behavioral traits were verified in the present analysis, demonstrating the convergence of this approach and most previous studies. The novel QTL identified on chromosome 5 were narrowed to a common core interval of 9 cM. A subsequently applied comparative genomics approach with a syntenic region identified in a study in humans narrowed this core interval even more to a 15 Mb region. For the selection of possible candidate genes, a semantic similarity comparison of gene ontologies was conducted revealing three very prominent candidate genes, encoding for enolase phosphatase 1 (Enoph1) and 17ß-hydroxysteroid dehydrogenases type 11 and 13 (Hsd17b11 and 13), respectively. These impressive results also demonstrated the power of gene ontology comparison on candidate gene prioritization.

All candidate genes revealed different expression levels in brain or liver between the parental strains, and for Enoph1, two non-synonymous co-segregating SNPs were verified in the parental strains, which is in line with recent publications. Further analysis confirmed differences in polyamines, which are regulated in anxiety and depression, but S-adenosylmethionine, the possible mediator of this result, revealed no variation. This leads to the conclusion that Enoph1 has no influence on S-adenosylmethionine levels and might have no influence on anxiety as well as depression by this mood enhancer.

Despite these results, Enoph1 still remains interesting as a candidate gene. It is possible that Enoph1 influences S-adenosylmethionine levels in the early postnatal phase and hence plays a role in epigenesis. The upcoming Enoph1 knockout mouse will provide deeper insights into the function of Enoph1. Upcoming research may resolve the question of what influence the genes Hsd17b11 and 13 have on steroid hormone regulation and anxiety as well as depression, since there are many unknown aspects. Taken together, all three candidate genes should be evaluated in further studies to identify the gene underlying the QTL. Additionally, future results of other QTL approaches with anxiety- and depression-related traits may provide the chance to narrow known QTL and simplify the selection of candidate genes.

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8 Appendix

8.1 Supplemental material

Supplement table S1. Name, primer sequences, fluorescent labels, expected product lengths, annealing temperature and polymerases used for the microsatellite genotyping (Q = Qiagen HotStarTaq; NEB = New England Biolabs Taq Polymerase; no annealing temperature = 60°C).

Marker name	Position (cM)	Forward primer	Reverse primer	5'-Label forward primer	Expected fragment lengths		Annealing temp.	Polymerase
					C57BL/6J	C3H/HeJ		
D1Mit64	5	AGTGCATTATGAAGCCCCAC	GTGTCTTTCAAATTTTAAAACAACCCATTTG	FAM	126	121		Q
D1Mit430	10	TATTAATGTTGAAGCCAGAAGCC	GTGTCTTCTTTAATCATCTCTGTGGCAAGG	HEX	121	131		Q
D1Mit169	15	CGCTGACTGCTACTTTATTATATTCC	GTGTCTTTCTGATTTACTGTCAATCAAGAGACC	TET	150	156		Q
D1Mit245	20,2	TGGTTACACAAGTCCAATACCG	GTGTCTTGGCCCAGGTCTATAAATAAGCC	FAM	151	163		Q
D1Mit236	25,7	ATACCCACCTAGCCTTTGTATAGG	GTGTCTTGGAAGAAGGCTCAGCAAGTG	HEX	150	143		Q
D1Mit161	27	ACCAGCCCTCCTTTTTTGTT	GTGTCTTCTTGCCTCTTCAGGCACCT	TET	121	123		Q
D1Mit303	34,8	GGTTTCTATTTCGGTTCTCGG	GTGTCTTTCTGTGCTGCAAAACAGAGG	FAM	131	125		Q
D1Mit251	38,1	TCTGTCCTTTCTGATGATTACTTCA	GTGTCTTATGGTGAAATATCACATGGCA	HEX	195	207		Q
D1Mit135	59,7	TTGATGACTTAAAAATGTCAATACTGA	GTGTCTTACACCCCTGCCTTAAAATATTT	TET	184	174	50°C	Q
D1Mit94	64	CGACTTCCCTTGATGTCCAT	GTGTCTTTTTGTGTTGTGCAGTCTGTCTG	FAM	163	223		Q
D1Mit139	65	CGACATTATCACTTCAGAGTTTGA	GTGTCTTGAGTTCCAGCCCACTGAGAG	HEX	249	237		Q
D1Mit446	70	TGAGTATATCATGAAGACAGCAACC	GTGTCTTACGTATTTACCTTGTTCTGAATTTTG	TET	175	141		NEB
D1Mit200	80	GCCATGTTCATGTACATAGGTAGG	GTGTCTTATGGATGGATGGTTTTCCTG	HEX	212	304		Q
D1Mit399	85	TTAGGGTATGGGAAGGGGAG	GTGTCTTTCATTTCCCAGTCATTGTGTG	TET	145	142		Q
D1Mit353	92,3	TACACTATGGGTATATGCTCACTATGC	GTGTCTTACACATGAACATACTCATATGCACA	FAM	125	109		Q
D1Mit206	95,8	TGAGGCACCTTTGTATTCAGC	GTGTCTTCCAGATGTCTTTGAACATTCTCC	HEX	130	125		Q
D1Mit292	107,3	GAACTGGAGGTTTGCTACTGC	GTGTCTTGGACATTGTTATCTCAGTTTTCTTC	FAM	208	205		Q
D1Mit155	112	ATGCATGCATGCACACGT	GTGTCTTACCGTGAAATGTTCACCCAT	HEX	259	223		Q
D2Mit1	1	CTTTTTCGTATGTGGTGGGG	GTGTCTTAACATTGGGCCTCTATGCAC	TET	131	127		Q
D2Mit365	17	GAGATCCCACTGATGATACAAGC	GTGTCTTAGATGTGCCCAAGGGTCC	HEX	109	113		Q
D2Mit369	27,3	GCCTCCATCAAAGGAAGACA	GTGTCTTTTTCTTCCCTGTCTATGTGATAAGG	TET	136	117		Q
D2Mit458	31,7	GTAGTTGAGGAAGACAATTGACACA	GTGTCTTAGTGCTGTCCTCTGGGCTTA	FAM	129	105		Q
D2Mit90	37	TCTTTTGTAAAGATTTGTTTCGTG	GTGTCTT TATGTCTAGGGTGTCCGATGC	HEX	93	107		Q
D2Mit37	45	TGTGCAAGCCAGAAAAGTTG	GTGTCTT GAAGGGGATTGTAAATTGGTACC	FAM	181	187		NEB
D2Mit100	47,5	GTGTTCCTAAGGTTGTATTTTGGC	GTGTCTTGAAATTTGACAATTGCTAGGTGC	HEX	121	133		Q
D2Mit101	52,5	ATAATTCCTGATTTGCTGTTTGTG	GTGTCTTACATGAAGCCTAGAGGGTGC	TET	199	227		Q
D2Mit398	57,9	GTACCTCTGGCTCCTGAGG	GTGTCTTTATTTTAAAAGTATAGGTGTGTGCCG	FAM	153	139		Q
D2Mit305	60,1	CTCAGAAAACATGCAATTGAGG	GTGTCTTATGAGTGCAAACCAAATAAAATTG	HEX	144	108		Q
D2Mit395	66,9	AGGTCAGCCTGGACTATATGG	GTGTCTTAGCATCCATGGGATAATGGT	TET	135	129		Q
D2Mit164	71	TCTCTGCTAATTAAGTTGAAGAGTGC	GTGTCTTACCAGTGTGTGTTTGTATGATGTG	FAM	148	120		Q
D2Mit498	80,9	GCAGCCTTTCCTTCCTTTCT	GTGTCTTCAGATAGAGCACTCAGACATACATACA	TET	129	121		Q
D2Mit285	86	TCAATCCCTGTCTGTGGTAGG	GTGTCTTTATGACACTTACAAGGTTTTTGGTG	FAM	145	155		Q
D2Mit263	92	ACTGAATCATCTCTCTCCTCAGC	GTGTCTTAGTTCAGTTCTTAGAACCCACAGC	HEX	147	141		Q
D2Mit226	96	TTTTTGCAACTTTGTTAAGAATTCC	GTGTCTTAAAACACCCTCCCACCCTT	TET	109	131		Q
D2Mit147	102	CATCCCTAAGACAAGCAACTCC	GTGTCTGTCACAATGTCCTTCTCCATCA	FAM	124	128	50°C	Q
D2Mit457	108	GACTTTCACATGAAAGTTGTTAGACC	GTGTCTTTAGTGATTGCACTTAATTGTATGCC	HEX	127	121		Q
D3Mit92	10,6	CCTCTGTTAGGATATCCAATCCC	GTGTCTTCTTGTGTCCCTCCACTTGGT	FAM	249	245		Q
D3Mit203	11,2	CTGAATCCTTATGTCCACTGAGG	GTGTCTTGGGCACCTGCATTCATGT	HEX	161	145		Q
D3Mit179	16,5	TTTCCACAGGGAACCATACTT	GTGTCTTAACACACTACCTATGTTTTCTTTCTCT	TET	157	134		Q
D3Mit333	22	CTCCCTCCCTTCCTCCTTC	GTGTCTTACAAAAGCAGAAGACTGATCCC	HEX	133	151		Q
D3Mit67	28	AGCATACATCATAGCCTAAAATGG	GTGTCTTGTAACTAGGGAGACAGCCCACTTG	TET	153	151		Q
D3Mit199	33,7	CCAGACCTCAGAAAGTGAGTCC	GTGTCTTACCATGACATTCATGCTATTGTG	FAM	148	121		Q
D3Mit339	38,3	TCTATATTTGGGGGGAAGGG	GTGTCTTGATTTAGTGTCAAAGGCTATGCA	HEX	155	125	50°C	Q
D3Mit74	41	TCATCGTAGCAATAGAAATCCTG	GTGTCTTCACCGTTTCCTGACCTCTGT	TET	159	155		Q
D3Mit101	47	CCTCTAGATGCATACATGTGCC	GTGTCTTGGTCAAGTTAAGTGTATTTTTTCCC	FAM	115	133		Q
D3Mit57	55	TCCAGTTACTTGGTGAACTCCA	GTGTCTTATATGTGTACATGTTCATGGTGTG	HEX	165	163		Q
D3Mit216	58,8	AGGACTGAAGAAACATACACATGC	GTGTCTTAGAAACATCTTGATTTTCAACAAGG	TET	129	149		Q
D3Mit14	64,1	ATTGCGGTTAAAGTTTGCTT	GTGTCTTTCCTGCAAATTGTCCTCTGA	FAM	177	205		Q
D3Mit257	70,3	CCTAGCGCAGGAATAGTTAACC	GTGTCTTACAAACAGAACAAAACAAAAAGTCC	HEX	135	227		NEB
D3Mit18	76,2	GAACAGTTCCCAGGTCCTCA	GTGTCTTCTGCCTTTAAATTCTGTCACCC	TET	238	219		NEB
D3Mit262	80,2	TTGTGTTTTTTATTGTTTGTTTTGG	GTGTCTTGAGGTAGAGAAATCTGACAGAGCC	FAM	142	146		Q
D3Mit116	84,9	TCACTGCCCATCTTTGTAACC	GTGTCTTCCCAGAGACCCGGAATAAGA	HEX	270	282		Q
D3Mit19	87,6	CAGCCAGAGAGGAGCTGTCT	GTGTCTTGAACATTGGGGTGTTTGCTT	TET	167	183		Q
D4Mit149	0	TGAATTCAGAAGGATGTGTGTATG	GTGTCTTATGTGAGAATCAACACCTGAGG	FAM	121	137		Q
D4Mit39	10,6	TCTTTTCTGCCCTCACAGCT	GTGTCTTGTCTATCTTGCCAATTTCAGGG	TET	179	175		Q
D4Mit286	14,5	ATGGGGTCTAGGAAAACATGG	GTGTCTTAAATTATGAGTATTTCACCTGAGTGTG	FAM	103	83		Q
D4Mit89	19,8	GTGGGCATTTTTTTTGTGGA	GTGTCTTTCCCAGATCCTCTCCCTCT	HEX	139	125		Q
D4Mit139	28,6	CAGCCGTAGAAGAGAAGTAATTTT	GTGTCTTATCAAACTGGGAAGAGCCAA	TET	159	151		Q
D4Mit87	31,4	ACAGGTAGGAATGGAGCCCT	GTGTCTTTCATCCCTTTGCCAAAGC	FAM	123	127		Q
D4Mit178	35,5	GCCCTGAAGGTAAATCAGTAACT	GTGTCTTGCTCAGGAGGTACATTGCCT	HEX	153	177		NEB
D4Mit27	42,5	GCACGGTAGTTTTTCCAGGA	GTGTCTTTGGTGGGCAGGCAATAGT	TET	157	125		Q
D4Mit153	45,5	ATATGGAGTCTGTGTGTGTGTGC	GTGTCTTCACTGAATTTCTATTGTTGGAATAGG	FAM	129	119		Q
D4Mit31	51,3	ACGAGTTGTCCTCTGATCAACA	GTGTCTTTAGCCAGAGCAAACACCAACT	HEX	129	119		Q
D4Mit76	55,7	TGAAGGAACCTGAAGCAAGG	GTGTCTTACCTCCCAGGAGTGTCCAG	TET	189	179		Q
D4Mit203	60	GAATTCTTCCTGGGCCTTTC	GTGTCTTCAAGAGCCCAGGTGTGGTAT	FAM	151	131	65°C	Q
D4Mit251	66	AAAAATCGTTCTTTGACTTCTACATG	GTGTCTTTTTAAAAGGGTTTCTTTATCCTGTG	HEX	123	135		Q
D4Mit32	69,8	CCCTGGATAAACGTCATTTAATTC	GTGTCTTATGGTTGGGTGTTACCAGGA	TET	155	191	50°C	Q
D4Mit233	75,5	TGGTCATGTGTGTCCATGC	GTGTCTTACTTCATGTAGCCAGGTGGG	FAM	184	174	65°C	Q
D4Mit42	81	CATGTTTGCCACCCTGAAAC	GTGTCTTCCTCACTTAGGCAGGTGACTC	HEX	109	101		Q
D5Mit146	1	TTAAATCTGAAGGTGTGGCTATAGC	GTGTCTTGAGATTGCAAGTAAAGTGAGAGAGG	TET	133	129		Q

(Continued on the next page)

Supplement table S1.

Marker name	Position (cM)	Forward primer	Reverse primer	5'-Label forward primer	Expected fragment lengths		Annealing temp.	Polymerase
					C57BL/6J	C3H/HeJ		
D5Mit348	8	CTGACCAGAACACAGCATAGTACA	GTGTCTTTTAAAATAGGAAAAGCATTCTTTCC	FAM	130	140		Q
D5Mit387	15	CCCCATGTATCTCTAGATTAACAATG	GTGTCTTGCACTCGTGTACATAACCAAATAC	HEX	181	189		Q
D5Mit352	20	CCCAGAGCCCACATCAAG	GTGTCTTTAGGTGGGTGTGTCTCTCCC	TET	120	127		Q
D5Mit106	26	GTCAGGCATGGTGATTCCAT	GTGTCTTATGGATGACTGTGAACATACAACT	FAM	170	160		Q
D5Mit183	29	TATAAAGATAATCAGGGCTTAAACTCG	GTGTCTTACCTCCACAACATGAGCACA	HEX	120	122		Q
D5Mit254	34	GTGCAGGCCTGAATTGAAAT	GTGTCTTCAAAGTGCCTGTGCATGTG	TET	143	131		Q
D5Mit201	42	GAGGACTCCTTCGATTTCCC	GTGTCTTTTCCTAAGCAGGAACTGACCA	FAM	117	111		Q
D5Mit18	45	CTGTAGTGGGTGGTTTTAAAATTG	GTGTCTTATGCCACTGGTGCTCTCTG	HEX	245	227		Q
D5Mit277	58	GTGTGTTTGTGCATGGGTATG	GTGTCTTACCATCGGGAAAAAATGTAGC	FAM	131	147		Q
D5Mit210	64	GATGGGTGCATTCATCCTG	GTGTCTTTGAAAGTGATTCCTCAGGGG	HEX	201	213		Q
D5Mit95	68	TGTTCTTGTCCATGTCTGATCC	GTGTCTTAACCAAAGCATGAAACAGCC	TET	123	141		Q
D5Mit99	80	CAGAAAAGAGAAAACGGAGGG	GTGTCTTTTCCTGCTGCCTGAAGTTTT	HEX	105	211		Q
D5Mit409	84	GACACAGTTTGGTCACTTGCA	GTGTCTTACACACTCTCTCTATTCCACTTTCTG	TET	205	221		Q
D6Mit138	0,68	GCTCTTATTAATGAAGAAGAAGGAGG	GTGTCTTCAAAGAAAGCATTTCAAGACTGC	FAM	118	142		Q
D6Mit83	3,5	TTCTGTAAATTGCTAATCTGTCCA	GTGTCTTTTGTATGCATTTAACAACTCAGGA	HEX	157	137		Q
D6Mit159	7	CATATTCAAGACGGAGACTAGTTCC	GTGTCTTCACATGAAACACATGCACACA	TET	123	147		Q
D6Mit268	15,6	AGTCAGAATATGGCAAGTCAGTG	GTGTCTTTTTCAGAGTCTTTCTTTCAGTATCTCC	FAM	130	117		Q
D6Mit274	20,5	GCAATGCCAAAATGTTCAAA	GTGTCTTTCCTTCTCCATTTACACTTACAACA	HEX	122	102		Q
D6Mit384	27,5	AATGCTTTATATGCAAACTACTCTCTC	GTGTCTTGAATATAGCAAGACAAGGGAGACA	TET	133	155		Q
D6Mit209	32,5	CTCCCCCTCTGTGTGATTGT	GTGTCTTTATTACACCAGACCCATGTGG	FAM	141	145		Q
D6Mit284	37,5	GGCTGCTGAGAAACAACCTC	GTGTCTTGAGTATTGAGCCAAATCCTCC	HEX	152	150	65°C	Q
D6Mit36	46	ACCATCTGCATGGACTCACA	GTGTCTTGTTGAAGAGGACGACCAAGTG	FAM	203	185		Q
D6Mit62	51	CTCACCCACACTCCTGTTAGC	GTGTCTTTGTGTGTGATAGACTTACTGGGG	HEX	297	173	65°C	Q
D6Mit219	63,6	AAATGTTGACTTTAATGAGGTAATTG	GTGTCTTTCACATATCCCTCAGACATGC	FAM	195	185		Q
D6Mit59	67	GCCATCCTTTGTAATAACAAACA	GTGTCTTCGTCTGGGAAAACCTCAAAA	HEX	175	185		Q
D6Mit15	74	CACTGACCCTAGCACAGCAG	GTGTCTTCCTGGCTTCCACAGGTACT	TET	267	202		Q
D6Mit374	74	TTCTGGCTCTTAACAGTCTGTCC	GTGTCTTTACATATGCCAATGATATTCTCCC	FAM	185	153		Q
D7Mit21	0,5	GGGTTGAACCTTACAGGGGT	GTGTCTTATCAAACCAGCCCAAGTGAC	HEX	135	139		Q
D7Mit191	3,4	TTGGGTTTGTACTACCTAGATACCTC	GTGTCTTCCTCTAGGGCTCTTGCACAC	TET	158	190		Q
D7Mit266	10	TCAGGGATGTCTTAAAACTGGG	GTGTCTTCGCTGTAAAGCGTATTCGTG	FAM	129	123		Q
D7Mit228	18	ATTCTTGGCCTTTTCTTGTAACA	GTGTCTTAAACCTCCACACTGACTTCCA	TET	155	147		Q
D7Mit229	23	GGTTCTCTTTCCTTGTTTGCC	GTGTCTTTACTGGTTACATCTGGTGGGTG	FAM	130	150		Q
D7Mit248	27,8	AATCAGGCAACTCAGGCACT	GTGTCTTTCCTTAGGTCTCCAGTGAAAGC	HEX	115	119		Q
D7Mit346	34	CTCCTTTTTGGTACATATATACACACA	GTGTCTTACACTGGAGAGCCAGGAGAA	TET	103	109	50°C	Q
D7Mit31	44	TTCAAACCATCCAGTAAGTCCA	GTGTCTTTTGGTGAACTGCTTCAATGC	HEX	253	233		NEB
D7Mit323	50	TTTCACCTTCTAATCCTACTTCCTG	GTGTCTTTGTCCAGAACAGGAAATAGAGTACC	TET	123	149		Q
D7Mit281	52,4	TTCCTCTACCTCCTGAGCCA	GTGTCTTGCCACAAGGAAGACACCATT	FAM	120	210		Q
D7Mit66	57,5	TTCACTCCCAGCCAGTCTCT	GTGTCTTTAACCAGGAAACACACGAACC	HEX	171	155		Q
D7Mit105	63,5	AGCAAAGTAAGGCAGACTTTGG	GTGTCTTAGGAGAGGCAGAACATGGAA	TET	267	249		Q
D7Mit259	72	CCCCTCCTCCTGACCTCTT	GTGTCTTGTCTCCATGGGAACCACACT	HEX	155	159		Q
D8Mit124	6	CAACTGTGTATCATAAACTGGGAA	GTGTCTTGAAGAATCACTCAGCAGTGTATGG	FAM	136	142		Q
D8Mit289	11	AAAAAGAAAAGAAGGCTTAGTAATGTG	GTGTCTTCTTGCTATTCATTGCAAAATTCC	HEX	159	125	50°C	Q
D8Mit63	15	TCTGGAACACAGTCCAATTCC	GTGTCTTATATGTGTGAGGGTTTTACCGG	TET	215	229		Q
D8Mit190	21	CTTTGTTGCTGTTTCATTCTGG	GTGTCTTAGTCATATACAAGGTCAACCTGAGC	FAM	145	123		Q
D8Mit339	23	ACCTATGGTACACACACATCGC	GTGTCTTCAAACATTTTTAGGCATTTAGATCC	HEX	129	113		Q
D8Mit231	31	TGCAAAGAAAAAAGTATCAAAATTG	GTGTCTTTGTGTCCTATTTGCAATGTAACTG	TET	149	167		Q
D8Mit135	37	TTGTTTTAAAAGGAAGGTCTATTTTAG	GTGTCTTCAGAGCCCACATGACAGAGA	FAM	207	181	50°C	Q
D8Mit80	41	TGCATTTGTCAGGGCTCTC	GTGTCTTATGACACATGAGCCTCCACA	HEX	115	141		Q
D8Mit271	57	GGCAGAACCACAGGTTGATT	GTGTCTTGGAATGAGGTTTGGGTCAAA	HEX	103	127		Q
D8Mit89	59	TGTTTTGAATCTGTTATTAGGTGTG	GTGTCTTGAGAGAAAGGAACAAATTTATCAAGG	TET	139	129		Q
D8Mit49	67	TCTGTGCATGGCTGTGTATG	GTGTCTTTGGTGTGCTGCTGATGCT	FAM	159	161		NEB
D8Mit13	67	CCTCTCTCCAGCCCTGTAAG	GTGTCTTAACGTTTGTGCTAAGTGGCC	HEX	105	98		Q
D8Mit280	72	CATGCAATTCCAATGTCAGTG	GTGTCTTTAGCACTCAATCAAACCCCC	TET	115	167		Q
D9Mit1001	15	ATATCAGGCATGCATTATGATCC	GTGTCTTTCTCTCTAGTGGGATTATCAACACA	HEX	129	139		Q
D9Mit2	17	GTGGTCTGCCCTCTTCACAT	GTGTCTTCAAAGCCAGTCCAACTCCAA	TET	182	189		Q
D9Mit328	23	CATTTACTGTCTCTCTTTCATTCTCTG	GTGTCTTCTTACATCTGGTCCACAAGAAGG	FAM	191	237		Q
D9Mit129	26	TTGTCTTTTAACCTCCTGGAGC	GTGTCTTTCCCATCTTTCTCCTTGTGG	HEX	139	157		Q
D9Mit162	30	ACCACCAAATACAACCACTTCC	GTGTCTTGACTGAACAATCAGGAGTATGGC	TET	147	129		Q
D9Mit208	36	GCCTCTCTTTCTTTAAACACTTTAAG	GTGTCTTCCTCCACACACCTGTTTGTG	FAM	119	93		Q
D9Mit269	43	TTTTTGGACTAATAGTCAACTGTGTAA	GTGTCTTAGGAAGACTGAAAACTTGTGGG	TET	183	155		Q
D9Mit110	48	CCAGAAGGGGTGTGTTTTGC	GTGTCTTCTACCCTCCTTTCTAGTTTTTGTCC	FAM	82	157		Q
D9Mit35	52	CCAGCGCACTGTTCTGATAA	GTGTCTTAGGTGCCTTCTGCTTTGAAA	HEX	131	119		Q
D9Mit53	57	ATTCATGTGTCTCCAAAATCCC	GTGTCTTCAAACTCTTGCTGGGTGTGA	TET	219	211		Q
D9Mit214	62	AGCACAGGAAAAGGACGCTA	GTGTCTTAACCTGTCTCTGTAAAACTATCTCCA	FAM	147	123		Q
D9Mit279	67	CTCCAGAAACTTGTCCGCTC	GTGTCTTAATTGAAACTGTATCTAAGGCATGG	HEX	153	143		Q
D9Mit18	71	TCACTGTAGCCCAGAGCAGT	GTGTCTTCCTGTTGTCAACACCTGATG	TET	187	217		Q
D10Mit49	2	GGAATTTACACTGGAATACAACCC	GTGTCTTGTGGGCATTTGCACTGTG	FAM	113	115		Q
D10Mit189	7	TGTGTAGGTATGTGTGTGCATAGG	GTGTCTTATCAGACAGCACCTGGGAAC	HEX	113	137		Q
D10Mit213	11	CTCCTCCTACTGATTGTCCCC	GTGTCTTGGGACAAACTTTTAAAAATTGCA	TET	157	143		Q
D10Mit86	17	TTTGCCTGTAACAAGCCAGA	GTGTCTTTTGAGGCTATCAGTTTAAAATCCC	FAM	163	157		Q
D10Mit126	21	ACATTCACAAAATGTGTATGTATGTG	GTGTCTTTGTTTTTCATTAATCTCTTGAGATGG	HEX	136	132		Q
D10Mit38	26,8	CGATGAGCCCTAACACCAAT	GTGTCTTCCTGTTACAAACTAAACCAAACCC	TET	173	203	50°C	Q
D10Mit130	31,5	TGCCACACAAACACCACC	GTGTCTTATTCATCAGTGTGAAATATGGCC	FAM	157	165		Q
D10Mit31	36	CATAAGGAGCACAGGCATGA	GTGTCTTCCCTCTACGTGCATGCTGTA	HEX	159	161		NEB
D10Mit42	44	GCATTCAGAAGCTGGAAAGG	GTGTCTTTGCCCAGCATATGTTTAAAGG	FAM	191	203		Q
D10Mit95	51	CCAGTCTCAAAACAACAACAAAC	GTGTCTTTTGCACCTAGATTGCCTGA	HEX	208	174		Q
D10Mit96	56	ATGTCCAAAACACCAGCCAG	GTGTCTTGGAAGTGATGGAGCTCTGTT	TET	160	134		Q
D10Mit233	62	GTGCTTTATATTGGAGATCATCACA	GTGTCTTGTCCCGAATTTCACATACATAGC	FAM	137	115		Q
D10Mit180	64	GACCTTCCTTTATACACAAGTCATAGC	GTGTCTTGTGGTACAGAACTTAGGTGTTTAATTG	HEX	141	213		Q
D10Mit103	70	TATGCCGACAATATTTCATTGC	GTGTCTTGCCTCTGCATACATACCAATACC	TET	149	151		NEB
D11Mit71	1,1	GCCATACCTGGTAGCGTGTT	GTGTCTTAATTTTCAGATGTAGCCATAAGCC	FAM	221	245		NEB

Supplement table S1.

Marker name	Position (cM)	Forward primer	Reverse primer	5'-Label forward primer	Expected fragment lengths		Annealing temp.	Polyme rase
					C57BL/6J	C3H/HeJ		
D11Mit2	2,4	TCCCAGAGGTCTCCAAGACA	GTGTCTTCCACAGTGTGTGATGTCTTC	HEX	129	144		Q
D11Mit295	11	GTTCTAAAATGCAAGTCCCTGG	GTGTCTTCTCTTTGATACCCCCACCCT	TET	119	123		Q
D11Mit163	16	AACCCTGCTATTGTGCTGCT	GTGTCTTCTAGAACACACATGCATGCTCA	FAM	144	162		Q
D11Mit296	20	TAGGGCATATTAAAATATAAAGGCTG	GTGTCTTCTGCACCAATGGTTTATATTTCC	HEX	127	103		Q
D11Mit86	28	TTGACATTGTGACAAAGACTTTCA	GTGTCTTAAGGCATCATGAGGTTTTTAGTG	TET	133	141		Q
D11Mit242	31	GAAGCCAGCAAGAAAAATGC	GTGTCTTCTGTCTGGTAGTGCAGCCAA	FAM	129	143		Q
D11Mit351	36	GTATGTGAGGGAGAGTACTCACATG	GTGTCTTTCTCAGTAACATGAGATATTCAGTGTG	HEX	117	111		Q
D11Mit29	40	TTGAGGCATGAGGGGATTAG	GTGTCTTTTTCCGTCATTGCTAAAGGG	TET	151	157		Q
D11Mit245	44,8	ATGAGACCATGCTCCTCCAC	GTGTCTTTTGTCCTCTGACCTTCACACC	FAM	159	147		Q
D11Mit356	50	GGCAAGCAACTTCTTCCATC	GTGTCTTTTCAGAAATTTGGGTATTAGAGTGG	HEX	103	123	50°C	Q
D11Mit285	52	CATGAATCCATCACCAGCAG	GTGTCTTTTTTTCAGTCATGCAGGCAG	TET	128	139		Q
D11Mit289	55	CTTTGGTTGGTTTTAAATGTTTTAA	GTGTCTTAAGGAGAAAGCAGATTCATACACA	FAM	133	126		Q
D11Mit198	61	TGAAAATATGCAGCCTCCG	GTGTCTTATCTGCAAAGGGATCTGGTG	HEX	293	305		Q
D11Mit258	65	AAACAGAGATAAACCACGGGG	GTGTCTTTGTGGAACTAACTCTCAGAAGGC	TET	137	177		Q
D11Mit214	70	CATACAGCCTTCAACAATGACA	GTGTCTTACTGCATACATGTGCACTCATG	FAM	156	142		Q
D11Mit104	79	CACATGATCATACACTGTTTCTCC	GTGTCTTGCCACGTGTTCTAACCTTCC	TET	163	169		Q
D12Mit103	1	ACTATGGTGAAATCATACCCACG	GTGTCTTATCAATGGATCTTTTTTGGTGG	FAM	140	142		Q
D12Mit185	11	TGGAACTAGAAATCCATGTTAAAGG	GTGTCTTACTCAGGTATTTGTGCAATTGG	TET	159	137	50°C	Q
D12Mit59	13	AGTGAAATTCAGAGCACAAAAGC	GTGTCTTACCCTATATCTCCATGGTACGTG	FAM	157	159		NEB
D12Mit222	18	TTTAAAAACAACAACAACAAAAAAGG	GTGTCTTATCTGGGTTTTGAAATAAGAGCC	HEX	121	127		Q
D12Mit112	22	CTTCAGGCCTCCCTGGTAC	GTGTCTTTGCCTCCAAATATACTCACAAGC	TET	161	143		Q
D12Mit210	28	CTGATGTGAAATTCACAAAGAACC	GTGTCTTTGGGGCCCACTCTACATTAG	FAM	157	167		Q
D12Mit52	32	CCATCTTCTGGCATTTTGCT	GTGTCTTAGACAGGAGGGTCCCAAAGT	HEX	147	125		Q
D12Mit158	38	CATTGGGCAATGGAATTTG	GTGTCTTATGAGAGAAAACCAGAAACAAAGG	TET	154	159		Q
D12Mit239	44	AAAGCATTTCTTGTTTTATGTAATGTG	GTGTCTTCATGCATCTGCAACTCGC	FAM	97	107		Q
D12Mit101	50	GCTTTTCCTTATCAAGATATGCG	GTGTCTTGCAGCAGAAAGAGAGGGAAA	HEX	177	125		Q
D12Mit17	55	TCCGAGTGTTGCTTCTCCTT	GTGTCTTCCTTAGATGCTCAAGGCTGG	TET	187	177		Q
D12Mit144	61	CCACACATGTGCAGACACAG	GTGTCTTCTGGCTCTAAACCTTAGCACTAGG	FAM	312	316		NEB
D13Mit303	7	AGTTCAAGTTTGAGACAGATTCAGG	GTGTCTTTTCTCTCGCTTCATAAAGTCCC	HEX	131	123		Q
D13Mit3	10	TCAGGCTCATCCCAGATACC	GTGTCTTTTTTTGCAGAGAACACACACC	TET	166	203		Q
D13Mit133	11	TAGACACTTAATTCTGTGATGAAATGG	GTGTCTTAGCAAAAGCCCCAGTTAGTG	FAM	124	118		Q
D13Mit275	16	TTAGCAAGGGAACAGAGAGAGG	GTGTCTTCAATCAAGGTATCCCTGTCTCC	HEX	115	123		Q
D13Mit18	18	TGTATCCAGCTCATCCTGATAGG	GTGTCTTACTTCCTTTGAACTTCATGACTTC	TET	203	197		Q
D13Mit179	30	GACCAATGCCCTACAATTTCA	GTGTCTTCAGAAGCAGTTTGTCTTTGTGG	HEX	103	89		Q
D13Mit248	34	TAAAGTAGAAGGCAGCATGAGTG	GTGTCTTACCCAAATGTTTTGGATCCA	TET	121	101		Q
D13Mit231	39	GCACGGAGGGAGAAATGTAA	GTGTCTTGTACTTAGGGACTCTTCAGCGTG	FAM	123	151		Q
D13Mit24	43	TGCATGACTGTGTAATGCTTTG	GTGTCTTGAAGAACTGGGGAAACTGAGG	HEX	213	173		Q
D13Mit159	47	CCCATTGTCCCTGTTCAGAT	GTGTCTTAAACCCACCATGAATTAAATGC	TET	149	167		Q
D13Mit147	49	CATCCAGGAAGGCAATAAGG	GTGTCTTCAAATGCACAGTGCCGAG	FAM	115	100		Q
D13Mit30	52	TTTTTGATGTGTATGCTTGTTGG	GTGTCTTAAAGAGAAGACGGGGAGGAG	HEX	115	119		Q
D13Mit213	59	GCCTGAAACTCTACATAAAATACATCC	GTGTCTTAGTTTCATTGCTTTAGTTACATTTTCA	TET	155	167		Q
D13Mit292	62	AAATGACATTTTTGTATGCACACA	GTGTCTTGAGACAGAGTAATGACCGAATGG	FAM	159	145		Q
D13Mit262	68	CTGCGGCTGTAGGTTAAGTATG	GTGTCTTAGGCTGCTGCTAACAGATGG	HEX	133	123		Q
D13Mit35	75	GATTTTCCAGGTAAGTGGCG	GTGTCTTCACATTCACTGTGAGTGCACA	TET	197	189		Q
D14Mit207	5,5	TCCAACTAGTCCCCCTCTACTT	GTGTCTTCTGTGACTATCTGTACAAGACCTGC	HEX	135	111		Q
D14Mit127	10	AAACTTTACCTACCAGTGTCAAGTTAG	GTGTCTTGTGTTGAACAACTCTATGTCTGTCTG	TET	154	156		Q
D14Mit259	19,5	TGGTGTCTCCTTCGGAATTT	GTGTCTTTAAATGTAAAAGGTAAAGGCAATGG	HEX	133	153		Q
D14Mit5	22,5	CACATGAACAGAGGGGCAG	GTGTCTTGTCATGAAGTGCCCACCTTT	TET	185	111		Q
D14Mit157	27,5	GGTTGACCTCTGACCTCCAC	GTGTCTTAATAGCACTGGAATTAAAAATGTGG	FAM	157	153		Q
D14Mit85	32,5	TCCCACATATGCACATACACG	GTGTCTTATTCTGATTGCAGATTCCGG	HEX	167	163		Q
D14Mit68	39	GTGGCATGCACAACCGTATA	GTGTCTTCCCTTTTGAGGTGCTTGTTT	TET	161	163		Q
D14Mit263	44,4	TGAGCACAGAGCCTATGTGG	GTGTCTTACAGAGAAATACCATGAAAACACC	FAM	129	125		Q
D14Mit106	48	CATAGGCTCTAGCGCTGACC	GTGTCTTATTGCATTGATGTCATAATTTCA	HEX	316	314		Q
D14Mit166	52	TGGGGTTAGAGTAACTAGAATATAGGG	GTGTCTTGGGGGCATTGTATGCTTAAA	TET	151	135		Q
D14Mit97	58	TCAGTCCAAACTCTGTTAATCTTCC	GTGTCTTCAGCTCCACATTTTTGCTCA	FAM	162	168		NEB
D14Mit266	60	ATGCACAGGATTGATCTGCA	GTGTCTTAGCATGACCTAAATAATGAGACCC	HEX	155	183		Q
D15Mit102	6,7	TATGGAACACACACAAGCATACA	GTGTCTTTGATCATTCATGAATAGGTTGAGG	TET	208	195		Q
D15Mit138	15,4	TTCAATTCCCTTTTGTCAAATG	GTGTCTTCAAGACCCTAGATTCAGTCTACCC	HEX	156	134		Q
D15Mit152	20,2	AAATGTAGGACTTACACAGTTTGTGC	GTGTCTTCAAAGTTTAGTGTCAGAACGAATACA	TET	113	89		Q
D15Mit229	22,2	AGAGTGATTATTTACAAGAAACACACA	GTGTCTTGATTAATGTTTAAAATCATGGCTGC	FAM	117	147		Q
D15Mit88	27,6	TAGCAATCACAGGAGGAATTAGG	GTGTCTTTTACTGAACTTAAGAACTGGAATCATT	HEX	216	208		Q
D15Mit209	32	TTGTGCTTCACTAGATGTAGACCA	GTGTCTTTTTTATAGTTGCACATAAGCAGCA	TET	134	113		Q
D15Mit156	39,1	CCCACATTCATGCACATATAGG	GTGTCTTAACAAATCAAGAACCAATTGGG	FAM	152	130		Q
D15Mit107	49	CAACACTTATACACTTGTGTCAGGG	GTGTCTTTCATGGTTGGAACAGCAGAC	TET	158	152		Q
D15Mit43	54,5	GAGTTTGGTTCGGTTGTAGAGG	GTGTCTTCTGGGTACCTCAGCTTTTGC	FAM	211	227		NEB
D15Mit79	66,2	CGAAACATTTGGGCACTTG	GTGTCTTCCCCATTCCTGAGTCTCTTG	TET	283	289		Q
D15Mit161	69,2	TCTGTTTTGTTTGTTCGTTTGC	GTGTCTTTAAAATCTCCCTGTATACAAGTCTGTG	FAM	135	109		Q
D16Mit107	3,4	ACCCCATGAGACTCAGCATC	GTGTCTTGAAAGCCTGAACACATGGGT	HEX	197	199		Q
D16Mit100	8,5	AGTCTTGTCCGCGTCAGAAT	GTGTCTTAAAAGGATTGCAGGGACTACTG	TET	154	160		Q
D16Mit101	17	TTATGAAATGTTTTATCTTTTGGGG	GTGTCTTCTCCAGATGTAGAAATTAAAATCTTGG	HEX	157	153		Q
D16Mit134	23,5	ATGGGAAGCAATCAGTAATAACTG	GTGTCTTACCCACATAGACATCATGGTATACACA	TET	157	165		Q
D16Mit12	27,6	GAACTCAGTAAGCTCTCTATGCCC	GTGTCTTGGAGGACTAGCAGGCTAGAGC	FAM	199	163		Q
D16Mit42	33	TAACCATCACATTCTTTTTCATGT	GTGTCTTTGTGGCATAAGGCAGGCT	HEX	135	159		Q
D16Mit30	36,5	GTGCACATACATACCACAGCG	GTGTCTTTCACTGCAGGGAGGTTCAG	TET	159	117		Q
D16Mit140	42,8	ATAGTTGAAAAAGTTGAACATGCG	GTGTCTTGAAAAGGTTAATGCTGGTCACC	FAM	157	171		Q
D16Mit139	43,1	GTATGTAAGGAATGGTCAAATTCTTG	GTGTCTTTCATTGTGATTGTGAAAGAATGC	HEX	155	179		Q
D16Mit27	45,5	AGAAAAGAATGAAAATCACGCA	GTGTCTTTAGAGACCTTTTGTCTGAAATCCA	TET	91	81		Q
D16Mit189	55,2	ACAGTGTTTGTTTGTTTGTTTGTG	GTGTCTTCAGTACAGGAAGTCTTTGCATCC	FAM	206	192	50°C	Q
D16Mit70	57	GGATCTATATGCTATAGAACCATTCA	GTGTCTTGTCATCAATTCCATTTCCTAATATAGA	HEX	196	176		Q
D16Mit86	66	TAATGTGGCAAGCAACCAAA	GTGTCTTGCATGTTTCCATGTGTCTGG	TET	135	129		Q
D17Mit164	4,1	AGGCCCTAACATGTAGCAGG	GTGTCTTTATTATTGAGACTGTGGTTGTTGTTG	FAM	143	133		Q

Supplement table S1.

Marker name	Position (cM)	Forward primer	Reverse primer	5'-Label forward primer	Expected fragment lengths C57BL/6J	Expected fragment lengths C3H/HeJ	Annealing temp.	Polymerase
D17Mit133	10,4	TCTGCTGTGTTCACAGGTGA	GTGTCTTGCCCCTGCTAGATCTGACAG	HEX	202	174		Q
D17Mit29	15,1	CATCTTTCCAGTCCAAATCTCC	GTGTCTTCTTCTGGCTTCCTCAACCC	TET	157	153		Q
D17Mit33	18,8	TGTTGGAGCTGAATACACGC	GTGTCTTCCAAACACCAGGGTCTCTGT	FAM	201	179		Q
D17Mit180	29,4	AGACACTGTCTAAAAACACAAGATGG	GTGTCTTTGTGTTCATATGCATGTGTGC	TET	155	157		Q
D17Mit20	34,3	AGAACAGGACACCGGACATC	GTGTCTTTCATAAGTAGGCACACCAATGC	FAM	187	177		Q
D17Mit119	38,5	CCTCCTGTTCTGAACTTCAGC	GTGTCTTTCGATGCAACCCAGTATAAAA	HEX	153	145		Q
D17Mit218	42	GGAGAAGATGGGAGAAAGGC	GTGTCTTCAAAGCATTTCCAAGCATAGG	TET	123	103		Q
D17Mit142	47,4	AATATATATATCCTGGAGCCAACACA	GTGTCTTACCTTTATGAAGTTATGCTGAGTATCA	FAM	154	128		Q
D17Mit76	54,6	CTCCTCACCCAGATTCTTGTAA	GTGTCTTTTTCGCAAGTTATTTTAACCCG	HEX	131	97		Q
D18Mit64	2	TCAGATTCACTGCTAAGTCTTTTC	GTGTCTTAGCAAGAAAAGCAGGTGAGG	TET	159	179		Q
D18Mit34	12	CACTGGATGACACAGCCTGT	GTGTCTTGATGTTTCCTTGGGTTTGTCA	HEX	141	149		Q
D18Mit119	16	AGATGCTTGTGAAACATACATATGTG	GTGTCTTGAGTGTATAGCGGACTTTTGGG	TET	162	136		Q
D18Mit177	20	CTGTAGTTTATCAGTTCACCCTGTG	GTGTCTTTGTGCTGTTAAACAAATATCTCTGG	FAM	179	175	50°C	NEB
D18Mit74	25	AGCCAGAGCTACAAAGTTTCAA	GTGTCTTGCTCTTGTAGAGCCATCATTCC	HEX	227	191		NEB
D18Mit124	32	CCCAAATGGGGTGTCTTTTA	GTGTCTTCTGCCACACATTTGTGTGTATG	TET	158	146		Q
D18Mit40	37	GGTAGGAGTCACTTTCCGTCC	GTGTCTTTTTTTGTGAGCATTTTTATACCATT	FAM	149	139		Q
D18Mit184	41	CACACATGTGTAGGTAGGTAGGTAGG	GTGTCTTCGCACAAGGACTACTGAAACA	HEX	179	134		Q
D18Mit186	45	AAGTGTTGGGCAAAGGCTAA	GTGTCTTCTTTAGTATAGTGTGCATGAGTGTGA	TET	133	115		Q
D18Mit48	50	TTGCACTCACAGGGCACAT	GTGTCTTTCAGAGTTTCCAGAAGACACCA	FAM	173	165		Q
D18Mit144	57	TAGGGTTTTTTTTTTCTTTTTCTCC	GTGTCTTGATAAAAAAATATGTTCACAAAACGC	HEX	187	184		Q
D19Mit59	0,5	CTCTAACTATCCTCTGACCTTCACA	GTGTCTTTTTTAAGCAGAACATTGAGGACC	TET	206	146		Q
D19Mit56	5	CTGAATGTGTATGTGTGCAAGTATG	GTGTCTTATTATGAATTCAAGACTAGCCTAGGA	FAM	145	137		Q
D19Mit128	10,9	GGCAGGAGAATGTATTCAGAAA	GTGTCTTTCCTCCAACCTGCTTCCTC	HEX	130	152		Q
D19Mit96	15	CTTAACTGCAGTTTTAAAAGACATTTG	GTGTCTTCATTTGAGAGAATGTTTGAACATACA	TET	128	120		Q
D19Mit106	22	CCTTTTTTTTTTTAACCAGACAGG	GTGTCTTATCAATGAATGAAGAACAAATAGTTTC	FAM	131	123		Q
D19Mit40	25	CAGGGTAGTATTGCAGATAATCAA	GTGTCTTAAAGTTTCTTTGTGTGTGCACG	HEX	119	113		Q
D19Mit119	27,5	CACCCACATACCTTGATT	GTGTCTTCTCTCTTTATCTCTCCTCTCTCT	TET	271	283		Q
D19Mit13	33	TCTGGCACAAAGAGTTCGTG	GTGTCTTCTTTTGCAGGAGCAGGTAGG	FAM	253	269		Q
D19Mit90	41	GTGGGAATCAATTTTAGTATGAACA	GTGTCTTGGATGCTTGATATCATGTACATACA	HEX	139	133		Q
D19Mit83	47	GACACATGCGGCATACAGTC	GTGTCTTCTTGCTCTGAGTATTTTAATGACTGC	TET	134	112		Q
D19Mit26	51	TTGTTACACAGCAAAATCCTGC	GTGTCTTTTGAGGAGTAAGGCAAAAAAGG	FAM	139	141	50°C	Q
D19Mit6	55	ATTAGTAAACTGACTCCCATGCG	GTGTCTTCTCATGAGTCCCCTGGGTTA	HEX	119	123		NEB
DXMit119	29,5	CTTTAACCATAATAATGGCCTTGC	GTGTCTTGGGTTCTGTGATCGCAAGTT	HEX	161	175		Q
DXMit16	37	CTGCAATGCCTGCTGTTTTA	GTGTCTTCCGGAGTACAAAGGGAGTCA	FAM	125	93		Q
DXMit170	41,5	TGCAGGCACTAACAGTGAGG	GTGTCTTTAGTTTCACTGTGCCATTGTATACA	HEX	123	129		Q
DXMit64	45	GGATCAGTTAGCAGGGAAAGG	GTGTCTTCACAGACTGAGAAGGCTGTCC	TET	141	121		Q
DXMit130	55	TTCATATCGCCCCAACCTAC	GTGTCTTTATTTTGAAACCTCTGCCATTT	HEX	175	153		Q

Supplement table S2. Sex of F2 mice and mutation in Pde6b gene screened in a subgroup (m = male; f = female; A = wild type allele; B = mutated allele; H = heterozygous).

Animal ID	Sex	Pde6b genotyp	Animal ID	Sex	Pde6b genotyp
1_1	f		13_4	m	
1_3	f		13_5	m	A
1_5	f		13_6	m	H
1_6	f		14_1	f	
2_1	m	H	14_2	f	H
2_2	m	H	14_3	f	
2_3	m		14_4	f	
2_4	m	B	14_5	f	H
2_5	m	H	14_6	f	
3_1	f		15_1	m	H
3_2	f	A	15_2	m	H
3_3	f		15_3	m	A
3_5	f	H	15_4	m	
4_1	m		15_5	m	
4_2	m	H	16_1	f	
4_3	m	B	16_4	f	H
4_4	m	H	17_1	f	H
4_5	m		17_2	f	H
5_1	f	A	17_3	f	A
5_2	f		18_1	m	A
5_3	f		18_2	m	
5_4	f		18_3	m	
6_2	m	A	18_4	m	H
6_3	m		19_1	f	A
6_4	m	H	19_2	f	A
6_5	m		19_3	f	
7_1	m	H	19_4	f	B
7_2	m	A	19_5	f	
7_3	m	A	20_1	m	B
7_4	m	B	20_2	m	A
7_5	m	A	20_3	m	H
8_1	m	H	20_4	m	
8_2	m	A	20_5	m	A
8_3	m	H	21_2	m	
8_4	m	H	21_3	m	H
8_5	m	B	21_4	m	
8_6	m		21_5	m	
9_1	f		22_1	m	
9_4	f		22_2	m	A
9_6	f	A	22_4	m	
11_1	f	H	22_5	m	H
11_3	f	H	23_1	f	B
11_5	f		23_2	f	H
11_6	f		23_3	f	
12_1	m		23_4	f	B
12_2	m	A	23_5	f	
12_3	m		23_6	f	A
12_4	m		24_1	m	H
12_5	m	H	24_2	m	B
12_6	m		24_3	m	A
13_2	m		24_4	m	B
13_3	m	B	24_5	m	A

Supplement table S2.

Animal ID	Sex	Pde6b genotyp	Animal ID	Sex	Pde6b genotyp
25_1	f		35_5	m	
25_2	f	H	36_1	f	
25_3	f	H	36_3	f	
25_4	f		36_5	f	
25_5	f	H	37_1	m	
25_6	f	H	37_2	m	A
26_2	f		37_3	m	A
26_3	f	B	37_4	m	H
26_4	f	B	37_5	m	
26_5	f		38_1	m	
27_1	m	B	38_2	m	B
27_2	m		38_3	m	
27_3	m	B	39_1	f	
27_4	m		39_2	f	A
28_1	f		39_3	f	
28_2	f		39_4	f	
28_3	f		40_1	m	
28_5	f	B	40_2	m	H
28_6	f		40_3	m	
29_1	m	A	40_4	m	A
29_2	m	H	40_5	m	
29_3	m		41_1	f	
29_4	m	A	41_2	f	
29_5	m	A	41_3	f	
30_2	f		41_4	f	
30_3	f	B	41_5	f	H
30_5	f		42_1	m	H
30_6	f		42_2	m	A
31_1	f		42_3	m	H
31_2	f		42_4	m	
31_3	f		42_5	m	H
31_4	f	H	43_1	f	
31_5	f		43_2	f	A
32_1	m		43_3	f	
32_2	m	A	43_4	f	B
32_3	m		43_5	f	
32_4	m	A	44_1	f	H
32_5	m		44_2	f	B
32_6	m		44_3	f	B
33_1	f		44_4	f	
33_3	f		44_5	f	
33_4	f		45_1	f	A
33_5	f		45_2	f	H
34_1	f		45_3	f	
34_2	f		45_5	f	
34_3	f		46_1	m	
34_4	f		46_3	m	H
34_5	f		46_4	m	
35_1	m		46_5	m	
35_2	m	H	47_1	f	
35_3	m		47_2	f	
35_4	m		47_3	f	A

(Continued on the next page)

Supplement table S2.

Animal ID	Sex	Pde6b genotyp		Animal ID	Sex	Pde6b genotyp
47_4	f	B		58_5	m	B
47_5	f	H		59_1	f	H
48_1	m	B		59_2	f	A
48_2	m	B		59_3	f	H
48_3	m			59_4	f	A
48_4	m			59_5	f	
48_5	m			60_1	f	H
49_1	m	B		60_2	f	B
49_2	m			60_3	f	
49_4	m	H		60_4	f	H
49_5	m			60_5	f	B
50_1	m	H		61_1	m	
50_2	m	H		61_2	m	
50_3	m	A		61_3	m	H
50_4	m			61_4	m	
50_5	m			61_5	m	A
51_1	m			62_1	m	
51_2	m	A		62_2	m	
51_3	m			62_3	m	B
51_4	m	B		63_1	f	
51_5	m	H		63_2	f	H
52_1	f			63_3	f	
52_3	f			63_5	f	
52_4	f	B		64_1	m	
52_5	f			64_2	m	
53_1	m	B		64_3	m	
53_2	m	H		64_4	m	
53_3	m	H		64_5	m	
53_4	m	B		65_1	f	
53_5	m	H		65_2	f	
54_1	f	A		65_3	f	
54_2	f			65_4	f	
54_3	f			65_5	f	
54_4	f	A		66_1	m	
54_5	f			66_2	m	
55_1	m			66_3	m	
55_2	m			66_4	m	A
55_4	m	H		66_5	m	
55_5	m	A		67_1	f	
56_2	f			67_2	f	
56_3	f			67_3	f	A
56_4	f			67_4	f	
56_5	f			67_5	f	
57_1	f			68_1	m	
57_2	f	H		68_2	m	
57_3	f			68_3	m	B
57_4	f	B		68_4	m	
57_5	f			69_1	m	
58_1	m	H		69_2	m	H
58_2	m	B		69_3	m	
58_3	m			70_1	m	
58_4	m	A		70_2	m	

Supplement table S2.

Animal ID	Sex	Pde6b genotyp	Animal ID	Sex	Pde6b genotyp
70_4	m		84_1	m	B
70_5	m		84_2	m	
71_1	f		84_3	m	H
71_3	f		84_4	m	
71_4	f		84_5	m	
72_1	m		85_1	f	
72_2	m	H	85_2	f	
72_3	m		85_3	f	
73_1	f		85_4	f	A
73_2	f		85_5	f	
73_3	f		85_6	f	
73_4	f		86_1	m	
73_5	f		86_2	m	B
73_6	f		86_4	m	
74_1	f		86_5	m	H
74_2	f		86_6	m	
74_3	f		87_1	f	B
74_4	f		87_2	f	H
74_5	f		87_3	f	A
74_6	f	A	87_4	f	
75_1	f	B	87_6	f	H
75_2	f	B	88_1	m	
75_3	f		88_2	m	
75_4	f		88_3	m	H
76_1	m	B	88_4	m	A
76_2	m		88_5	m	H
76_3	m		88_6	m	
76_4	m	B	89_1	m	
76_5	m		89_2	m	
77_1	m	H	90_1	m	
77_2	m	A	90_2	m	
77_3	m	B	90_3	m	A
77_4	m		90_4	m	
77_5	m	H	90_5	m	A
78_2	f		91_1	f	
78_3	f		91_2	f	
78_4	f	H	91_3	f	
79_3	f		91_4	f	A
79_4	f		91_5	f	
80_2	f		91_6	f	B
80_4	f	B	92_1	f	
80_5	f		92_3	f	A
81_1	m		92_5	f	
81_2	m	H	93_1	m	H
81_4	m	B	93_2	m	
81_5	m		93_3	m	H
82_3	f	H	93_5	m	
82_5	f	H	93_6	m	A
83_1	m	A	94_2	f	H
83_3	m	A	94_3	f	
83_4	m	H	94_4	f	
83_5	m	A	95_1	f	

(Continued on the next page)

Supplement table S2.

Animal ID	Sex	Pde6b genotyp	Animal ID	Sex	Pde6b genotyp
95_2	f		107_3	f	
95_3	f	A	107_4	f	H
95_4	f		107_5	f	
95_5	f		108_1	m	
96_1	m		108_3	m	H
96_2	m	A	108_4	m	H
96_3	m		108_5	m	
96_5	m	H	109_1	f	
97_1	m	B	109_3	f	B
97_4	m	H	109_4	f	
97_5	m		109_5	f	H
98_1	f	A	109_6	f	
98_2	f		110_1	f	H
98_3	f		110_2	f	H
98_4	f		110_3	f	
98_5	f	H	110_4	f	
99_2	m	H	110_5	f	
99_3	m		111_1	m	H
99_5	m		111_2	m	B
100_1	m		111_3	m	
100_2	m	A	111_4	m	B
100_3	m		111_5	m	A
100_4	m	H	112_2	f	
100_5	m		112_3	f	
100_6	m		112_4	f	
101_1	f		112_5	f	A
101_4	f	H	113_1	f	B
102_1	f		113_2	f	H
102_2	f		113_3	f	
102_3	f	H	113_4	f	H
102_4	f		113_5	f	
103_1	m		114_4	m	A
103_2	m	A	115_1	f	B
103_3	m	H	115_3	f	
103_4	m	A	115_4	f	
103_5	m	B	115_5	f	H
104_1	f		116_1	m	A
104_2	f		116_2	m	
104_3	f	H	116_3	m	H
104_4	f		116_4	m	
104_5	f	H	116_5	m	
105_1	f	B	117_1	m	
105_2	f		117_2	m	A
105_3	f	B	117_3	m	A
105_4	f		117_4	m	H
105_5	f		117_6	m	
106_1	m		118_1	f	
106_2	m	A	118_2	f	A
106_3	m	H	118_3	f	
106_4	m		118_4	f	H
106_5	m		118_5	f	H
107_2	f	A	119_1	m	

Supplement table S2.

Animal ID	Sex	Pde6b genotyp
119_2	m	H
119_3	m	H
119_5	m	
120_2	f	A
120_3	f	
120_5	f	
121_1	f	
121_2	f	
121_3	f	H
121_4	f	H
121_5	f	
122_1	f	
122_3	f	
122_4	f	B
123_1	m	H
123_2	m	A
123_3	m	B
123_4	m	A
123_5	m	
124_2	m	H
124_3	m	H
124_4	m	
124_5	m	

A

B

	5' flanking sequence		3' flanking sequence
E122G09	......TTTTTCTAAAACTTTACTAG	gene trap cassette	GAAACGTTAATCAAGAAGGG......
E237D04	......AAAACCCCTTACTCTTGGCT	gene trap cassette	GGGGATGTGGCTTTGCTAGC......
E261G04	......GGTAGCATGAGCCTATAATG	gene trap cassette	TCAGCACTCAGGTTTCAGGC......
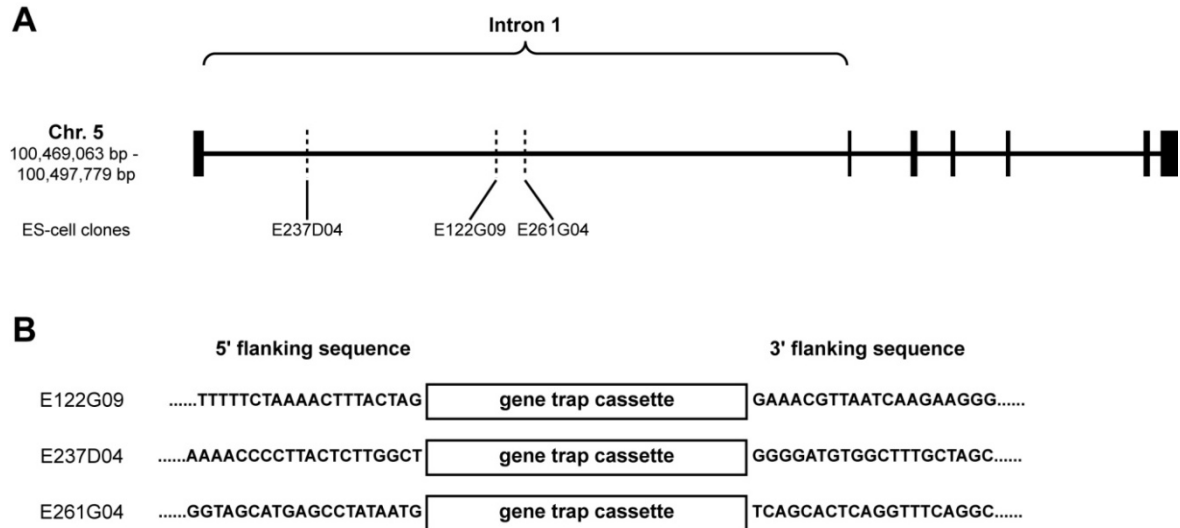
Supplement figure S1. Genomic positions of verified gene trap clones. (A) Exon-Intron structure of the Enoph1 gene. Exons are marked by vertical lines and introns lying between them. Gene trap positions of the three verified clones (E122G09; E237D04; E261G04) are marked by dotted lines. (B) The genomic sequence flanking 5' and 3' the gene trap insertion is presented for all ES-cell clones.

8.2 Publication

Barth A, Bilkei-Gorzó A, Drews E, Otte DM, Diaz-Lacava A, Varadarajulu J, Turck CW, Wienker TF, Zimmer A (2011) QTL Associated with Stress Responses: Identification of Candidate Genes. *PLoS Genet* submitted.

Drews E, Rácz I*, Lacava AD*, **Barth A***, Bilkei-Gorzó A, Wienker TF, Zimmer A (2010) Quantitative trait loci contributing to physiological and behavioural ethanol responses after acute and chronic treatment. *Int J Neuropsychopharmacol* 13(2):155-69.
* equal contribution

8.3 Declaration

I hereby declare that I prepared this thesis entitled: "Identification of genetic factors involved in the regulation of stress" entirely by myself except where 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.

8.4 Acknowledgement