Hunting Anxiety Genes

A consomic survey to unravel the genetics of avoidance behaviour in mice

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(met een samenvatting in het Nederlands)

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(mit einer Zusammenfassung in deutscher Sprache)

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General Introduction

Anxiety Disorders

Anxiety is mostly described as an unpleasant emotion that is associated with apprehension and worries and usually is accompanied with physiological sensations such as heart palpitations, nausea, chest pain, shortness of breath, or tension headache. It is generally accepted that anxiety consists of somatic, cognitive, emotional, and other behavioural components which will always be linked to each other in some way making it impossible to unravel e.g. only the behavioural aspects without the influence of the other components (Rosen & Schulkin 1998).

Anxiety can be divided into six different disorders, namely, generalized anxiety disorder (GAD), social anxiety disorder (SAD), post traumatic stress disorder (PTSD), obsessive compulsive disorder (OCD), panic disorder and various specific phobias. GAD is an anxiety disorder characterized by over-excessive, uncontrollable and usually irrational worries about normal everyday events. Daily function of people suffering from GAD is often interfered by their over concernedness about everyday matters. Requirement for diagnosis of GAD is that symptoms are consistent and on-going, persisting for at least 6 month. SAD is an anxiety disorder comparable to generalized anxiety but only regarding social situations where one is evaluated by others, i.e. during public speaking and can be related to shyness. A severe and on-going emotional reaction resulting from exposure to extreme stress and / or trauma is called PTSD. Clinically, such events involve actual or threatened death, serious physical injury, or a threat to physical and/or psychological integrity, to a degree that usual psychological defences are incapable of coping with the impact. Intensity and duration of the experience, and the individual itself involved all influence the severity and presence of PTSD (Diagnostic & Statistical Manual of Mental Disorders, 2000). OCD is an anxiety disorder usually characterized by a patient's obsessive, distressing and intrusive thoughts resulting in compulsions that attempt to 'neutralize' the obsession. The World Health Organization describes it as one of the top 10 most disabling illnesses in terms of lost income and diminished quality of life (Kohn et al. 2004). Panic disorder is a psychiatric disorder characterized by reoccurring panic attacks and either significant behavioural change or at least a month of ongoing worry about the implications or concern about having other attacks. Specific phobias

or simple phobias are well-known anxiety disorders that can cause significant stress and impairment in patients. Frequently obligating stimuli that evoke phobic responses are among others hight (acrophobia), spiders (arachnophobia), snakes (ophidiophobia), and enclosed spaces (claustrophobia). Phobias are characterized by a persistent fear of a particular stimulus that causes an extensive fear response, usually in combination with significant stress (Gelernter et al. 2003).

Terms anxiety and fear are often confused and discussions about the correct definition of the terms remain unresolved. In this thesis anxiety is defined as representing the response to potential unspecific danger while fear refers to the response to a present danger, being cue- or stimulus- specific (Ohl et al. 2008). Notably, anxiety is not necessarily a pathological emotion. Large variations exist in the range of what is still classified as being normal. Anyhow, high levels of anxiety have higher potential to become pathological in both humans and laboratory rodents (Belzung & Griebel 2001a; Ohl et al. 2008; Rosen & Schulkin 1998). To understand the mechanisms behind anxiety-related behaviour and the development of pathological forms of anxiety disorders, it is important to know how normal variations in anxiety-related behaviour in humans are regulated. Therefore, the genetic background of anxiety-related behaviour in mice is investigated. Future translation into the human situation will gain more insight in the pathways and mechanisms involved in normal anxiety behaviour and its pathological forms.

The laboratory mouse as an animal model for anxiety disorders

In biomedical research, rodents, and in recent years specifically mice are often used as models for anxiety disorders in humans. Although, it is usually assumed that rodents experience fear, anxiety and also other psychological traits the same way as humans do, this assumption has never been proven. However, neurobiological pathways and hormone regulatory systems as well as other physiological parameters influencing psychological traits, have shown to be analogue, if not homologue in rodents and humans. For example, distinct behavioural patterns in mice have been shown to indicate anxiety (Ohl 2003; Olivier et al. 1994). It can therefore be assumed that rodents are anxiety models with translational value for the human situation.

The mouse has been used as a laboratory animal for over a century and for a wide variety of different research purposes. Behaviour has been extensively studied in mice over years, especially to investigate neurobiological and neurophysiological parameters, and the pathways and mechanisms involved in complex behavioural processes and disorders. Ultimately, findings in the mouse are translated to the human situation to help prevent behavioural disorders and find new and better therapeutics. These behavioural patters are accompanied by high sympathic nervous activity representing a homology between humans and mice (Hall 1936).

Further, it has been shown that rodent behaviour that is classified as high-anxiety in behavioural tests can be modulated by anxiolytic drugs used for treatment of anxiety disorders in humans (Millan 2003; van Bogaert et al. 2006).

Behavioural indicators for mouse anxiety

Behavioural tests are essential to characterize the neurobiological mechanisms underlying behavioural disorders, evaluate those animal models, and study the effects of potential new pharmacological treatments in rodents. Every test-system is designed to measure one or more species-specific behavioural pattern, which in turn refers to a distinct motivational system (sometimes called behavioural dimension). Generally, testing of anxiety-related behaviour in mice is based on their characteristic avoidance behaviour towards novelty (neophobia) (Belzung & Le Pape 1994; Treit & Fundytus 1988; Barnett 1967). When mice are introduced to novelty, their behaviour is regulated by the conflict between the drive to explore the novel stimulus, and at the same time to avoid it as being potentially dangerous. Typically, mice start to explore the most protected part of the novel environment, e.g. a novel area along the sidewalls surrounding (and protecting) it, a behaviour which is called thigmotaxis. The mouse behaviour first is aiming at assessing the risk to further explore the novel stimulus/environment. The characteristic stretching of their body (stretched attend) to explore the close surroundings therefore is classified as risk assessment behaviour (Blanchard & Blanchard 1989; Rodgers 1997; Weiss et al. 1998; Cruz et al. 1994). It is thought that this behavioural function is an active defence pattern (Blanchard et al. 2003), thus being closely related to anxiety. Notably, risk assessment has been shown to be independently regulated from avoidance behaviour (Cruz et al. 1994; Ohl et al. 2001b; Rodgers & Johnson 1995). Moreover, Grewal et al. (1997) and Blanchard et al. (1990) have shown that risk assessment behaviour is probably more sensitive to anxiety-modulating pharmacological treatment then avoidance behaviour. The readout of anxiety-related behaviour in all test for anxiety may be confounded by the overall activity of the animal tested (Belzung 1999): exploration for example is inhibited by anxiety, but still object exploration tests are indirectly indicating anxiety (Crawley & Goodwin 1980; Handley & Mithani 1984; Pellow et al. 1985). It has further been shown that anxiolytic treatment can suppress the inhibition of exploration in mice and other rodents (Belzung & Le Pape 1994; Belzung & Griebel 2001a; Rodgers et al. 1992).

Elevated plus maze (EPM)

The EPM was first introduced by Pellow et al. (1985) and is one of the most often used behavioural test for unconditioned anxiety-related behaviour. The EPM is an elevated plus-shaped runway, with two open opposite arms and the other two protected by surrounding sidewalls. The four arms cross in a centre area where the

mouse is placed at the beginning of the test. Avoidance of the open, elevated areas indicates avoidance behaviour, i.e. anxiety (Montgomery 1955; Pellow et al. 1985; Rodgers et al. 1997a).

Open field (OF)

The oldest test for unconditioned anxiety behaviour is the OF, being introduced by Hall in 1936 and was first used in rats as a brightly lit circular area surrounded by protective sidewalls. Today, a wide variety of open field tests has been used that vary in shape, size, enrichment, light conditions, and testing time, varying from two minutes to several hours. Mostly, mice and rats are tested for 5 to 10 minutes, just like in the EPM (Pellow et al. 1985). The mouse is either placed in the centre or at the side of the set-up when the test starts. Anxiety is indicated as a measurement of avoidance towards the unprotected (middle) area, but information about locomotor alterations can be revealed as well (Clement et al. 2002; Crabbe et al. 1999; Prut & Belzung 2003).

The modified hole board (mHB) as a multidimensional test

As described above, the conventional tests are usually directed towards only one type of motivational dimension, e.g. either anxiety or locomotor activity. Therefore, several individual tests have to be used subsequently (test battery) to behaviourally phenotype animals and/or to dissect motivational systems. This however results in the use of large numbers of rodents since the separate tests should be performed in naive animals in order to avoid interference between the tests. Unfortunately, this approach is also time-consuming and expensive (Ohl et al. 2001a). In order to overcome these problems, a multidimensional behavioural test paradigm was designed by Ohl et al. (2001a), called the modified hole board test (mHB). This test allows to asses for multiple dimensions of behaviour in only one single test (Ohl et al. 2001b), thereby helping to reduce the number of animals and improving behavioural phenotyping in the behavioural genetics studies (Laarakker et al. 2006).

The mHB paradigm is a test for unconditioned behaviour. It is based on a combination of the traditional hole board and an open field test but takes the rich behavioural repertoire of rodents into account. It can not only detect a wide range of behaviours like anxiety, locomotion, exploration, risk assessment, arousal, social affinity and different cognitive processes, but also enables the investigator to distinguish between the different motivational systems of the animal. The mHB test is validated, among others, using diazepam in C57BL/6 and BALB/c mice that clearly show anxiolytic effects in terms of sensitivity and specificity for the tested pharmacological compounds. Notably, anxiolytic effects can be separated from sedative effects and general alterations in for example exploration using the mHB (Douglas et al. 1998; Ohl et al. 2001a; Ohl 2003). Further, group housed animals can be tested in the mHB in the presence of their cage mates in order to avoid separation stress.

One-dimensional versus multidimensional behavioural testing

Different research groups have shown that anxiety is a complex trait and Henderson et al. (2004) proved that at least five dimensions are involved, which are: (i) suppression of activity in mildly and (ii) threatening environments, (iii) rearing, (iv) avoidance of a novel area, and (v) autonomic activity. These dimensions also seem to correlate genetically more or less with each other and QTLs that were found all influenced at least two of these dimensions (Henderson et al. 2004). Mostly, test batteries are used (van Gaalen & Steckler 2000; Rogers et al. 1997; Turri et al. 2001) to get information about multiple behavioural dimensions by testing one animal in various behavioural tests in a row. Other approaches try to omit test batteries since the separate tests and the test order may influence the results. Henderson et al. (2004) argue that pilot studies using counter balanced orders of test batteries show only few of such consequences, but there is still lot of discussion between research groups about this. In this study, the multidimensional approach of the mHB has been chosen for behaviourally characterising different mouse strains.

Inbred strains

Behaviour and associated physiological, metabolic, and neuro-endocrine traits usually develop due to a combined action of a variety of genetic and environmental factors (Crabbe et al. 1999; Kafkafi et al. 2005; Kotb et al. 2008; Willis-Owen & Valdar 2009). This complex development obstructs the research in genetic background of these behavioural traits in men. For identifying the genes underlying these multidimensional traits (i.e. cognitive, emotional, and other behavioural disorders), animal models can be used. For effectively identifying the involved genes, two limiting conditions are important: i) the availability of inbred strains that are clearly contrasting for the behaviour of interest and the related physiological, metabolic, and neuro-endocrine parameters (Figure 1); ii) a genetic and a physical map with sufficient localized genetic markers and genes has to be available (Figure 2). Both, the laboratory rat and mouse suffice these limiting conditions; in fact the genomes of both species are completely sequenced (Hubbard et al. 2002; Marra et al. 1999; Wade et al. 2002).

In a population, inbreeding occurs when animals breed that are related more then the average. The result is that the number of individuals that are heterozygous for allele pairs declines and the number of individuals that are homozygous for alleles increases (Dasmahapatra et al. 2008; Khlat & Khoury 1991). A measure of inbreeding is the inbreeding-coefficient (F). F is the fraction of the originally heterozygous genes that have become fixed in a homozygous state during the breeding process, and thus, increased with every next generation of inbreeding. The increase in F per generation (ΔF) is depended upon the degree of consanguinity of the ancestors. A term frequently used in laboratory animal science is inbred

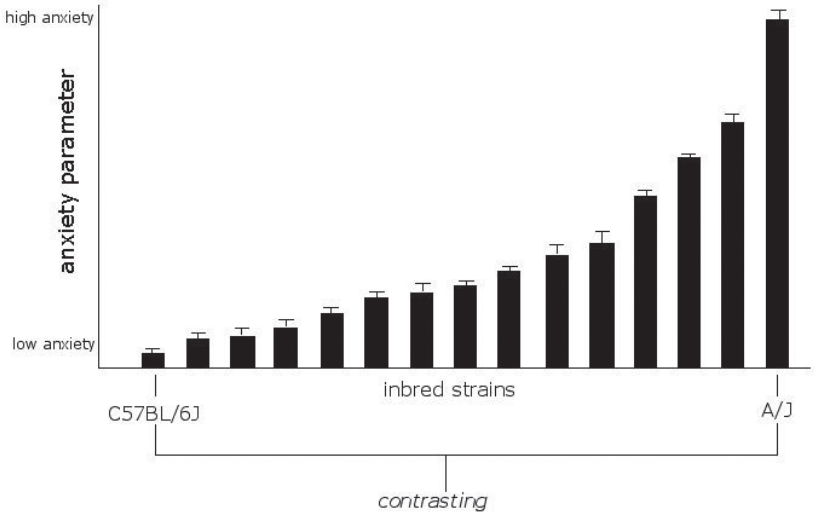


Figure 1 *Contrasting inbred strains. Inbred strains that are more contrasting with respect to a certain phenotype, i.e. anxiety, are more suitable to use for a genetic analysis regarding this phenotype.*

strain. Animals of an inbred stain can basically be seen as monozygous twins. A strain is called an inbred strain after a minimum of 20 successive generations of either brother x sister mating, or mating between offspring x youngest parent. At that point, on average, 98.4% of the total number of originally heterozygous loci has become fixed in a homozygous state. During the breeding process of an inbred strain, the measure of inbreeding increases per generation (ΔF) is $1/(2N)$, where N is the total number of breeding animals (in this case N = 2; since there are theoretically only 2 animals needed per generation, a brother and a sister). Further, for laboratory animals, the following formula gives a good indication of the inbreeding coefficient:

$$Ft = 1 - (1 - \Delta F)t$$

In Table 1, the F-values of the first 20 generations of successive brother x sister mating are indicated. The above mention formula was used to calculate these values. The true F value after 20 generations of brother x sister breeding (F = 0.984) differs slightly from the theoretical value F (0.997). This can be explained by the fact that the theoretically formula states that the reproduction capability of the heterozygous animals is the same as for the homozygous ones. This of cause does not need to be the case for the real breeding. Further, spontaneous mutations are not taken into account, which will likely be happening sometime during the 20 generations of inbreeding (Figure 3).

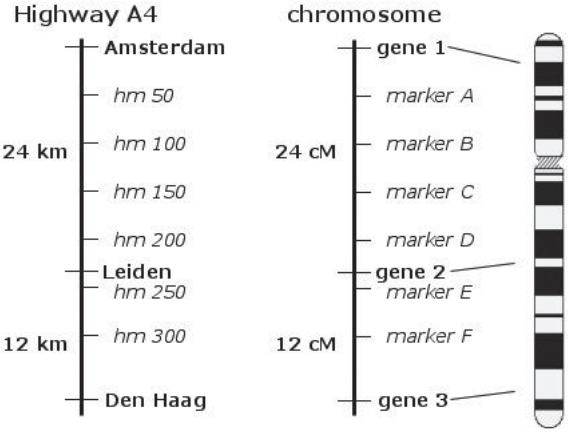


Figure 2 *Genetic and physical map of a chromosome. A genetic map is basically a road-map of a chromosome. Here the comparison is made between a highway (Highway A4 between Amsterdam and Den Haag) and a chromosome. Cities are replaced by genes, and hectometer signs (hm) by genetic markers. The distances are displayed in centimorgan (cM) in stead of kilometers.*

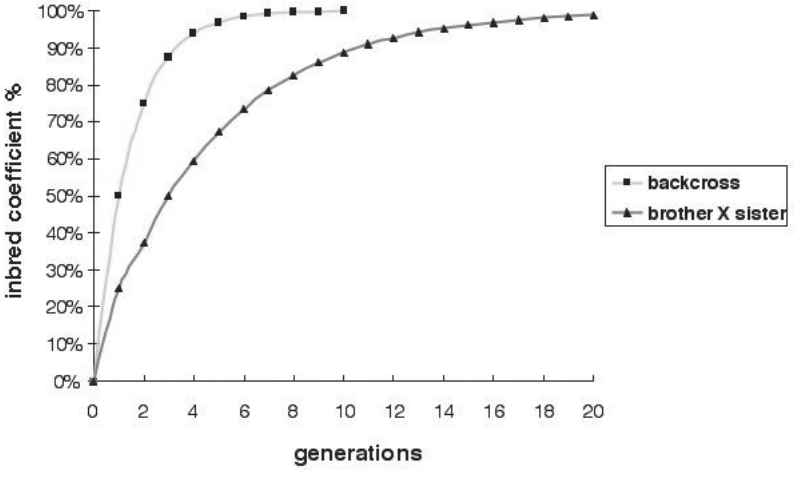


Figure 3 *Increase of the inbred coefficient per generation.*

Tabel 1 *Inbred coëfficient (F) of the first twenty generations.*

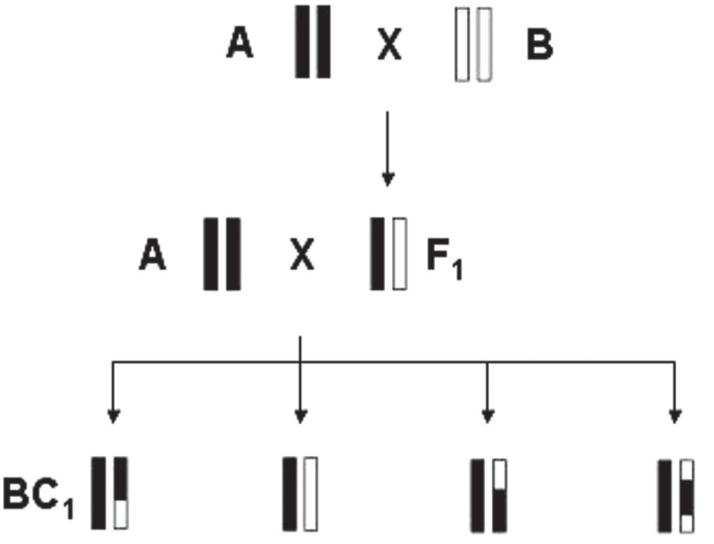
brother x sister mating	
generation	F-value
0	0
1	0.250
2	0.438
3	0.578
4	0.684
5	0.763
6	0.822
7	0.867
8	0.900
9	0.925
10	0.944
11	0.958
12	0.968
13	0.976
14	0.982
15	0.987
16	0.990
17	0.992
18	0.994
19	0.996
20	0.997

Inbreeding is usually harmful and causes inbreeding depression (Brewer et al. 1990; Marks 1986; Margulis & Altmann 1997). This is the decrease of immunity, fertility, and so on, which is the consequence of inbreeding. Two causes can be pointed out; first the occurrence of harmful recessive traits, when the number of homozygous alleles increases, also the number of harmful homozygous recessive alleles increases which can cause genetic disorders. The other problem is that every organism contains genes or alleles that are less fortune, but this is usually compensated with other alleles. During inbreeding the effects of these hidden, unfortunate alleles are being expressed when other compensating alleles become deleted during inbreeding. This is also why inbreeding in humans is thought to be harmful and therefore prohibited.

Conventional genetic analysis

There are different methods for the genetic analysis of multifactor traits by using inbred strains. One possibility is to cross two contrasting strains and then intercross the F1 generation to produce an F2 generation, or just make a backcross between the F1 and one of the parental strains (Figure 1). In order to identify the genes involved, so-called segregating laboratory animal populations have to be used (either backcross- and/or F2 generation). The animals of these generations are then tested for a specific characteristic (phenotyping) and typified for a large amount of genetic markers (mostly DNA-markers) that are spread over the whole genome (genotyping). With the help of a variety of statistical methods, genes that are involved in the trait of interest can be identified, thus via this procedure a variety of behavioural traits have already been mapped (Baile et al. 2008; Donner et al. 2008; Middeldorp et al. 2008; Valdar et al. 2006). The above described, traditional method for genetic analysis is still used regularly, however the disadvantage is that the backcross- and F2 generations are not permanent genetic systems, which means that they have to be produced again every time a genetic analysis has to be done. Furthermore, every animal of a backcross- or F2 population is unique, which means that there are no replicates available. Therefore, researchers started producing permanent genetic systems (Figure 4).

Figure 4 *Segregating populations: (a) Back cross (BC1) on parental strain A. (b) F2-generation obtained by brother x sister mating of the F1-generation.*



Permanent Genetic Systems

The first permanent genetic systems were already produced in 1948; the so-called congenic strains (Figure 5; Figure 6) (Snell 1964). These were followed by the recombinant inbred strains (Figure 7) in 1971 and fifteen years later the production of recombinant congenic strains (Figure 8) was completed (Bailey 1971; Demant & Hart 1986).

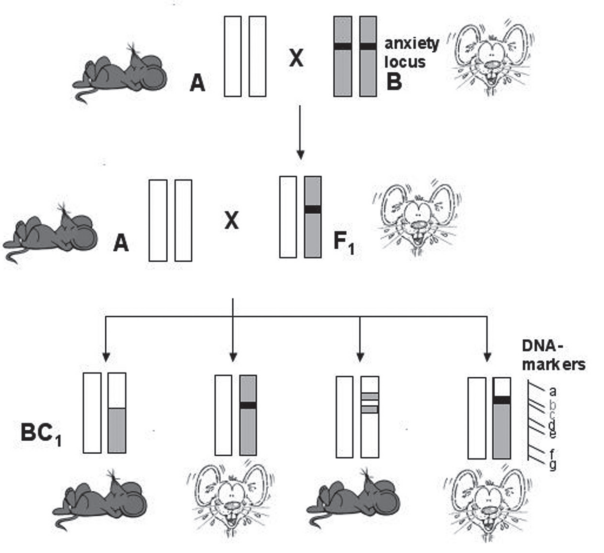


Figure 5 *Breeding scheme for a congenic line.*

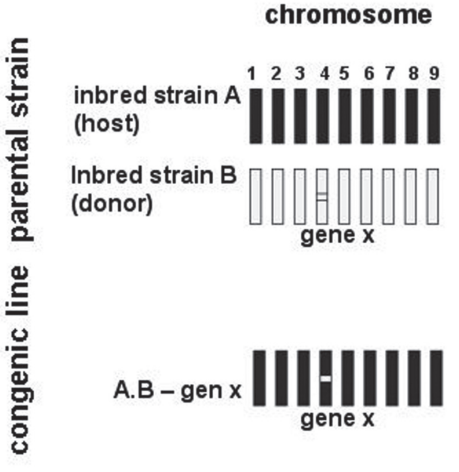


Figure 6 *Congenic line for gene 'x' obtained from two parental inbred strains.*

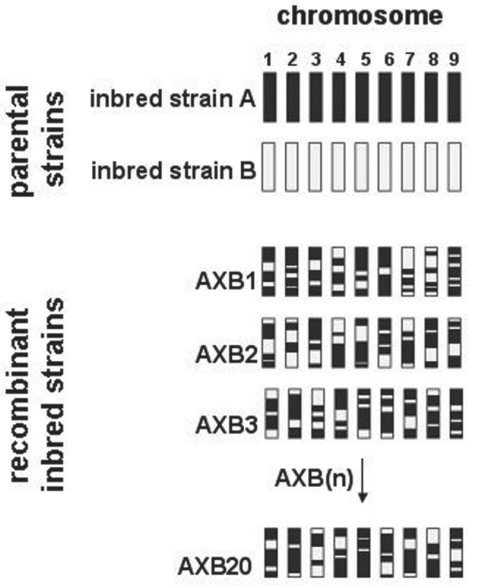


Figure 7 *Recombinant inbred strains produced by repeated intercrossing.*

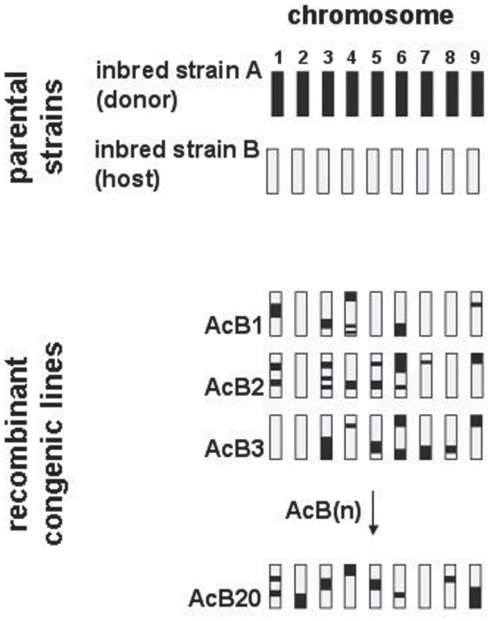


Figure 8 *Recombinant congenic lines produced by repeated backcrossing to the host strain.*

A relatively new method in the genetic laboratory animal science is the use of the chromosome substitution strains, also called consomic strains (Figure 9). These strains are made by inserting one whole chromosome of an inbred strain (donor strain) into the genome of another inbred strain (host strain). Such a set of chromosome substitution strains is a permanent genetic system. The genetic research of this thesis will be based on the use of the here described chromosome substitution strains, where the C57BL/6J mouse inbred strain functions as a host, and the A/J as a donor strain.

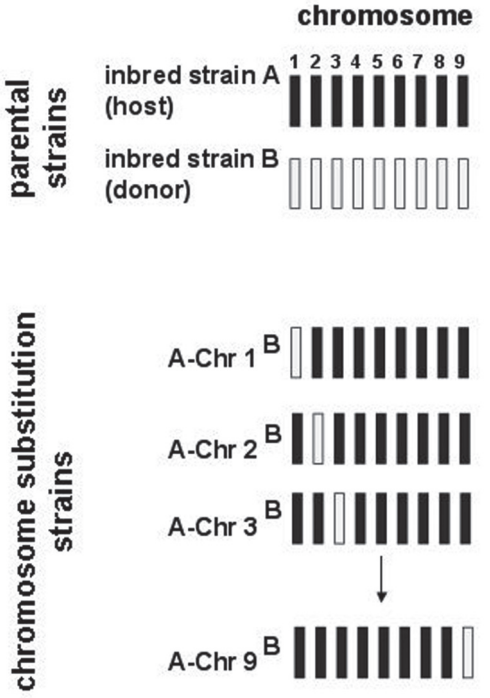


Figure 9 *Chromosome substitution strains. A full length chromosome from a donor inbred strain is transferred into the genetic background of a host inbred strain.*

Production of Chromosome Substitution Strains

A complete panel of chromosome substitution strains of the mouse consists of 21 strains; the rat panel contains 22 strains. Each of these strains originates from the same two parental strains (donor and host strain), but in every strain a different chromosome (which means one of the autosomes or the X- or Y- chromosome; Figure 10) of the host strain is replaced with the corresponding chromosome of the donor strain. If required, an extra strain can be made where the ones of the donor strain replace the mitochondria of the host strain. After all, not only the

nucleus contains DNA, but also the mitochondria of a cell, which is exclusively inherited by the mother. This type of consomic strain is called a conplastic strain. When making chromosome substitution strains, the principle of 'marker associated breeding' is applied, this means that the selection of the breeding animals is based on DNA-markers. It takes about 2-3 years to make a complete panel of chromosome substitution strains.

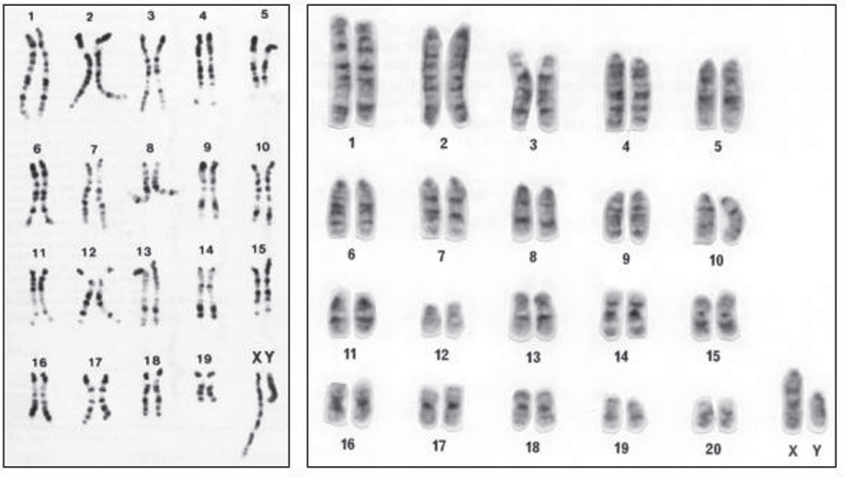


Figure 10 *Mouse karyogram (left), rat karyogram (right)*

To develop for example a chromosome-3-substitution strain (Figure 11), first a (AxB)F1-generation (N1) is made, followed by backcrossing the offspring with strain A (host strain). In each generation, animals are identified that contain an intact chromosome 3 of strain B (donor strain). These animals are so-called heterosomic for chromosome 3 and are used as parental animals to create the next generation. By genotyping the offspring for a large amount of DNA-markers of chromosome 3, selection of such heterosomic animals is possible. After 10 generations of backcrossing (including the first, AxB, crossing; N1 – N10), a male and a female mouse that are both heterosomic for chromosome 3 are mated. Statistically, this will result in 25% of homosomic offspring containing two intact chromosomes 3 of inbred strain B. This offspring will be used to produce a stable consomic strain. The above-described way to produce a chromosome substitution strain can be used to substitute the autosomes and the X-chromosome of an inbred strain and 10 generations of backcrossing are necessary. However, when every generation just animals are selected that lost 50% or more of the rest donor genome, so all chromosomes exclusively chromosome 3 in this example, 6 – 7 generations of backcrossing would be sufficient.

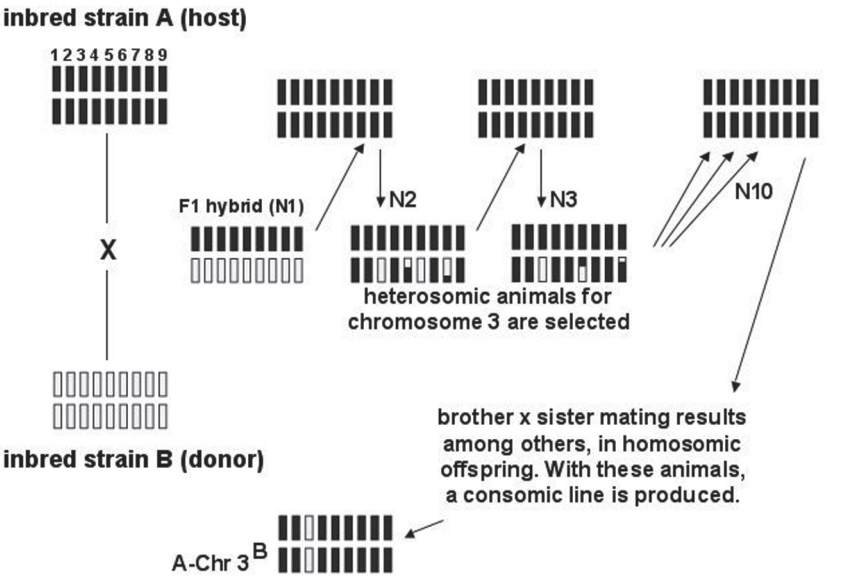


Figure 11 *Schedule that shows how to produce chromosome substitution strains.*

A Y-chromosome substitution strain can be produced without genotyping the offspring. Every generation, male animals are selected as parent for the next generation. However, when polymorphic DNA-markers are available for the pseudo-autosomal region (the region on the Y-chromosome that is able to be recombined with the corresponding regions on the X-chromosome), than the offspring should be genotyped for these DNA-markers.

Nomenclature Rules for Chromosome Substitution Strains

Chromosome substitution strains are designated to the complete name of the host strain, followed by a hyphen, 'Chr', a space, and an Arabic number of the chromosome that is substituted. This number has a superscript: the strain symbol, which means the abbreviated annotation of the donor strain. Thereafter, a forward slash is followed by the laboratory registration code of the laboratory that made the strain or keeps it. In case that a couple of animals of a certain consomic strain are transported to a different laboratory, the panel or strain has to be regarded as a sub-strain and a registration code of the second laboratory is added to the original name.

C57BL/6J-Chr1^{A}/NaJ is an example of the consomic strain where chromosome 1 of the A/J (A; donor strain) is substituted in the host strain background of the

C57BL/6J strain. The strain was created in the Laboratory of Nadeau (Na) and a couple of animals have been brought to the Jackson Laboratory (J), where the strain is kept as an inbred strain (Nadeau et al. 2000; Singer et al. 2004; Singer et al. 2005).

Cross between Chromosome Substitution Strain and Host Strain

The abbreviation QTL means 'Quantitative Trait Locus'. In fact a QTL is a chromosomal region where most likely a gene is positioned that has a certain influence on a specific trait. Finding QTLs on a chromosome using a set of consomic strains is easily done by finding the chromosome substitution strain that differs significantly for a specific trait (Belknap 2003) from the original host strain (Figure 12). In order to determine the exact position of the QTL on the chromosome, a relatively small segregating population between the relevant chromosome substitution strain and the host strain is made. In this segregating population, the chromosome of interest has to be genotyped for polymorphic DNA-markers (15-20 markers), which contrasts to the conventional method where a total genome scan has to be performed.

In genetic research, a genetic analysis is more effective when enough DNA-markers are available for several chromosomes. These DNA-markers should be polymorphic between the two parental strains. It is known that if two inbred strains are not related to each other, the chance on polymorphisms is larger. The A/J and C57BL/6J are distantly related (Atchley et al. 1988; Beck et al. 2000).

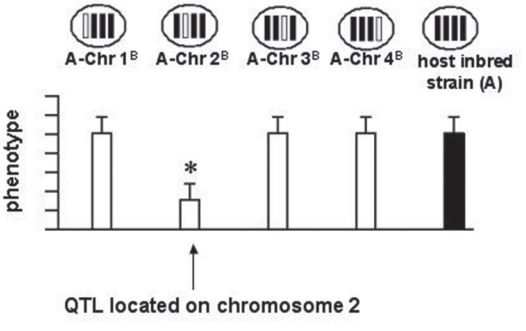


Figure 12 *A QTL is linked to a chromosome. The chromosome substitution strain that is significantly different (*) compared to the host strain contains one or more QTLs for the trait of interest (Figure modified from Singer et al. 2005).*

Sets of Chromosome Substitution Strains

Several institutions are producing murine chromosome substitution strains. In a joint project, Nadeau and Lander and colleagues made 21 consomic strains, using the C57BL/6J as a host strain and the A/J as donor strain (Singer et al. 2004; Singer et al. 2005). This strain is, via Charles River Laboratories, commercially available at the Jackson Laboratory (Bar Harbor, ME, USA) (Singer et al. 2004). The two American research groups are also creating a set where the A/J functions as host strain and the C57BL/6J as the donor strain (Nadeau et al. 2000). They will also make two sets of chromosome substitution strains where the 129S1/SvImj and the C57BL/6J are used as parental strains (Nadeau et al. 2000). Besides, there are plans to make an additional panel of chromosome substitution strains where the C57BL/6J as host strain and a strain of the Mus musculus castaneus are combined (Denny & Justice 2000). These last consomic strains are an example of so-called inter-subspecies chromosome substitution strains. In France, consomic strains between the C57BL6/J (host strain) and the Mus spretus (donor) are also made under guidance of Guénet (Guénet & Bonhomme 2003). This is an example of inter-species consomic strains. In the Czech Republic, Forejt et al. have made inter-subspecies consomic strains between the Mus musculus musculus donor strain and the C57BL/6J is used as a host strain (Gregorova & Forejt 2000), and in Japan, researchers under supervision of Shiroishi are doing the same, using Mus musculus molossinus as a donor strain (Oka et al. 2004).

Chromosome substitution strains are also available for the rat. This is done by the lab of Jacob and collegues in the USA (Cowley et al. 2004a). Two complete panels are available, where the BN/SsNHsdMcwi is used as a donor strain and either the SS/JrHsdMcwi or the FHH/EurMcwi as a host strain. These two panels are commercially available via Charles River Laboratories. There are also a couple of individual consomic strains available of both, rat (Yagil et al. 2002) and mouse (Belknap 2003; Matin et al. 1999). A complete overview of every available chromosome substitution strain, and the ones that are in the process of being produced is shown in Table 1. For rat and mouse, many Y-chromosome substitution strains are available. However, since these lines have been made over many decennia, these consomic strains are not mentioned anymore in Table 1.

The A/J and C57BL/6J Parental Strains

The consomic panel based on the A/J and C57BL/6J parental strains is very popular under (mouse) geneticists. One of the reasons for this is that these strains are commercially available at the Jackson Laboratory, so geneticists do not need to breed their own animals, something that's very time consuming, expensive and also brings quite some risks along. Researchers usually do not like breeding their own animals because the chance of a genetic contamination is quite large when several

inbred strains are kept in the same institute at the same location, which is the case when keeping chromosome substitution strains. The Jackson Laboratory is a well-established institute with a good genetic monitoring system, so contamination between inbred strains is considered nihil.

Furthermore, chromosome substitution strains of the mouse, based on the A/J and C57BL/6J inbred strains are very vulnerable to geneticists because of the great differences in susceptibility to infections and different chronic diseases (Table 2) (Marshall et al. 1992; Nadeau et al. 1995). In fact, the A/J and C57BL/6J are the two best characterized and frequently used inbred strains of the mouse. The genome of both strains is completely sequenced: the A/J by Celera Genomics (USA) and the C57BL/6J by the Mouse Sequencing Consortium (Waterston et al. 2002). The two strains differ, among others, in susceptibility to induction of Alzheimer disease, atherosclerosis, gallstone development, diabetes, hypertension, and several forms of cancer (Marshall et al. 1992). A/J and C57BL/6J mice do not only differ in susceptibility to the cause of infectious diseases that occur in Third World countries but also for the ones that occur in our Western society (like Mycobacterium tuberculosis and Salmonella enterica species) (Nadeau et al. 1995).

The two strains seem to differ in behavioural aspects; distinctive for the A/J is its non-aggressive behaviour within the strain (Brodkin et al. 2002), while aggression is observed often in C57BL/6J mice, especially between male animals (Popova et al. 1993). Several studies have showed that the A/J strain is very anxious, whereas the C57BL/6J is not (Clement et al. 2002; Laarakker et al. 2008; Singer et al. 2005). Furthermore, the strains differ in their responds to benzodiazepines (i.e. diazepam and alprazolam; two important anxiolytics) C57BL/6J also seem to be more susceptible to cocaine addiction then A/J mice (Gill & Boyle 2003; Mathis et al. 1995; Rayburn et al. 2002).

Tabel 2 *Difference in susceptibility between A/J and B6 with respect to a number of infectious and chronic diseases.*

Disease	**A/J**	**C57BL/6J**
Parasite infections		
Echinococcosis (genus: Echinococcus)	resistant	susceptible
Encephalito zoonosis (genus: Encephalitozoon)	resistant	susceptible
Entamoebiasis (genus: Entamoeba)	resistant	susceptible
Filariasis (genus: Brugia)	resistant	susceptible
Giardiasis (genus: Giardia)	susceptible	resistant
Malaria (genus: Plasmodium)	susceptible	resistant
Schistosomiasis (genus: Schistosoma)	susceptible	resistant
Taeniasis (genus: Taenia)	susceptible	resistant
Toxoplasmosis (genus: Toxoplasma)	resistant	susceptible
Trichomoniasis (genus: Trichomonas)	resistant	susceptible
Trypanosomiasis (genus: Trypanosoma)	susceptible	resistant

Disease	A/J	C57BL/6J
Bacterial infections		
Bronchitis (Pseudomonas bronchitis)	susceptible	resistant
Diphtheria (Corynebacterium diphtheria)	susceptible	resistant
Lepra (Mycobacterium leprae)	resistant	susceptible
Listeriosis (Listeria monocytogenes)	susceptible	resistant
Veteran disease (Legionella pneumophila)	susceptible	resistant
Tuberculosis (Mycobacterium tuberculosis)	resistant	susceptible
Typhus (Rickettsia typhi)	susceptible	resistant
Salmonella (genus: Salmonella)	resistant	susceptible
Virale infections		
Herpes (Herpes simplex virus type 1; MuHV-1)	susceptible	resistant
AIDS (mouse leukaemia virus)	resistant	susceptible
Maloney virus	susceptible	resistant
Fungal infections		
Candiasis (Candida albicans)	susceptible	resistant
Coccidioidomycose (Coccidioides immitis)	resistant	susceptible
Histoplasmosis (Histoplasma capsulatum)	resistant	susceptible
Chronice diseases		
Alzheimer	resistant	susceptible
Asthma	susceptible	resistant
Atherosclerosis	resistant	susceptible
Colon cancer	susceptible	resistant
Diabetes	resistant	susceptible
Galstenen	susceptible	resistant
Gespleten verhemelte	susceptible	resistant
Hydrocephalus	resistant	susceptible
Hypertension	resistant	susceptible
Long cancer	susceptible	resistant
Obesity	resistant	susceptible
Osteoporose	susceptible	resistant

Considerations

Compared to the conventional genetic analysis systems, the use of chromosome substitution strains has major advantages when investigating the relation between genes and complex biological mechanisms of diseases. These advantages are:

i) Assignment of QTLs to chromosomes is simply done by phenotyping a panel of chromosome substitution strains and the host strain.
ii) The QTL can be localized on the chromosome using a relative small progeny between the consomic strain and the host strain. Just a few (10 – 20) DNA-markers are needed for the characterization.
iii) Compared to the conventional systems, QTLs with smaller effects can be detected.
iv) It is possible to study more animals of one genotype, since the animals are not unique, which is the case when using a conventional system. The combination of genes in such a segregating population (backcross- or F2-generation) cannot be reproduced easily.
v) Using consomic strains, congenic strains can be produced in just two backcrosses. It takes minimal five backcrosses when the traditional procedure of making congenic strains is applied, even when DNA-markers are used to select specific strains.
vi) It is possible to make quick double-consomic strains to study QTL-QTL interactions (= epistasis). These are strains where, compared to the host strain, two chromosome pairs are exchanged. The assumption is that the two separate QTLs already have an affect.

However, if there are two loci on different chromosomes that need to interact with each other to express a certain phenotype, but do not show any effect separated from each other, chromosome substitution strains cannot be used to identify those QTLs. In this case other permanent genetic systems have to be used to localize those QTLs.

Comparing the different chromosome substitution strains to the host strain identifies chromosomes that contain at least one QTL. Unfortunately, it is impossible to distinguish if the chromosome contains one or more QTLs. In order to do so, an extra crossing is necessary. But it should be clear that these two limitations do not outweigh the many advantages.

Haplotype mapping as an In Silico Alternative and Complementation

In silico mapping (also known as haplotype mapping) is a powerful computational-based method for predicting chromosomal regions regulating phenotypic traits. The discovery based on the distribution of single nucleotide polymorhisms (SNPs) in different inbred mouse strains of an alternating mosaic pattern of relatively large, typically 1-2 Mb, genomic regions (blocks) of low or high polymorphic variation (SNPs) is the foundation for this approach. Regions that are poor in polymorphic markers have been designated as regions of common ancestry. Haplotype blocks

are conceptually defined as genomic segments harbouring sets of coupled polymorphisms that reflect a common ancestral origin. Genome-wide association studies involving the correlation of a phenotype, for instance, disease prone and disease resistant, over a wide selection of different inbred mouse strains to patterns of genetic variation in these same strains have provided powerful indications of potential candidate regions. The genes underlying QTLs are likely, although not necessarily, to be found in regions of different ancestry among pairs of differentially affected strains, while a given phenotype observed in common in different strains will be likely be controlled by a region that is held in common between these strains. The approach requires that well-standardized parental phenotype data are available for many inbred strains.

Applied to QTL analysis, knowledge of the haplotype block structure within a QTL interval (1-LOD support interval) may focus on a particular sub region within the QTL interval (Figure 13). There are several examples in the literature that have demonstrated the value of this type of combined approach.

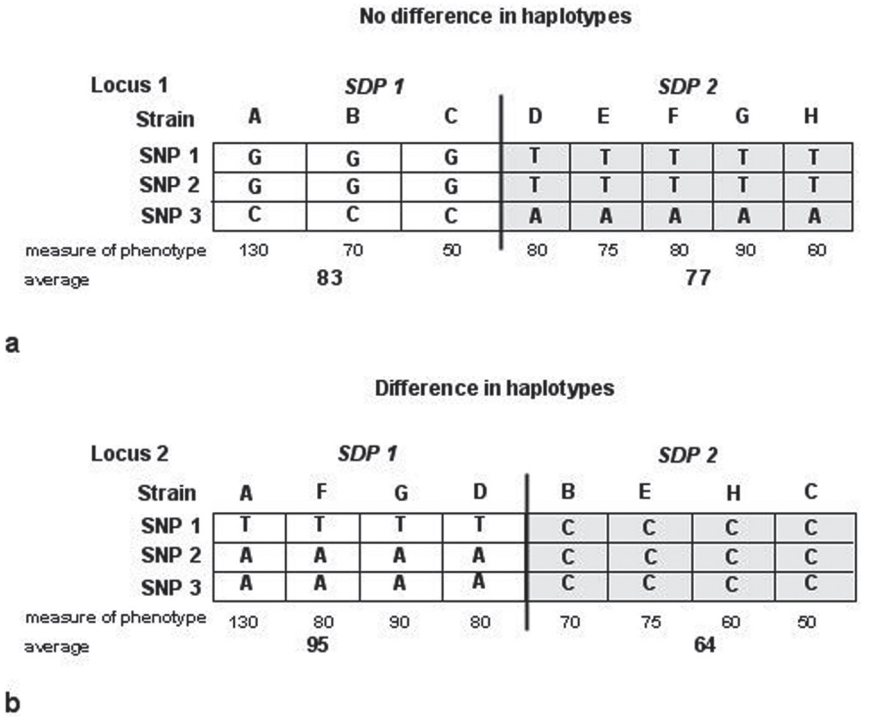


Figure 13 *Haplotype analysis (adapted from DiPetrillo et al. 2005): (a) Strain distribution pattern (SDP) across three SNPs in eight strains at locus 1. Strains A, B and C share an SDP, whereas mice D, E, F, G, and H share a different SDP. Haplotype analysis first uses the SDP to search for common haplotypes among inbred strains, and then associate these with a phenotype. The average value for the phenotype is 83 at SDP1 and 77 at SDP2, so the haplotype is not linked to the phenotype. (b) Contrasting to the situation in (a), the average phenotype for SDP1 at locus 2 is 95 versus 64 for SDP2, meaning that the phenotype is linked to the haplotype.*

Outline of the Thesis

Aim and general approach

The primary aim of this thesis is to detect QTLs for mouse avoidance behaviour, i.e. anxiety towards novelty. This aim was approached by using consomics, which is a first step in the identification of genes underlying the behavioural trait of interest. This knowledge is of crucial importance for successful treatment of pathological variants of anxiety in both human and animals since the genes identified may serve as new therapeutic targets for novel anxiotytics.

Although, anxiety disorders are treated by a variety of pharmacological compounds, still treatment success is not satisfying and often side effects can be observed (Belzung 2001). Up to now, no major breakthrough in the search for more specific treatment strategies was obtained. In recent years, some efforts have been taken to identify genes underlying anxiety disorders (Clement et al. 2002; de Mooij-van Malsen et al. 2008). While a variety of QTLs for various anxiety related behaviours were identified, only very few candidate genes could be confirmed, probably among others, due to methodological difficulties in identifying behavioural phenotypes in animal models used. In this study a multidimensional phenotyping approach was used, allowing for testing a variety of motivational systems in parallel, dissecting motivational systems in mice and, thus, controlling for factors potentially confounding anxiety related behaviour.

To elucidate the genetic mechanisms underlying those behavioural differences, further analyses with a complete commercially available set of mouse chromosome substitution strains (available at the Jackson Laboratory in Maine, USA) were performed. The progeny of a F2-intercross between the host strain and the consomic line of interest was used to perform the QTL analysis. This QTL analysis was aimed at finding candidate genes and predicts involvement of these genes in anxiety related behaviour. The results of this thesis will form the basis for future work that directs towards the use of knockout strategies and micro-array analyses to assess the contribution of these candidate genes in relation to anxiety related avoidance behaviour. Bioinformatics studies using publicly available online databases were used to predict possible candidate genes beforehand and obtained data will be published in a database to be available for other researchers (PhenomeNetwork).

This strategy can lead to the prediction of possible brain pathways of candidate genes that are involved and how this could relate to the human situation. Homologies between mice and humans or other species will be studied to find correlations.

Outline of the thesis

Chapter 1 is an introductory chapter where both male and female mice of the inbred strains A/J and C57BL/6J (parental strains of the consomic strain which was used later) are behaviourally characterized in the mHB. Since we are

interested in anxiety-related behaviour, and especially in the genetic background of this behavioural dimension, we analysed whether both strains differ with respect to anxiety and at the same time show a robust phenotype.

In the following chapter (**chapter 2**) we address a statistical method to reduce the number of laboratory animals used in behavioural genetic experiments. This method specially refers to the use of chromosome substitution strains, since this is the genetic approach we had planned to use in the genetic experiments described in this thesis. In fact, the multiple phase approach that we suggest is a form of sequential analysis, which was already suggested by Russell and Burch (1959, reprinted 1992) in 1959 as one method of reducing the number of laboratory animals.

This two-stage approach is integrated in **chapter 3**. Here the total commercially available consomic panel of the mouse is tested in the mHB to find chromosomes that are responsible for variations in anxiety related behaviour with respect to novelty. Several chromosomes seem to contain at least one QTL for anxiety related avoidance behaviour. Chromosome 19 was chosen to be the most interesting for our purposes since no pleiotropic effect of locomotion was detected. Being interested in statistics, we also showed that it is worthwhile to perform the consomic survey with both univariate and multivariate (bivariate) analyses and to use the two-stage approach as suggested in the previous chapter.

Mouse chromosome 19 contains the gene for the alpha 2A-adrenoceptor which is known to be involved in acute stress responses, both in laboratory animals and humans. It has also been shown that this receptor plays a critical role in regulating acute neuropsychological stress responses and, ultimately, stress coping behaviour. **Chapter 4** is a pharmacological study that investigates whether the alpha A2-adrenoceptor gene, assigned to mouse chromosome 19, is a possible candidate gene for avoidance behaviour in the mHB test. It was tested if this pathway is involved in the regulation of anxiety/arousal by applying an agonist and an antagonist of the alpha 2A-adrenoceptor to mice from a consomic strain (CSS19), and the corresponding donor (A/) and host (B6) strains.

In chapter 5 the QTL analysis of male progeny from an F2-intercross between C57BL/6J and CSS19 is outlined. The analysis resulted in significant QTLs for anxiety related avoidance behaviour and several positional and functional candidate genes were found. By combining the results from the QTL analysis with the mHB-data from the two strains (CSS19 strain + host strain) and the haplotype block structure of A/J and C57BL/6J, it was possible to focus on a particular sub-region within the QTL interval.

In the last chapter (**chapter 6**) F2 animals from two crosses (between C57BL/6J and CSS1, and between C57BL/6J and CSS19) were screened for QTLs and for candidate genes involved in the regulation of circulating total cholesterol levels. We studied blood plasma total cholesterol levels because numerous claims have been made concerning the relationship between this parameter and anxiety disorders.

A final evaluation of the results is presented in the general discussion. Finally, future research and limitations of the behavioural and genetic studies is discussed.

Chapter 1

Behavioural characterisation of A/J and C57BL/6J mice using a multidimensional test

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submitted

Abstract

Up to 25% of all adults will experience an anxiety-related disorder during their lives. Treatment of these disorders is still difficult en the exact mechanisms and pathways behind anxiety disorders remain to be elucidated. Although evidence exists for genetically based susceptibility of human psychiatric diseases, candidate genes have rarely been identified up to now. Chromosome substitution strains with a high-anxiety profile may be a potent tool to search for candidate genes in mice, which could then be translated to the human situation. In this paper we investigate whether the A/J and C57BL/6J strains differ in various motivational systems, but especially in anxiety related behaviour from each other. These strains form the parental strains in such chromosome substitution lines. Young adult individuals from both genders of A/J and C57BL/6J strains were behaviourally phenotyped using a multidimensional test. In addition, an acute, aversive stimulus was applied to the animals to validate the stability of their behavioural phenotype. It could be concluded that B6 and A/J mice differ with respect to almost all motivational systems, with male mice showing a more pronounced phenotypic strain difference than female mice. is the high anxiety phenotype of male A/J mice could further be shown to represent a primary characteristic. Therefore, these chromosome substitution strains may be an interesting tool to search for genes that affect anxiety-related behaviour in mice.

Keywords

Anxiety, behaviour, corticosterone, magnesium, mice, modified hole board, strain differences, tyrosine hydroxylase

Introduction

The term anxiety comprises a combination of essential emotions like fear, apprehension and worries, which are often accompanied with specific physical phenomena. Anxiety, which leads to a characteristic autonomic response, serves as a protective reaction in threatening situations. However, up to 25% of the adults will experience extensive chronic anxiety and suffer from one of the six anxiety disorders (generalized anxiety disorder; social anxiety; phobias; obsessive compulsive disorder; posttraumatic stress disorder and panic disorder) (Belzung & Griebel 2001a; Gordon & Hen 2004; Ohl 2003;). In both animals and humans it has been shown that inter-individual differences exists with respect to the genetic susceptibility to develop extreme or pathological anxiety. However, candidate genes for anxiety disorders in humans have rarely been identified yet. Data from several studies suggest that genetic variation explains about 90% of the behavioural variation, so rodent inbred strains form a useful tool to investigate quantitative trait loci for complex behaviour like anxiety-related behaviour (Flint 2003; Kas & van Ree 2004; de Mooij-van Malsen et al. 2008; Ohl 2004). Therefore, we aim at analysing the genetic characteristics of mice with a high anxiety phenotype. Such rodent models are based on the assumption that animals experience anxiety the same way humans do. Since anxiety is an evolutionary old emotion and neuronal circuits regulating anxiety are comparable in animals and humans, rodent models are of high translational value to anxiety research (Belzung & Griebel 2001a; Belzung et al. 2001b). However, especially the translation of animal behaviour to psychopathological conditions in humans remains to be difficult (Ohl 2004).

Classical behavioural tests for rodents often are focussed on one specific behavioural parameter, such as avoidance behaviour or locomotor activity. Due to their one-dimensional design, these tests are insufficient in discriminating between anxiety and exploration. The distinction between these two parameters is difficult and it is usually questionable whether an animal avoids a certain area because it is too anxious to approach it, or avoids it because it is to inactive to reach this area (Ohl et al. 2001b). Many studies in anxiety research use test batteries containing specific tests for different behavioural parameters to analyse this potential interaction. Since the separate tests can influence each other in this type of test paradigm, we used a multidimensional test, the modified hole board (mHB) test (Ohl et al 2001a). The mHB basically is a combination of the traditional hole board and the open field test (for review see Lister 1990 and Ohl et al. 2001a) allowing to test for multiple dimensions of unconditioned behaviour, such as anxiety, exploration, risk assessment, locomotor activity, social affinity, and physiological arousal, using just one single test. (Ohl et al. 2001a; Ohl 2004). The reliable and valid identification of behavioural phenotypes is essential for the search of candidate genes being involved in a behavioural trait of interest and it might be hypothesised that false positive phenotyping results account for the lack of knowledge about the genetic background of anxiety-related behaviour.

Especially useful types of inbred strains are consomic or chromosome substitution strains (CSS). These are inbred lines with a C57BL/6J genome as background, developed by the group of Nadeau (Nadeau et al. 2000). In each strain one chromosome of the C57BL/6J background has been substituted for a chromosome of the A/J strain. This means that there are a total of 21 different lines (19 autosomes and 2 sex chromosomes substituted) available that can be commercially purchased from The Jackson Laboratory (Bar Habor, USA). In this study A/J and C57BL/6J inbred mouse strains were behaviourally characterized by use of the mHB test. We compared the behaviour of the animals before and after an aversive stimulus (intra-peritoneal (ip) injection with saline) to test for the robustness of the behavioural phenotype determined in the mHB. In addition, physiological parameters were investigated, which have been suggested to correlate with certain behaviours. Cholesterol receptors have been suggested to play a role in activity in mice (Elder et al. 2008b) and in humans it is known that anxiety patients suffer form higher plasma cholesterol levels then normal controls (Peter et al. 1999). The influence of magnesium has often been discussed and it is suggested that elevation in plasma magnesium levels may reduce anxiety related phenotypes, as well as depression (Poleszak et al. 2004; Poleszak et al. 2005b). Corticosterone levels that are known to be involved in the short term stress responds (Amico et al. 2004; Aroya-Milshtein et al. 2004), are measured before and after an aversive stimulus. Glucose has shown to counteract the corticosterone response and might therefore reduce the acute stress response (Bell et al. 2002). Adrenal tyrosine hydroxylase (TH) levels are measured to detect long term stress effects (Osterhout et al. 2005; Zhu et al. 2005). This can help to unravel the behaviour of A/J and B6 inbred mice and the support the studies of robustness of their phenotypes.

Materials & Methods

Ethics

The protocol of the experiment was reviewed by the scientific committee of the Department of Animals, Science & Society, Utrecht University, the Netherlands, and approved by the Animal Experiments Committee of the Academic Biomedical Centre, Utrecht, The Netherlands. The Animal Experiments Committee based its decision on 'De Wet op de Dierproeven' (1996) and on the 'Dierproevenbesluit' (1996); both are available online (http://www.nca-nl.org/).

Animals

This study was performed with naive A/J (N=9 per gender) and C57BL/6J (N=9 per gender) mice from both genders that were all purchased from The Jackson Laboratory (Bar Harbor, USA). The mice were 4–6 weeks upon arrival. All animals were housed at the animal facility of Utrecht University, division of Laboratory Animal Science for 2 weeks to habituate prior testing. Testing took place in the same room where the mice were housed under standard laboratory conditions

(12h: 12h light: dark, lights on at 7 p.m.; 22˚C; 50% humidity and ad libitum access to water and standard mice chow). All mice were housed in social groups (3 per cage) in Macrolon-2 cages, enriched with a tissue, a shelter and a hand full of Enviro-dry™ bedding material. During the habituation period, all mice were handled at least 4 times a week for a few minutes by the person who performed the behavioural experiments. Handling included picking up the animal at the tail base, placing it on the hand or arm and restraining it by hand for a few seconds at random times of the day.

Modified hole board (mHB) test

The behavioural tests were all performed in the mHB. This test set-up has been published by Ohl et. al in 1998 and is a combination of an open field and a hole board test. The mHB basically consists of a gray PVC box (1.5 x 0.5 x 0.5 m) that is divided into two compartments by a transparent partition, perforated with 120 holes (3 mm diameter). The smaller compartment (0.5 x 0.5 x 0.5 m) is used as a group compartment and is enriched with a tissue, where all cage mates of the tested animal are placed. The group compartment allows the tested animal to have auditory, olfactory and visible contact to its known conspecifics in order to prevent isolation stress. The experimental compartment (1 x 0.5 x 0.5m) consists of two areas, one protected area, or the box, that is surrounded by the protective walls of the set-up, and an unprotected area, or the board. The box is divided into 12 rectangles (15 x 20 cm) with black lines in order to be able to determine locomotor activity. The board (60 x 20 cm) is placed right in the middle of the experimental compartment, and contains 20 PVC cylinders (3 x 3 cm), straggled over the board in 3 indented rows. Further, the board is lit with an extra red-light lamp (80 W), in a way that the board is illuminated by approximately 35 lux, while the box is only 1–3 lux. Furthermore, a familiar and a new object were placed in the box area of the experimental compartment in order to include a cognition test. The behavioural testing was performed between 10 am and 2 pm under red-light conditions and all behavioural tests were videotaped from right above the experimental compartment and from the side through the partition wall.

Anxiety-related behaviour is indicated by the parameters 'time on board', 'latency until the first board entry', and 'number of board entries'. The number of 'stretched attends' and the 'latency until the first stretched attend' are a measure of risk assessment. The latency times and total numbers of several parameters indicate undirected exploratory behaviour. The parameters we studied in the mHB test are 'rearing in the box' and 'rearing on the board' as well as the 'exploration of the holes'. For each of those parameters, the number of events and the latency until the first time event were measured. The parameters 'hole visit' and 'unfamiliar object exploration' are used to describe the motivational system 'directed exploration'. Object recognition was tested by the application of a familiar object in the behavioural test set-up. Line crossings were counted to make a statement about the activity of an animal. Behaviours like self-grooming,

defecation and urination indicate the arousal state of an animal. There are several other parameters measured in the mHB that could not be assigned to one specific motivational systems because they indicate different behavioural dimensions. For example, immobility may indicate the state of arousal or overall activity as well as anxiety-related behaviour.

Experimental protocol

For assessing object recognition of the mice in the mHB, a blue dice was placed in all cages 72 hours prior to behavioural testing as a familiar object. This object was removed again 24 hours before testing. When starting the behavioural testing, all mice of one cage were placed in the group-compartment of the mHB in order to allow habituation for 10 minutes. The familiar object, and a new object (a purple glass stone, about the size of a dice) were placed in the mHB, as shown in Figure 1.

The mice were subsequently placed in the experimental compartment as described in Figure 1 and allowed for free exploration for 5 minutes while a trained observer scored the behaviour by hand, using the computer software Observer 4.1 (Noldus, Wageningen, The Netherlands). Between mice, the test set-up was cleaned with a damp towel with only water.

30 minutes after the test, about 50 – 100 µl blood was collected by tail incisions (Dürschlag et. al, 1996) for determination of plasma corticosteron levels. Blood samples were collected in EDTA coated microvettes (Saerstedt, Nümbrecht, Germany). Seven days later the same test procedure was repeated, but this time all animals received an inter-peritoneal injection with 0.2 ml NaCl (0.9 %; B. Braun, Melsungen, Germany) 30 minutes before being placed in the box compartment of the mHB.

3 hours after testing, all animals were sacrificed by decapitation and brains, adrenal glands and trunk blood were collected for further determination of activated immediate early genes (c-fos), tyrosine hydroxylase (TH) activity, and glucose and cholesterol levels, respectively. Brains were frozen in 2-methylbutane on dry-ice and stored at –80˚C, while adrenals were stock-frozen in a Tris-HCl buffer in liquid nitrogen and also stored at –80˚C. Trunk blood was collected in lithium-heparin coated tubes (0.5 ml; Greiner Bio-One) and plasma samples were stored at –30˚C until further analysis. After sacrificing the female animals, vaginal smears were taken to determine in which phase of the oestrous cycle each animal was during the test to control for the potential effects due to the oestrous cycle.

Endocrine stress responses

Corticosterone

In order to determine the acute stress response, plasma corticosterone levels were determined by radio-immunoassays (RIA), according to the protocol of the Coat-A-Count® Rat Corticosterone kit (DPC®, Los Angeles, CA). RIAs were performed according the manufacturers protocol, using EDTA plasma from blood collected 30 minutes after each behavioural test.

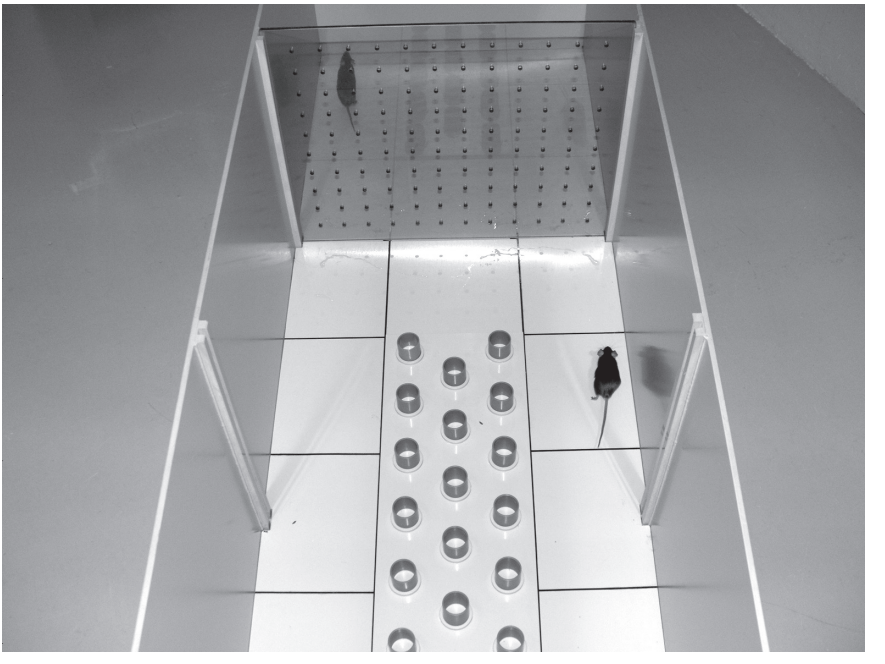

Figure 1 *The modified hole board (mHB) set-up.*

Tyrosine hydroxylase

Adrenal glands were directly removed after animals were decapitated, separately stock frozen in liquid nitrogen in 1.0 ml Tris-HCl buffer (5 mmol, pH 7.2; Boeringer/ HCl, 37 % pa; Merck) and stored until used at –70 ˚C (maximum storage: 3 month).

At the day of assay, according to a modification of the method described by Witte and Matthaei (1980), the adrenals were thawed above ice and kept on ice at all times during the analysis. Each adrenal was transferred to 150 µl fresh Tris-HCl buffer, homogenized (Eppendorf homogenizer) and centrifuged for 30 minutes (4 ˚C; 14,000 rpm; Eppendorf/ Merck).

The supernatant was removed and the pellet was dissolved in reaction mix (10 µl Na acetate buffer, 1.2 M, ph 6.0; 4 µl Brocresin (Dopa-decarboxylase inhibitor; Smith Nephew), 1.5 mM; 4 µl BH4 (L-tetrahydrobiopterin), 7.5 mM in 1 mM 2-Mercaptoethanol; 2 µl Catalase (1:10 in Na-acetate, pH 6.0) and Tyrosine-C14 solution (50 µl Ci / ml, 450 µl Ci / mmol)) and incubated for 30 minutes at 37°C shaking with uncovered lids in a thermo mixer (Eppendorf/ Merck). The same incubation procedure was done with the blanks containing only 20 µl reaction mix and 20 µl homogenate (a mix of all samples together), and for a Dopa-14C-recovery sample containing 20 µl reaction mix, 20 µl Dopa-14C-solution (375 µl Na-acetate buffer, 1.2 M, pH 6.0; Dopa-carrier solution; 5 µl Dopa-14C, 50 µl Ci / ml). The vials were then placed on ice and the reaction was stopped by adding 30 µl of 10 % trichloroacetic acid (TCA, pa; Merck) and 20 µl Dopa-carrier-

solution (1 mg Dopa / 100 ml H2O) to all. 20 µl Tyrosine-14C-substrate solution was added to the blanks and samples and were left to stand on ice to precipitate the proteins. After 10 minutes, the vials were centrifuged for 5 minutes at room temperature (14,000 rpm; Eppendorf/ Merck).

Meanwhile, columns were prepared by transferring Al2O3 (0.4 g Al2O3 act II-III, ICN; 5 ml 0.2 M NH4- acetate buffer, pH 6.1; Sigma) in a Pasteur pipette stopped with glass wool and washed once with 2.0 ml 0.2 M NH4 acetate buffer (pH 6.0). Before the columns ran dry, 100 µl of the incubation solution was brought on top of it and 1 ml 0.2 M NH4 acetate buffer (pH 6.0; Sigma) was added immediately. Columns were washed three times with 1 ml of dH2O and three times with 1 ml of Acetic acid (pH 3.6). 500 µl HCl-Methanol (1:1 (v/v) HCl, 1 mol/ l: methanol) is added. All eluates were discarded.

Column eluates were collected in scintillation vials (20 ml; glass with screw caps; Packerd) after adding 2 ml of HCl-Methanol. 6 ml of scintillation cocktail (Ultimagold liquid cocktail; Packerd) is added and mixed well. Radioactivity was determined for 10 minutes in a scintillation counter (program 14C) or until 25 % precision is reached (Liquid scintillation analyzer, Tricarb; Packerd). As a Tyrosine-14C-standard, 20 µl Tyrosine- 14C-substrate solution (190 µl Tyrosine, 0.135 mM; 10 µl Tyrosine- 14C (0.111 µmol/ ml)) with 2 ml HCl-Methanol and 6 ml scintillation cocktail was used, and for the Dopa- 14C-standard 20 µl Dopa-14C-solution with 2 ml HCl-Methanol and 6 ml scintillation cocktail was used.

The amount of Tyrosine converted was calculated as follows; factor F is the Dopa- 14C-standard (cpm) divided by the difference of the Dopa-recovery (cpm) and the blank (cpm) multiplied by the Tyrosine- 14C-standard (cpm). Then, the blank (cpm) is multiplied by the Tyrosine concentration, multiplied by 2, and by 20 and subtracted from factor F. This is the converted Tyrosine in pMol per h per adrenal. The percentage of the recovery of the columns is calculated by subtracting the blank from the Dopa recovery and dividing it by the Dopa-standard multiplied by 100.

Metabolic & Physiological parameters

Cholesterol

Cholesterol concentrations were measured photometrical in the collected trunk blood, following the protocol of an enzymatic colorimetric test (CHOD/PAP method, Roche Diagnostics, Mannheim, Germany). For the determinations a Cobas-MIRA analyzer (ABX, France) was used. Furthermore, cholesterol calibration sets (1.29, 2.59, 5.18 and 10.36 mmol/l; Sigma) were used as standards and control serum Precinorm U (Roche, Manheim, Germany) was used for quality control.

Glucose

An enzymatic UV test (HK/G-6PDH method, ABX Diagnostics, France) was used for the determination of glucose levels in the collected trunk blood. A calibrator for automated systems (CFAS, 2.625, 5.25, and 10.5 mmol/l, Roche Diagnostics,

Almere, The Netherlands) was used as standard and the same analyzer and quality control serum were used as described for the cholesterol determinations.

Magnesium
Magnesium levels were determined in lithium-heparin plasma in a photometric test using xylidyl blue (Test combination kit cat.nr. A11A01646, ABX Diagnostics, Montpellier, France). As a positive control serum Precinorm U (catnr 171743, Roche, Mannheim, Germany) was used and for calibration AXB Calibrator (catnr A1100108; lot nr 7379891 since 19-1-2005, 1.09 mmol/l). All samples were analyzed on a Cobas-MIRA analyzer (ABX, France) that was programmed for magnesium.

Vaginal smears

Vaginal smears were made by fixating the female mice and very gently flushing the vagina by pipetting 10 μl of sterile NaCl (0.9%) up and down to isolate cells. This cell solution was pipetted onto an object glass to determine the oestrous cycle under a light microscope. This procedure was once done after 2 weeks of housing and again after one month. This was done in a pilot experiment with 8 animals before the actual experiments described in this paper started, using sur plus mice.

Statistics

All statistical experiments were performed in SPSS® for Windows according to Petrie and Watson (1999). Furthermore, all analyses were carried out two-sided with tests calculating exact p-values. Continuous data (latency and relative duration of behavioural parameters, plasma parameters, and tyrosine-hydroxylase levels) were described by means and standard deviations (SD). Normality of these data was checked per group by Kolmogorov-Smirnov one sample test. Discrete or ordinale data (frequencies of behavioural parameters) are represented by medians and interquartile range (IQR).

Continuous data were first analyzed for normal-distribution using the Kolmogorov-Smirnov test. This was done per experimental group and led to the conclusion that several parameters were not distributed parametrically. All experimental groups of these non-normal distributed parameters were rank-transformed (Conover & Iman 1982). Following, homoscedacity was tested by the Levene's test. If this criterion was not fulfilled, all parameters that were not ranked yet were transformed as well and the Levene's test was repeated. Data that were rank-transformed and still did not obey the Levene's criterion were analyzed non-parametrically by the Kruskal-Wallis test, using the original non-transformed data-set. Data that were either ranked or original non-transformed normally distributed data that did obey the Levene's criterion were analyzed by ANOVA. Behavioural continuous data and values of the corticosterone determinations were tested for significant differences by repeated measures ANOVA. All other plasma- and stress response-values were analyzed by multivariate ANOVAs. For this, data

that were ranked are also used as transformed data-set in the statistical analysis. If the ANOVA detected significant effects, groups means were further compared. Between-subject post-hoc comparisons (strain- and gender differences) were done with either independent Student's t-tests for normally distributed data with separate variance estimates for which SPSS® uses the Welch correction. Or, for non-normally distributed data, the same comparisons were performed using a Mann-Whitney U test. Within-subject post-hoc comparisons were made using a paired Student's t-test when the difference between the two compared groups was normally distributed or, if not, Wilcoxon-Rank-Sum test was used.

Discrete data on the ordinal scale were fist rank-transformed and then checked for homoscedacity using the Levene's test. If the Levene's test criteria were fulfilled, the ranked data were analyzed with a repeated measure ANOVA (frequencies of behavioural data). When the Levene's test was significant, the original non-ranked ordinal data-set was analyzed non-parametrically using a Kruskal-Wallis test. Post-hoc comparisons were made with either Wilcoxon Rank Sum tests for paired data (treatment effect) or Mann-Whitney U tests for unpaired data (strain- and gender differences).

Due to multiple comparisons the greater risk of Type I errors was taken into account and the significance level of all post-hoc comparisons was pre-set at alpha = $1- (1- 0.05)^{1/C}$ where C is the number of meaningful comparisons multiplied by the number of parameters per motivational times two (because the were measurements at two time points). This approach leads to different p-thresholds for various motivational systems measured in the mHB. In all other cases, the probability of a Type I error $P<0.05$ was taken as threshold level of significance.

Results

Behaviour

Significant differences for the parameter 'board' were found between the two strains A/J and C57BL/6J (B6) in the number of board entries. For the male mice there was only a strain difference after the i.p injection, females differed only in baseline levels. B6 mice performed more board entries. Both strains spend the same time on the board, indicated by the fact that no significance was detected for the parameter 'relative duration'. Also, no difference for the latency until the first board entry was detected.

Analysis of the total numbers of stretched attends shows significant differences between strains, being gender independent. Both, before and after an application procedure, A/J and B6 mice differed significantly: for all experimental conditions, A/J mice showing significantly more risk assessment then B6 mice. Further, the A/J female mice of both treatment groups and male mice of the baseline testing group had a shorter latency time until the first stretched attend compared to B6 mice.

Analysis of variance for the number of rearing in the box revealed significant strain and treatment effects. However, these could not be confirmed in the post-hoc analysis. The number of rearing on the board showed a treatment effect in the ANOVA, but this could not be confirmed either during post-hoc testing. The analysis of the number of holes that were explored by the mice revealed significance, but only a significant strain difference in the male mice after i.p. injection was maintained in the post-hoc testing. Analysis of the latency times of the described parameters only showed a strain effect in the ANOVA for the latency until the first hole exploration but non of the post-hoc values were significant.

Analysis of the measurements of the parameter 'hole visit' did not show any significant differences after post-hoc analysis. The ANOVA showed overall differences between stains and treatments, and gender x treatment and gender x stain x treatment interactions. Kruskal-Wallis analysis also showed an overall difference for the latency until the first hole visit. Further, male and female mice at baseline levels and females after i.p. injection revealed significant strain differences in the number of unfamiliar object exploration attempts. In general, B6 showed higher numbers of exploration attempts then A/J mice. Neither the latency time until the first object exploration, nor the relative duration mice spend exploring the unfamiliar object revealed in significant differences in the post-hoc analysis.

Significant strain differences were found for the number of explorations and for the latency until the first exploration attempt of the familiar object. For the latency, there was a strain-treatment interaction as well. At the same time, there was a significant interaction between gender, strain and treatment according to the repeated measures ANOVA (P=0.015). Male B6 mice had a significantly shorter latency time until the fist object exploration then A/J mice, but this is only true at baseline measures. At the same time, female B6 mice did significantly more explorative attempt compared to female A/J mice, regardless of the treatment condition.

Analysis of variances showed a significant treatment effect for the number of total line crossings but this could not be confirmed during post-hoc testing. Furthermore, a significant (P=0.002) gender-treatment interaction was detected by the ANOVA. Significant strain effects were found for all experimental groups, B6 mice performing always more line crossings then A/J mice. Strain differences for the latency until the first line crossing were only found for the baseline tested groups but a clear trend is visible for the groups after tested after ip-injection.

For self-grooming strain differences for the number of grooming were found in the ANOVA, however these could not be detected anymore after post-hoc analysis. For the parameter defecation, a significant difference could be found for the number of boli between the female mice after baseline testing. Further an overall significance after baseline testing was found in the Kruskal-Wallis analysis for the latency of the first defecation but this was not significant anymore after Dunn-Sidak correction in the post-hoc analysis. Gender differences were detected for the number of urinations in the ANOVA and overall differences in both treatment groups in the Kruskal-Wallis test but again could not be confirmed

after post-hoc testing.

Since mice were tested in presence of their cage mates, it was possible to measure social affinity of the different experimental groups. Significant strain effects were found for the number of group contacts in the ANOVA but this was not confirmed in the post-hoc analysis. Clearly, B6 had more often contact with their cage mates then the A/J strain. The Kruskal-Wallis test also showed significant effects for the mice after baseline testing but this could not be confirmed by post-hoc analysis. No treatment effects could be detected.

With respect to male mice tested before an ip-application it was found that A/J mice spent relatively more time on being completely motionless then B6 mice. In this same group A/J mice showed significantly shorter latency times until the first time of being immobile compared to the other strain. Counting the number of box entries, gender, strain, treatment, and gender-treatment interaction effects could be measured significantly in the ANOVA. Significant strain differences were detected in both male and female mice at baseline levels and in female mice after ip injection. Generally, female mice made more box entries then males and B6 more then A/J mice. Lastly, the ANOVA detected a significant effect for the number of jumps the animals made but this was not found after post-hoc analysis.

Endocrine stress response

No significant effects were detected for plasma corticosterone levels. Female B6 mice had significantly higher TH-levels then female A/J mice and at the same time female A/J mice had higher values then male A/J mice.

Metabolic & Physiological parameters

Strain differences for both male and female mice were detected for magnesium levels, with B6 mice having higher magnesium plasma concentrations then A/J mice. Significant positive correlations were found between the number of board entries and the plasma magnesium levels after the second mHB testing; animals that made more board entries at the same time had higher magnesium levels (Figure 2).

For plasma glucose levels, gender, strain and gender-strain interaction effects were found to be significant. The post-hoc test showed differences for both strains, strain differences were only detected in female mice. Female mice in both strains had significantly lower glucose concentrations in their blood then male mice and at the same time female B6 mice had higher concentrations of glucose then female A/J mice. For cholesterol, the only effect that could be detected was a gender difference for A/J mice, the male animals having higher cholesterol levels then the females.

Brain magnesium

Total brain magnesium levels were determined three hours after modified hole board testing. The p-values shown in table 4 indicate that there was neither a

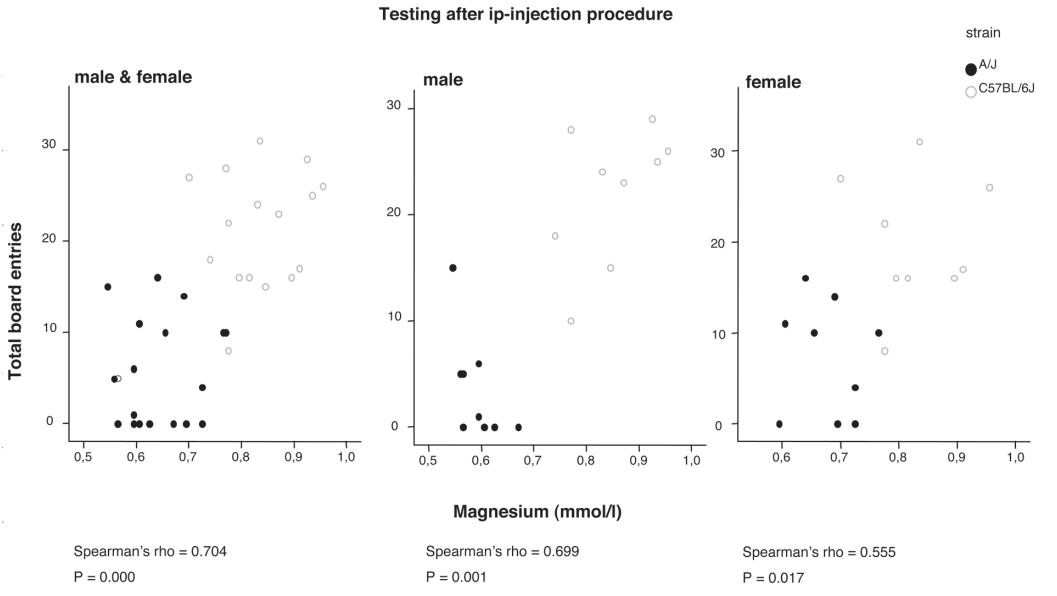

Figure 2 *Spearman's rank correlation between total board entries and plasma magnesium levels in male and female A/J and C57BL/6J mice tested in the mHB after an ip-injection procedure.*

significant strain nor gender difference detectable for total brain magnesium levels in A/J and C57BL/6J mice.

Oestrous cycle

The oestrous cycles of the female mice was not synchronized between female animals during behavioural testing.

Discussion

Overall, A/J and B6 mice differed in almost all motivational systems, supporting earlier studies by for example Brodkin et al. (1998), Hefner & Holmes (2007), and Thifault et al. (2001). A/J mice are generally less active than animals from the B6 strains, which makes it at the first sight difficult to conclude whether differences in anxiety-related behaviour represent a true high-anxiety phenotype or are secondary to differences in for example activity. If A/J mice indeed are more anxious than B6 animals, it could be expected that they would be more sensitive to an aversive stimulus than the potentially less anxious B6 mice. Interestingly, locomotor and exploration related parameters remained grossly unaffected by the ip injection in both strains. In contrast, the number of board entries revealed gender-specific

effects: the baseline strain difference in female mice disappeared after applying the aversive stimulus, while male mice did show a significant strain effect only after application of the aversive stimulus. These results indicate that the anxiety related profile of both strains is primary modulated by an aversive stimulus and the resulting behaviour indicates that A/J mice indeed are more anxious that B6 animals. Furthermore, A/J mice seem to be more sensitive to arousal then B6 mice, which is expressed by the larger number and longer duration of grooming, higher defecation and longer immobility behaviour and a shorter latency until the first time of showing immobility, confirming the profound strain differences reported by other studies (table 1-3).

Table 1 *Behavioral differences (parameters measured on an ordinale scale) between A/J and C57BL/6J mice in the modified hole board test under basal conditions and after an intra-peritoneal injection*

	Strain						
	A/J (A)		C57BL/6J (B6)				
Measure	♂♂	♀♀	♂♂	♀♀	**G**	**S**	**GxS**
Anxiety p< 0.0021							
Board entries							
Basal	0(5.5)	7(7)	14(8.5)	19(5)	0.069	**0.000**	0.628
After i.p.	1(5.5)	10(12.5)	24(10.5)	17(10.5)			
Basal vs after i.p.	1.0000	0.3980	0.0077	0.9057			
Risk assessment p< 0.0032							
Stretched attends							
Basal	15(15.5)	20(12.5)	0(0)	0(0)	0.330	**0.000**	0.634
After i.p.	14(15)	6(10.5)	0(0)	0(0)			
Basal vs after i.p.	0.1731	0.0077	0.3173	1.0000			
Undirected exploration p< 0.0011							
Rearings in the box							
Basal	13(21.5)	14(10.5)	35(7.5)	41(15)	0.471	**0.000**	0.313
After i.p.	20(27.5)	15(26.5)	43(15)	48(15.5)			
Basal vs after i.p.	0.1551	0.5541	0.0506	0.1415			
Rearings on the board							
Basal	0(0)	0(0.5)	0(0.5)	0(0)	0.956	0.956	0.052
After i.p.	0(0)	0(4.5)	0(3)	0(0)			
Basal vs after i.p.	0.3173	0.1088	0.1088	0.3173			

The high-anxiety phenotype of A/J mice is further confirmed by their object recognition behaviour: A/J mice explore the unknown object significantly later than their B6 counterpart, indicating a higher avoidance towards the novel object, i.e. anxiety. As no difference was found for the frequency and duration of object recognition, we can further conclude that A/J and B6 mice do not differ with respect to cognitive performance. Thus, the overall anxiety-related strain differences are unlikely to be secondary to cognition but seem to represent a primary anxiety related strain difference. Models of impaired fear memory in other test-settings have been studied by Schimanski and Nguyen (2005), showing that A/J mice when compared to other strains, including B6, show impaired fear memory performance, a characteristic which is well known for high anxiety individuals.

				Post hoc comparisons[2]			
ANOVA[1]				♂♂ vs ♀♀		A vs B6	
T	**GxT**	**SxT**	**GxSxT**	**A**	**B6**	♂♂	♀♀
0.002	**0.008**	**0.019**	**0.010**	0.0753	0.0129	0.0032	**0.0009**
				0.2950	0.5354	**0.0005**	0.0025
0.000	0.082	**0.002**	**0.020**	0.4797	0.3173	**0.0002**	**0.0001**
				0.1682	1.0000	**0.0001**	**0.0015**
0.002	0.474	0.366	0.834	0.9295	0.1321	0.0035	0.0023
				0.5354	0.4522	0.0031	0.0026
0.023	1.000	1.000	0.680	0.1456	0.1449	0.1449	0.1456
				0.2249	0.2249	0.2249	0.2249

[Table 1 continued]

	Strain						
	A/J (A)		C57BL/6J (B6)				
Measure	♂♂	♀♀	♂♂	♀♀	**G**	**S**	**GxS**
Holes explored							
Ba*sal*	0(10.5)	23(21)	22(20)	39(17.5)	**0.039**	**0.000**	0.230
After i.p.	2(12.5)	28(31)	50(12)	35(35)			
Basal vs after i.p.	0.5002	0.3454	0.0077	0.0687			
Directed exploration p< 0.0016							
Holes visited							
Basal	0(0)	0(0.5)	1(2)	0(1.5)	0.746	**0.003**	0.314
After i.p.	0(0)	0(0)	0(1)	0(1.5)			
Basal vs after i.p.	0.3173	0.3173	0.0180	0.7150			
Explorations of unfamiliar object							
Basal	1(2)	2(2.5)	5(2.5)	8(4)	**0.009**	**0.000**	0.051
After i.p.	1(4)	3(2.5)	6(2.5)	9(3)			
Basal vs after i.p.	0.1730	0.2367	0.2863	1.0000			
Memory p< 0.0014							
Explorations of familiar object							
Basal	1(3.5)	3(2.5)	4(3.5)	7(3)	0.079	**0.000**	0.643
After i.p.	1(2)	3(2.5)	6(2)	6(3.5)			
Basal vs after i.p.	0.4631	0.1282	0.2863	0.1422			
Locomotor activity p< 0.0032							
Line crossings							
Basal	16(31)	28(34)	123(39)	141(31)	0.778	**0.000**	0.979
After i.p.	43(51)	29(38.5)	159(30)	125(40)			
Basal vs after i.p.	0.0382	0.0687	0.0687	0.1097			
Arousal p< 0.0009							
Self-grooming							
Basal	1(2)	1(2.5)	1(1)	1(1)	0.111	**0.000**	0.106
After i.p.	4(4.5)	2(1.5)	0(0.5)	1(1.5)			
Basal vs after i.p.	0.1763	0.8658	0.1775	0.2012			
Number of boli							
Basal	0(2)	1(0)	0(0)	0(0.5)	0.653	**0.000**	0.875
After i.p.	1(2.5)	0(2)	0(0)	0(0)			
Basal vs after i.p.	0.5541	0.4631	0.3173	0.1797			
Urination							
Basal	0(1)	0(0)	0(0)	0(0)	**0.002**	0.167	0.167
After i.p.	0(1)	0(0)	0(0.5)	0(0)			
Basal vs after i.p.	1.0000	1.0000	0.5930	1.0000			

ANOVA[1]				Post hoc comparisons[2] ♂♂ vs ♀♀		A vs B6	
T	GxT	SxT	GxSxT	A	B6	♂♂	♀♀
0.003	**0.009**	**0.002**	0.260	0.0123	0.1020	0.0021	0.0270
				0.2026	0.7570	**0.0007**	0.0068
0.029	**0.029**	0.468	**0.046**	0.6339	0.0758	0.0098	0.4658
				0.3173	0.4448	0.0652	0.1037
0.068	0.303	0.583	0.776	0.2904	0.0066	**0.0011**	**0.0003**
				0.9286	0.0397	0.0043	**0.0004**
0.200	0.250	0.444	0.273	0.4150	0.0359	0.0091	**0.0006**
				0.1847	0.6541	0.0020	**0.0006**
0.019	**0.002**	0.254	0.158	0.5652	0.1331	**0.0003**	**0.0003**
				0.4797	0.0931	**0.0005**	**0.0003**
0.234	0.609	0.139	0.051	0.5161	0.5556	0.3899	0.2007
				0.2531	0.0087	0.0011	0.4299
0.465	0.079	0.673	0.842	0.7440	0.1449	0.0285	**0.0054**
				0.5682	0.3173	0.0580	0.0285
0.957	0.957	0.483	0.483	0.0275	0.3173	0.1250	1.0000
				0.0662	0.1449	0.5354	1.0000

[Table 1 continued]

	Strain						
	A/J (A)		C57BL/6J (B6)				
Measure	♂♂	♀♀	♂♂	♀♀	**G**	**S**	**GxS**
Social Affinity p< 0.0021							
Group contacts							
Basal	7(4.5)	4(4.5)	11(3.5)	12(2.5)	0.917	**0.000**	0.320
After i.p.	5(6)	5(3.5)	12(3.5)	12(4.5)			
Basal vs after i.p.	0.9442	0.3270	0.1282	0.9528			
Other behavioral parameters p< 0.0009							
Box entries							
Basal	14(9.5)	17(6.5)	30(13)	47(9)	**0.047**	**0.000**	0.273
After i.p.	17(9)	18(13)	44(12)	45(15)			
Basal vs after i.p.[3]	0.0663	0.7353	0.0117	0.9528			
Immobility							
Basal	5(5)	1(8.5)	0(0)	0(0)	0.237	**0.000**	0.974
After i.p.	5(10.5)	2(10.5)	0(0.5)	0(0)			
Basal vs after i.p.	0.7353	0.7532	0.5930	1.0000			
Jumpings							
Basal	0(0)	0(0)	0(0.5)	0(0)	**0.041**	0.155	0.155
After i.p.	0(0)	0(0)	0(2.5)	0(0)			
Basal vs after i.p.	0.3173	1.0000	0.1797	1.0000			

Results (median and, in parenthese, the interquartile range) are for 9 animals per group.

[1]P values in repeated measurements ANOVA after ranking of the data with main between-subject factors strain and gender and within-subject factor treatment. S indicates effect of strain; G, effect of gender; SxG, interaction; I, effect of injection; GxI, interaction; SxI, interaction; SxGxI, interaction. Significant effects (P<0.05) are indicated in bold characters.

[2]P values in Mann-Whitney U Wilcoxon Rank Sum W test. Significant differences are indicated in bold characters. Threshold p-values corrected for the number of parameters per motivational system, number of comparisons and the two time points measured are indicated behind each motivational system.

[3]P values in Wilcoxon Matched-pairs Signed-ranks test. Significant differences are indicated in bold characters. Threshold p-values corrected for the number of parameters per motivational system, number of comparisons and the two time points measured are indicated behind each motivational system.

ANOVA[1]				Post hoc comparisons[2] ♂♂ vs ♀♀		A vs B6 ♂♂	A vs B6 ♀♀
T	**GxT**	**SxT**	**GxSxT**	**A**	**B6**		
0.318	0.581	0.750	0.239	0.2284	0.0972	0.0077	0.0033
				0.9291	0.7891	**0.0019**	**0.0019**
0.001	**0.002**	0.695	0.204	0.2675	0.0030	**0.0003**	**0.0003**
				0.9292	0.6906	**0.0007**	**0.0003**
0.621	0.950	0.966	0.636	0.3969	0.3173	0.0009	0.0015
				0.8931	0.1449	0.0028	0.0015
0.144	0.144	0.876	0.876	1.0000	0.1456	0.1456	1.0000
				0.3173	0.0662	0.2026	1.0000

Table 2 *Behavioural differences in latency until the 1st time a behaviour was shown between A/J and C57BL/6J mice in the modified hole board test under basal conditions and after an intra-peritoneal injection*

	Strain			
	A/J (A)		C57BL/6J (B6)	
Measure	♂♂	♀♀	♂♂	♀♀
Anxiety p< 0.0021				
Board entry *				
Basal	203.3 (116.2)	112.0 (109.6)	43.5 (41.1)	17.3 (17.4)
After i.p.	143.6 (122.5)	150.3 (128.8)	58.1 (98.0)	28.0 (27.4)
Basal vs after i.p.[3]	0.265	0.295	0.704	0.271
Risk assessment p< 0.0032				
Stretched attend				
Basal	22.5 (25.6)	6.9 (2.9)	262.2 (95.3)	300.0 (0.0)
After i.p.	105.5 (145.9)	84.5 (122.8)	213.3 (133.9)	300.0 (0.0)
Basal vs after i.p.	0.510	0.008	0.125	1.000
Undirected exploration p< 0.0011				
Rearing in the box *				
Basal	103.4 (100.7)	57.7 (67.3)	22.2 (12.2)	23.5 (15.7)
After i.p.	19.2 (12.2)	25.2 (20.3)	31.2 (26.1)	17.8 (7.1)
Basal vs after i.p.	0.031	0.105	0.450	0.287
Rearing on the board				
Basal	300.0 (0.0)	291.6 (16.7)	282.8 (38.6)	300.0 (0.0)
After i.p.	279.6 (41.1)	257.8 (75.7)	273.3 (53.1)	294.7 (15.9)
Basal vs after i.p.	*0.169*	*0.152*	0.274	1.000
Hole explored *				
Basal	203.0 (116.7)	112.4 (109.4)	30.5 (13.5)	17.7 (17.4)
After i.p.	161.1 (113.9)	150.5 (128.6)	59.6 (97.2)	28.9 (26.9)
Basal vs after i.p.	0.462	0.296	0.400	0.240
Directed exploration p< 0.0016				
Hole visited				
Basal	284.7 (45.9)	278.8 (48.1)	199.0 (74.4)	280.1 (35.5)
After i.p.	257.1 (74.6)	296.4 (10.8)	258.5 (89.2)	255.4 (68.6)
Basal vs after i.p.	1.000	0.500	0.012	0.490
Exploration of unfamiliar object *				
Basal	217.4 (111.72)	147.3 (100.5)	36.7 (25.5)	24.0 (16.2)
After i.p.	114.3 (115.6)	111.7 (118.3)	47.9 (35.3)	18.6 (13.6)
Basal vs after i.p.	0.083	0.096	0.479	0.484

ANOVA[1]/ Kruskal-Wallis							Post hoc comparisons[2] ♂♂ vs ♀♀		A vs B6	
G	S	GxS	T	GxT	SxT	GxSxT	A	B6	♂♂	♀♀
0.075	**0.000**	0.917	1.000	0.133	0.485	0.949	0.106	0.097	0.003	0.032
							0.911	0.397	0.122	0.022
0.000 #							0.107	*0.471*	**0.000**	***0.000***
0.008 #							0.745	*0.206*	0.122	***0.002***
0.677	0.545	0.761	1.000	0.958	0.122	0.322	0.274	0.845	0.042	0.172
							0.457	0.168	0.234	0.325
0.220 #							*0.471*	*0.471*	*0.471*	*0.471*
0.629 #							*0.576*	*0.471*	*0.718*	*0.329*
0.053	**0.000**	0.782	1.000	0.301	0.273	0.896	0.109	0.102	0.002	0.032
							0.856	0.383	0.059	0.022
0.008 #							*0.735*	0.013	*0.010*	*0.929*
0.413 #							*1.000*	*0.555*	*0.735*	*0.135*
0.094	**0.000**	0.301	1.000	0.820	0.081	0.297	0.181	0.230	**0.001**	0.006
							0.964	0.042	0.132	0.046

[Table 2 continued]

	Strain			
	A/J (A)		C57BL/6J (B6)	
Measure	♂♂	♀♀	♂♂	♀♀
Memory p< 0.0021				
Exploration of familiar object *				
Basal	209.9 (108.2)	144.7 (92.3)	29.9 (17.6)	21.0 (11.0)
After i.p.	137.1 (131.3)	123.2 (130.1)	77.7 (106.1)	20.1 (19.3)
Basal vs after i.p.	0.201	0.425	0.192	0.895
Locomotor activity p< 0.0021				
Line crossing				
Basal	62.6 (96.1)	45.5 (95.9)	3.8 (1.8)	4.0 (1.5)
After i.p.	13.4 (18.1)	39.9 (97.6)	4.2 (1.6)	3.0 (0.8)
Basal vs after i.p.	0.068	*0.096*	0.259	0.253
Arousal p< 0.0009				
Self-grooming *				
Basal	193.3 (10.9)	183.8 (87.5)	245.1 (57.0)	210.7 (63.1)
After i.p.	204.7 (87.1)	143.5 (72.2)	200.6 (123.0)	160.8 (88.3)
Basal vs after i.p.	0.774	0.286	0.312	0.043
Defecation				
Basal	197.9 (132.3)	146.3 (106.8)	300.0 (0.0)	235.8 (127.5)
After i.p.	232.3 (125.5)	222.6 (110.2)	208.9 (137.1)	300.0 (0.0)
Basal vs after i.p.	0.674	0.035	0.084	0.500
Urination				
Basal	224.5 (109.4)	300.0 (0.0)	281.6 (55.2)	300.0 (0.0)
After i.p.	223.6 (115.9)	300.0 (0.0)	269.0 (64.8)	300.0 (0.0)
Basal vs after i.p.	0.725	1.000	0.602	1.000
Social Affinity p< 0.0021				
Group contact *				
Basal	49.2 (63.1)	40.2 (46.7)	14.9 (7.4)	15.7 (5.6)
After i.p.	20.0 (21.4)	87.5 (121.8)	16.4 (18.8)	14.0 (9.1)
Basal vs after i.p.	0.214	0.314	0.837	0.664
Other behavioral parameters p< 0.0009				
Immobility				
Basal	74.7 (95.9)	148.5 (128.8)	292.9 (21.3)	300.0 (0.0)
After i.p.	178.4 (132.3)	120.7 (110.2)	229.3 (123.6)	300.0 (0.0)
Basal vs after i.p.	0.204	0.892	0.049	1.000

ANOVA[1]/ Kruskal-Wallis							Post hoc comparisons[2] ♂♂ vs ♀♀		A vs B6	
G	S	GxS	T	GxT	SxT	GxSxT	A	B6	♂♂	♀♀
0.261	**0.000**	0.302	1.000	0.849	**0.041**	0.368	0.188	0.218	**0.001**	0.004
							0.824	0.145	0.307	0.045
0.001 #							*0.685*	0.809	0.104	*0.011*
0.010 #							*0.436*	0.067	0.166	*0.004*
0.147	0.474	0.925	1.000	0.598	0.392	0.524	0.841	0.242	0.230	0.466
							0.124	0.441	0.936	0.657
0.008 #							0.376	*0.471*	*0.082*	*0.082*
0.249 #							0.863	*0.206*	0.711	*0.082*
0.024 #							*0.082*	*1.000*	*0.188*	*1.000*
0.048 #							*0.206*	*0.471*	*0.365*	*1.000*
0.168	0.097	0.961	1.000	0.535	0.645	0.810	0.738	0.800	0.144	0.155
							0.138	0.733	0.707	0.108
0.000 #							0.187	*1.000*	***0.000***	*0.002*
0.007 #							0.330	*0.082*	0.412	*0.002*

[Table 2 continued]

	Strain			
	A/J (A)		C57BL/6J (B6)	
Measure	♂♂	♀♀	♂♂	♀♀
Jumping				
Basal	300.0 (0.0)	300.0 (0.0)	251.8 (99.5)	300.0 (0.0)
After i.p.	266.7 (64.7)	300.0 (0.0)	286.3 (41.0)	300.0 (0.0)
Basal vs after i.p. 0.081		1.000	0.297	1.000

Results (median and, in parenthese, the interquartile range) are for 9 animals per group.

[1]P values in repeated measurements ANOVA (parameters that are not normally distributed were first ranked; p-values in italics) of the data with main between-subject factors strain and gender and within-subject factor treatment. S indicates effect of strain; G, effect of gender; SxG, interaction; T, effect of injection (treatment); GxT, interaction; SxT, interaction; SxGxT, interaction. Significant effects (P<0.05) are indicated in bold characters.

[2]P values in Independent Samples Student's t-test when data are normally distributed and Mann-Whitney U Wilcoxon Rank Sum W test (p-values in italics) for parameters without normal distribution. Significant differences are indicated in bold characters; italics characters were ranked due to not normal distribution. Threshold p-values corrected for the number of parameters per motivational system, number of comparisons and the two time points measured are indicated behind each motivational system.

Table 3 *Behavioural differences in relative time (% duration) a behaviour was shown between A/J and C57BL/6J mice in the modified hole board test under basal conditions and after an intra-peritoneal injection*

	Strain				
	A/J (A)		C57BL/6J (B6)		
Measure	♂♂	♀♀	♂♂	♀♀	**G**
Anxiety p< 0.0021					
Time on Board					
Basal	8.2 (10.6)	20.3 (13.6)	13.9 (7.7)	17.5 (8.2)	0.017
After i.p.	9.1 (10.7)	19.8 (18.7)	15.7 (10.5)	19.3 (7.7)	
Basal vs after i.p.[3]	0.861	0.938	0.744	0.310	

ANOVA[1]/ Kruskal-Wallis							Post hoc comparisons[2] ♂♂ vs ♀♀		A vs B6	
G	S	GxS	T	GxT	SxT	GxSxT	A	B6	♂♂	♀♀
0.233 #							*1.000*	*0.471*	*0.471*	*1.000*
0.092 #							*0.206*	*1.000*	*0.329*	*1.000*

[3]P values in Paired Samples Student's t-test when _'s (=basal vs. after i.p.) of data are normally distributed and Wilcoxon Matched-pairs Signed-ranks test (p-values in italics) for _'s of parameters without normal distribution. Significant differences are indicated in bold characters. Threshold p-values corrected for the number of parameters per motivational system, number of comparisons and the two time points measured are indicated behind each motivational system.

* Parameters indicated with '*' were ranked in the repeated measures ANOVA due to different variances in the Levene's test.

\# Data indicated with '#' were analyzed non-parametrically with a Kruskal-Wallis test because variances still differed according to the Levene's test after ranking. If these data are represented in italics they were not normally distributed (according to Kolmogorov-Smirnov criteria) as well. Significant effects are indicated in bold characters (P<0.05).

With one exception, all P-values are exact P-values calculated with the Exact-test. Values determined by the Kruskal-Wallis test are simulated according to the Monte-Carlo method.

ANOVA[1]/ Kruskal-Wallis						Post hoc comparisons[2] ♂♂ vs ♀♀		A vs B6	
S	GxS	T	GxT	SxT	GxSxT	A	B6	♂♂	♀♀
0.459	0.196	0.694	0.868	0.765	0.899	0.052	0.345	0.211	0.613
						0.158	0.438	0.209	0.926

[Table 3 continued]

	Strain				
	A/J (A)		C57BL/6J (B6)		
Measure	♂♂	♀♀	♂♂	♀♀	**G**
Directed exploration	p< 0.0016				
Exploration of unfamiliar object *					
Basal	0.7 (0.7)	1.6 (1.1)	1.4 (0.4)	2.8 (1.4)	**0.000**
After i.p.	0.7 (0.9)	1.4 (0.9)	0.8 (0.6)	1.4 (0.3)	
Basal vs after i.p.	0.972	0.388	0.219	0.020	
Memory p< 0.0021					
Exploration of familiar object					
Basal	0.8 (0.8)	1.6 (1.3)	1.6 (0.7)	1.8 (1.1)	**0.026**
After i.p.	0.6 (0.7)	0.6 (0.4)	0.5 (0.3)	1.3 (0.6)	
Basal vs after i.p.	0.710	0.034	**0.001**	0.166	
Arousal p< 0.0009					
Self-grooming					
Basal	5.5 (9.6)	3.8 93.2)	1.7 (3.3)	1.3 (1.1)	0.0
After i.p.	6.1 (10.5)	3.6 (1.4)	3.7 (6.2)	1.6 (1.2)	0.1
Basal vs after i.p.	0.886	0.845	0.456	0.670	

	Average duration of a board entry
Risk assessment	Total number of risk assessments
	Latency until the first risk assessment
Undirected exploration	Total number of rearing in the box
	Latency until the first rearing in the box
	Total number of rearing on the board
	Latency until the first rearing on the boar
	Total number of hole explorations
	Latency until the first hole exploration
Directed exploration	Total number of holes visited
	Latency until the first hole visited
	Total number of unfamiliar object explorans
	Latency until the first unfamiliar object exration

ANOVA[1]/ Kruskal-Wallis						Post hoc comparisons[2] ♂♂ vs ♀♀		A vs B6	
S	**GxS**	**T**	**GxT**	**SxT**	**GxSxT**	**A**	**B6**	♂♂	♀♀
0.033	0.371	**0.012**	0.098	0.057	0.285	0.053	0.003	0.118	0.051
						0.122	0.013	0.761	0.799
0.054	0.607	1.000	0.591	0.811	**0.015**	0.117	0.576	0.039	0.690
						0.971	**0.002**	0.562	0.010
6 #						0.632	0.708	0.286	0.040
6 #						0.493	0.346	0.552	0.004

Stretched body posture, including hind-paws

Rearing on hind-paws in the box

Rearing on hind-paws on the board

Exploration of a cylinder (hole) on the board

Nose-poking into a cylinder (hole) on the board

Exploration of the unfamiliar (new) object

[Table 3 continued]

Results (median and, in parenthese, the interquartile range) are for 9 animals per group.

[1]P values in repeated measurements ANOVA (parameters that are not normally distributed were first ranked; p-values in italics) of the data with main between-subject factors strain and gender and within-subject factor treatment. S indicates effect of strain; G, effect of gender; SxG, interaction; T, effect of injection (treatment); GxT, interaction; SxT, interaction; SxGxT, interaction. Significant effects ($P<0.05$) are indicated in bold characters.

[2]P values in Independent Samples Student's t-test when data are normally distributed and Mann-Whitney U Wilcoxon Rank Sum W test (p-values in italics) for parameters without normal distribution. Significant differences are indicated in bold characters; italics characters were ranked due to not normal distribution. Threshold p-values corrected for the number of parameters per motivational system, number of comparisons and the two time points measured are indicated behind each motivational system.

As shown in table 4, a variety of parameters from the blood plasma were determined. These specific parameters were chosen based on the literature demonstrating the correlation of magnesium, glucose, and cholesterol to anxiety- and locomotor-related phenotypes in animals as well as in humans (Desrumaux et al. 2004; Elder et al. 2008a; Jakovljevic et al. 2007; Poleszak et al. 2008; Seelig 1994; Surwit & Schneider 1993).

Strain differences in magnesium, glucose and cholesterol levels were found as well as gender differences for glucose and cholesterol, indicating a genetic differences between the two strains: Paigen (1995) and Stylianou et al. (2006) found quantitative trait loci for high HDL cholesterol sensitivity in several inbred mouse strains and the existence of related genes that for example affect atherosclerosis development and other groups described the genes of obesity resistant A/J and obesity-prone C57BL/6J mice (Adan et al. 2006; Brockmann & Bevova 2002; Owada et al. 2006).

Plasma corticosterone levels measured 30 minutes after stimulation have been shown to give a reliable indication of the acute hormonal stress response (Hodge et al. 2002). Neither exposition to the novel testing environment (mHB), nor the ip-injection revealed significant hormonal stress responses in the two strains tested, indicating that both stimuli were only mildly stressful to the animals. This conclusion is underlined by the fact that the behavioural response to the ip-injection in both strains was only subtle.

Thyrosine-hydroxylase (TH) levels were measured in the adrenals to possibly indicate a state of chronic stress. Female A/J mice had significantly higher TH levels then male A/J mice and female B6 mice had significantly higher levels then female B6 mice. Higher TH levels indicate a chronic stress response, however from this cannot be concluded whether the aversive stimulus influenced the TH values or the mHB test itself. Interestingly, strain differences were found for plasma magnesium levels with A/J mice having significantly lower levels then B6 mice. These strain differences were previously found by Chollet et al. (2000) but several times lower

[3]P values in Paired Samples Student's t-test when _'s (=basal vs. after i.p.) of data are normally distributed and Wilcoxon Matched-pairs Signed-ranks test (p-values in italics) for _'s of parameters without normal distribution. Significant differences are indicated in bold characters. Threshold p-values corrected for the number of parameters per motivational system, number of comparisons and the three two points measured are indicated behind each motivational system.

* Parameters indicated with '*' were ranked in the repeated measures ANOVA due to different variances in the Levene's test.

\# Data indicated with '#' were analyzed non-parametrically with a Kruskal-Wallis test because variances still differed according to the Levene's test after ranking. If these data are represented in italics they were not normally distributed (according to Kolmogorov-Smirnov criteria) as well. Significant effects are indicated in bold characters (P<0.05). With one exception, all p-values are exact p-values calculated with the Exact-test. Values determined by the Krusal-Wallis test are simulated according to the Monte-Carlo method.

then our measurements. This difference might be explained by age differences of the animals used in the two studies. At least for rats it is known that age has great influence on plasma magnesium levels (Chan et al. 1992). Remarkably, the plasma magnesium levels were positively correlated to the number of board entries, one of the anxiety-related parameters in the modified hole board test. These findings correspond to the research of Poleszak and collegues, who demonstrated the anxiolytic effect of magnesium treatment in mice (Poleszak et al. 2004; Poleszak et al. 2005a; Poleszak et al. 2005b; Singewald et al. 2004).

Table 4 *Differences in plasma values and stress response between A/J and C57BL/6J mice in the modified hole board test under basal conditions and after an intra-peritoneal injection*

	Strain			
	A/J (A)		C57BL/6J (B6)	
Measure	♂♂	♀♀	♂♂	♀♀
Trunk blood plasma (mmol/ l)				
Magnesium	0.6 (0.0)	0.7 (0.1)	0.8 (0.1)	0.8 (0.1)
Glucose	10.2 (1.4)	8.1 (0.8)	11.5 (0.8)	9.7 (1.0)
Cholesterol	2.2 (0.4)	1.6 (0.1)	2.2 (0.2)	2.9 (2.7)
Stress response				
Corticosteron (from tail blood plasma; nmol/l) *				
after baseline	740.6 (197.7)	676.2 (276.5)	768.4 (151.6)	777.8 (199.8)
after ip	760.0 (211.9)	929.2 (320.4)	774.7 (150.6)	759.7 (109.3)
baseline vs after ip	0.796	0.134	0.855	0.696
Adrenal Tyrosine-Hydroxylase				
(nmol/ h/ adrenal)	1.6 (0.4)	2.5 (0.5)	2.8 (1.2)	3.2 (0.5)
Brain				
Magnesium				
(mmol/g DW[$])	0.1 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)

Results (median and, in parenthese, the interquartile range) are for 9 animals per group.

[1]P values in repeated measurements ANOVA (parameters that are not normally distributed were first ranked; p-values in italics) of the data with main between-subject factors strain and gender and within-subject factor treatment. S indicates effect of strain; G, effect of gender; SxG, interaction; T, effect of injection (treatment); GxT, interaction; SxT, interaction; SxGxT, interaction. Significant effects (P<0.05) are indicated in bold characters.

[2]P values in Independent Samples Student's t-test when data are normally distributed and Mann-Whitney U Wilcoxon Rank Sum W test (p-values in italics) for parameters without normal distribution. Significant differences (P<0.0167) are indicated in bold characters; italics characters were ranked due to not normal distribution.

ANOVA[1]/ Kruskal-Wallis							Post hoc comparisons[2] ♂♂ vs ♀♀		Post hoc comparisons[2] A vs B6	
G	**S**	**GxS**	**T**	**GxT**	**SxT**	**GxSxT**	**A**	**B6**	♂♂	♀♀
0.149	**0.000**	0.670					**0.002**	0.589	**0.000**	**0.000**
0.000	**0.000**	**0.022**					**0.002**	**0.001**	0.031	**0.003**
0.000 #							***0.002***	*0.024*	*0.931*	*0.089*
0.862	0.923	0.658	0.974	0.101	0.135	0.199	0.578	0.914	0.742	0.405
							0.205	0.819	0.868	0.166
0.001 #							**0.000**	0.422	0.021	**0.016**
0.277	0.653	0.974					0.446	0.446	0.776	0.732

[3]P values in Paired Samples Student's t-test when _'s (=basal vs. after i.p.) of data are normally distributed and Wilcoxon Matched-pairs Signed-ranks test (p-values in italics) for _'s of parameters without normal distribution. Significant differences (P<0.0167) are indicated in bold characters.

* Parameters indicated with '*' were ranked in the repeated measures ANOVA due to different variances in the Levene's test.

\# Data indicated with '#' were analyzed non-parametrically with a Kruskal-Wallis test because variances still differed according to the Levene's test after ranking. If these data are represented in italics they were not normally distributed (according to Kolmogorov-Smirnov criteria) as well. Significant effects are indicated in bold characters (P<0.05).

With one exception, all p-values are exact p-values calculated with the Exact-test. Values determined by the Krusal-Wallis test are simulated according to the Monte-Carlo method.

$ dry weight

Since animals were group housed during the experiment, possible effects of testing order was controlled by taking it as co-factor in the analysis of variance. However, for all data the p-values that indicated for this testing order was in all cases 1,000 (data not shown), proving that testing order did not confound the behaviour of the mice in the mHB.

Due to their phenotypical differences and their phylogenetical relation, A/J and B6 mice form an excellent panel for various permanent genetic systems like recombinant inbred (Fortin et al. 2001; Toth et al. 1999) and consomic strains (Bevova et al. 2005; Brockmann & Bevova 2002; Cowley et al. 2003; Cowley et al. 2004a; Cowley et al. 2004b; Fernandez-Teruel et al. 2002; Liang et al. 2002; Nadeau et al. 2000) but also AcB/BcA recombinant congenics and congenic strains form an in demand system for genetics research (Gill & Boyle 2005; Thifault et al. 2001).

In conclusion, B6 and A/J mice differ with respect to almost all motivational systems. Differential analysis of their behavioural response towards an aversive stimulus reveals that the profound strain difference with respect to anxiety related behaviour is a primary characteristic. Therefore, chromosome substitution strains, also called consomics, derived from B6 and A/J as parental strains may be an interesting tool to search for genes that affect anxiety-related behaviour in mice. Due to the found gender differences, with male showing a more pronounced phenotypic strain difference than female mice, future genetic analysis will be performed in male mice. Moreover, the complex statistical calculation necessary to design a quantitative genetic approach will be based on the parameter 'latency to the first board entry' since this anxiety-related parameter appeared to be the most robust of those parameters indicating anxiety in mice.

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Chapter 2

Reducing the number of animals used in behavioural genetic experiments using chromosome substitution strains

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Abstract

Chromosome substitution strains (also called consomic lines or strains) are strains in which a single, full-length chromosome from one inbred strain — the donor strain — has been transferred onto the genetic background of a second inbred strain — the host strain. Based on the results obtained from behavioural tests with the two parental strains, the minimum number of animals from each of the host and consomic strains that are required for a successful behavioural genetic analysis can be estimated. Correct application of statistical knowledge can lead to a further reduction in the number of animals used in behavioural genetic experiments using chromosome substitution strains.

Keywords

Animal welfare, behavioural genetics, chromosome substitution strain, mice, QTL, reduction

Introduction

A quantitative trait locus (QTL) is a position on the genome that is associated with genetic differences for a quantitative trait. Over the past decade, methods for genome analysis of animal models have been developed to identify and locate QTLs. Chromosome substitution strains, also called consomic strains, can accelerate the identification and mapping of QTLs. Chromosome substitution strains are produced by transferring a single, full-length chromosome from one inbred strain — the donor strain — onto the genetic background of a second strain –- the host strain — by repeated backcrossing (Singer et al. 2004). Because the host and donor

strains are genetically very diverse, the consomic panels (a set of chromosome substitution trains) can be used as a general genetic discovery tool. Therefore, panels of chromosome substitution strains are an advantage to researchers studying genes effecting developmental, physiological and behavioural processes. The Division of Laboratory Animal Science, Utrecht University, is specifically interested in behavioural genetic research and plans to use a commercially available set of mouse chromosome substitution strains (van Lith 2005). Determination of the number of animals required per strain of both the host strain and the consomic strain for the genetic analysis is one of the most important and difficult decisions one has to make. Based on the results obtained from behavioural tests with the two parental strains, the minimum number of animals from the host and consomic strains that are required for a successful behavioural genetic analysis can be estimated. This paper demonstrates that, by using statistical knowledge in a correct way, it is possible to reduce the number of laboratory animals used in behavioural genetic experiments using chromosome substitution strains.

Materials & Methods

The protocol of the experiment was approved by the Animal Experiments Committee of the academic Biomedical Centre, Utrecht, and peer-reviewed by the scientific and ethical committee of the Department of Animals, Science & Society, Utrecht University.

Animals

This study was performed using naive male mice from two commercially available mouse inbred strains: A/J, the donor strain, and C57BL/6J, the host strain (n = 9 per strain; The Jackson Laboratory, Bar Harbor, USA). The mice were 4–6 weeks old at arrival, and were housed in a room of the laboratory animal facility at the Department of Animals, Science & society (Utrecht University) for two weeks for habituation before the behavioural testing started; testing was carried out in the same room. The animals were socially housed in Macrolon–2 cages (three mice per cage) and maintained under a reversed light: dark cycle (white light: 1900h–0700h; red light: 0700h–1900h) with food and water available ad libitum. In each cage, animals were provided with a shelter, tissues (Kleenex®: Kimberly–Clark Professional V, Ede, The Netherlands) and paper shreds (EnviroDri®: Tecnilab–BMI BV, Someren, The Netherlands) as environmental enrichment. Humidity was kept at a constant level of 50% and the ambient temperature was maintained at 21.0 ± 2.0°C. During the habituation period all mice were handled at least four times per week for a few minutes by the person who performed the behavioural experiments (MC Laarakker), this included picking up the animal at the tail base and placing it on the hand or arm, and restraining it by hand for a few seconds at random times of the day.

The modified hole–board test

The behavioural tests were performed using a modified hole–board (mHB) test (Figure 1) (Ohl 2003). The mHB test combines the features of an open–field and a hole–board test. In summary, the mHB consists of an opaque grey polyvinylchloride (PVC) box (150 × 50 × 50 cm, length × width × height) that is divided into two compartments by a transparent partition perforated with 20 holes each 3 mm in diameter. The smaller compartment (50 × 50 × 50 cm, length × width x height) is used as a group compartment and is enriched with a tissue (Kleenex®: Kimberly–lark Professional BV, Ede, The Netherlands); all cage mates of the tested animal are placed in this group compartment, allowing the tested animal both olfactory and visible contact in order to prevent isolation stress. The experimental compartment (100 × 50 × 50 cm, length × width × height) consists of two areas, one protected area — the box — which is surrounded by the protective walls of the set-up, and an unprotected area — the board. Black lines divide the box into 10 rectangles (20 × 15 cm, length × width) and 2 squares (20 × 20 cm, length × width). The board (60 × 20 × 0.5 cm, length × width × height) is placed in the centre of the experimental compartment, and contains 23 PVC cylinders (3 × 3 cm, diameter × height), positioned across the board in three rows. The board is lit with an additional red light lamp (80 W), such that the board is illuminated with approximately 35 lux, whereas the box is only illuminated with 1–3 lux. Behavioural testing was performed between 1000h and 1400h under red light conditions; all behavioural tests were videotaped from above the experimental compartment and from the side through the partition wall. At the start of the behavioural test, all three mice from one cage were placed in the group compartment of the mHB for 10 min in order to allow for habituation. The mice were subsequently placed in the experimental compartment one at a time and allowed free exploration for 5 min while a trained observer (MC Laarakker) scored the behaviour by hand, using the program Observer 4.1 (Noldus, Wageningen, The Netherlands). The test set-up was cleaned with water and a damp towel between each mouse.

As in previous studies (Ohl et al 2001; references cited in Ohl 2003), several parameters for anxiety–related behaviour (e.g. latency until the first board entry and number of board entries), locomotion, exploration, risk assessment, memory, arousal, immobility and social affinity were measured. However, only the results for the two parameters 'latency until the first board entry' and 'number of board entries' have been presented here.

Statistical analyses

All statistical analyses were carried out using the SPSS® computer program (version 15.0.1). Two-tailed probabilities were estimated throughout. Continuous data (parameter 'latency until the first board entry') were summarised as means ± standard error of the mean (SEM), whereas discrete data on the ordinal

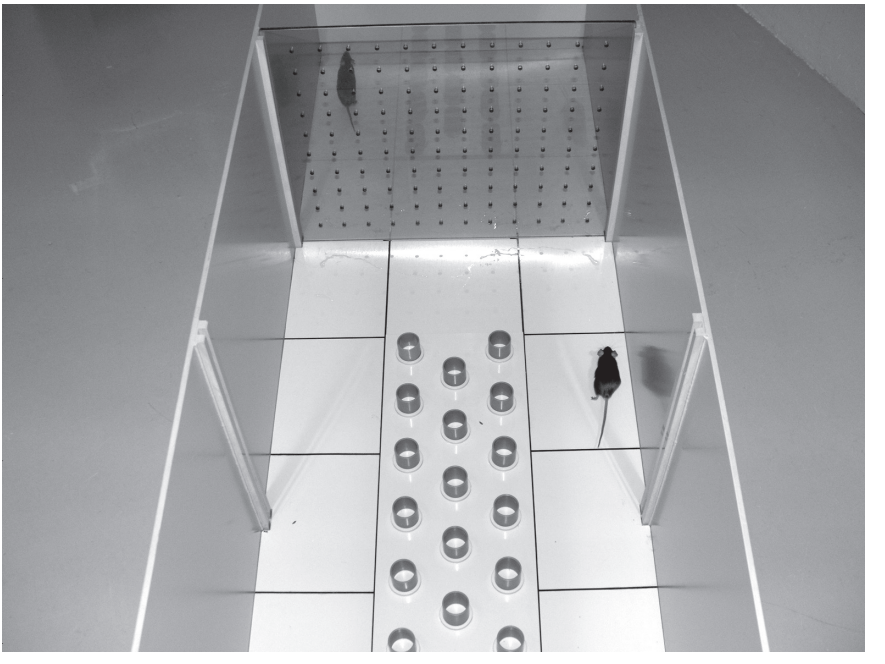

Figure 1 *The modified hole board. The experimental animal is placed in the testing-compartment, containing the hole board in the centre. In case of socially housed mice, the group mates are placed in the group-compartment behind the partition to prevent isolation stress.*

scale (parameter 'number of board entries') were presented as medians with the interquartile range (IQR). The Kolmogorov-Smirnov one-sample test was used to check normality of the continuous data; all continuous results within the A/J and C57BL/6J strains were normally distributed. Significant differences in the continuous data between A/J and C57BL/6J mice were calculated using the unpaired Student's t-test. The unpaired Student's t-tests were performed using pooled (for equal variances) or separate (for unequal variances) variance estimates. The equality of variances was tested using an F test. For the unpaired Student's t-test with separate variance estimates, SPSS uses the Welch correction. The significance of differences for the ordinal data between A/J and C57BL/6J mice was calculated using the Mann-Whitney U test.

Results

Parental strains

Nine mice from the strains A/J and C57BL/6J were used as donor and host strains respectively. Based on former mouse strain comparisons (Ohl et al. 2001) two discriminating parameters were selected: (1) latency until the first board entry and (2) number of board entries. Significant differences between the two parental strains were found for both parameters (see below). The obtained strain differences prompted the investigation into the chromosomal location of the QTL involved by testing a set of chromosome substitution strains between the A/J and C57BL/6J strains.

Latency until first board entry

The parameter 'latency until the first board entry' was considered to be a continuous variable. The mean ± standard error of the mean (SEM) for this parameter were:

C57BL/6J = 43.5 ± 13.7 s (n = 9, all male);
A/J = 203.3 ± 38.7 s (n = 9, all male).

Using a two-tailed unpaired Student's t-test with Welch correction, there was a significant difference between the two strains and the parameter 'latency until the first board entry', t = 3.89, df = 9.97, P = 0.003.

Using two standard behavioural tests, the open–field test and the dark–light transmission test, Singer et al. (2004) found that there are probably 3–4 non-linked (i.e. located on different chromosomes) QTLs present per parameter. If the effects of these QTLs are assumed to be additive and that every QTL has the same magnitude of effect, then this means that a chromosome substitution strain with n = 9 animals (all male) can have a mean of 83.5 ± 20.0 s, assuming that the four QTLs are non-linked. The mean value for a consomic strain containing one QTL

$$43.5 + \left(\frac{203.3 - 43.5}{4} \right) \approx 83.5$$

was calculated using the following equation (and see Figure 2):

The standard error of the mean (based on n = 9) for a consomic strain with one

$$13.7 + \left(\left[\frac{38.7 - 13.7}{203.3 - 43.5} \right] \times (83.5 - 43.5) \right) \approx 20.0$$

QTL (linear interpolation; Figure 3) was calculated as follows:

In order to compare the chromosome substitution strain with its host strain, α has to be adjusted because of a greater probability of a Type 1 error attributable to multiple comparisons. Belknap (2003) proposes a value of between 0.003 and 0.004. A chromosome substitution strain cannot be expected to differ significantly from the host strain (C57BL/6J) with respect to the parameter 'latency until the first board entry' when n = 9 animals per strain are used (two-tailed Student's t-test, t = 1.65, df = 16, P = 0.119). Therefore, in order to obtain a significant result, the number of animals (n) was increased. If n is doubled, the results for the host strain and the consomic strain (e.g. for chromosome 1) become:

C57BL/6J = 43.5 ± 9.4 s (n = 18, all male);
C57BL/6J–Chr1A/NaJ (the consomic strain) = 83.5 ± 13.7 s (n = 18, all male).

Using an unpaired two-tailed Student's t-test with Welch correction, P = 0.022 (t = 2.40, df = 34), which, according to Belknap (2003), was not significant. The results were statistically significant when n = 27 animals per strain were used, when P = 0.004 (t = 2.97, df = 52). The predicted results were: C57BL/6J = 43.5 ± 7.6 s (n = 27, all male); C57BL/6J–Chr1A/NaJ = 83.5 ± 11.1 s (n = 27, all male). This suggested that behavioural testing should start with 27 mice of the C57BL/6J host strain and 6 mice per chromosome substitution strain, that is, to start with a ratio of 4.5:1 (27:6), as suggested by Belknap (2003). This experiment predicted the following results:

C57BL/6J = 43.5 ± 7.6 s (n = 27, all male);
C57BL/6J–Chr1A/NaJ = 83.5 ± 25.3 s (n = 6, all male);
P = 0.053 (t = 2.02, df = 31).

With a value of P < 0.053, then for the chromosome substitution strain for which this is the case (assuming 4 consomic strains are found, and using the parameter 'latency until the first board entry'), 21 additional male animals would be tested. After behavioural testing, the statistical analysis would be repeated, but with 27 animals for both the chromosome substitution strain and the host strain (C57BL/6J), it is most likely that a value close to P = 0.004 will be obtained.

Board entries

The parameter 'board entries' was considered to be a discrete variable on the ordinal scale. See Table 1 for the number of board entries made by the A/J and C57BL/6J mice, and the median with the IQR. For this parameter the two inbred strains were significantly different (two-tailed Mann-Whitney U test, U = 7.5, P = 0.0032). Assuming there were also four QTLs with equal affects involved for this parameter then the results for the chromosome substitution strain and the host

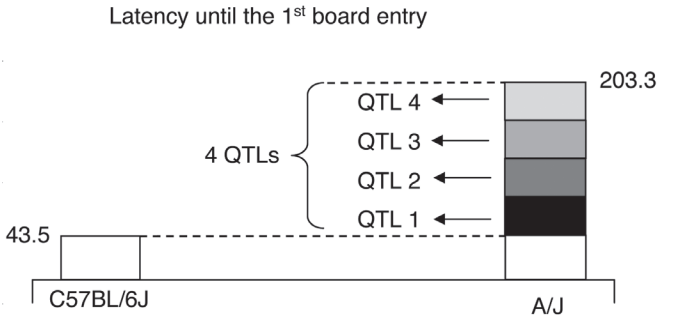

Figure 2 *Calculation of the mean value for a consomic line containing one QTL using the behavioural data obtained for the parameter 'latency until the first board entry'.*

strain could be calculated (see Table 2).

Estimating the median for a consomic strain with one QTL was calculated as follows:

$$\left(\frac{14 - 0}{4} \right) = 3.5$$

Because an odd number of animals was used, the median was either 3 or 4. Considering the most unfavourable situation (which means the difference between host strain and consomic strain is smaller) a median of 4 was chosen. The IQR was estimated as follows:

$$5.5 + \left(4 \left[\frac{8.5 - 5.5}{14} \right] \right) \approx 6.36$$

Because the numbers of board entries were counts and nine animals were used, the IQR became 6.25 or 6.5; therefore, similar to 6.36. Again, the most

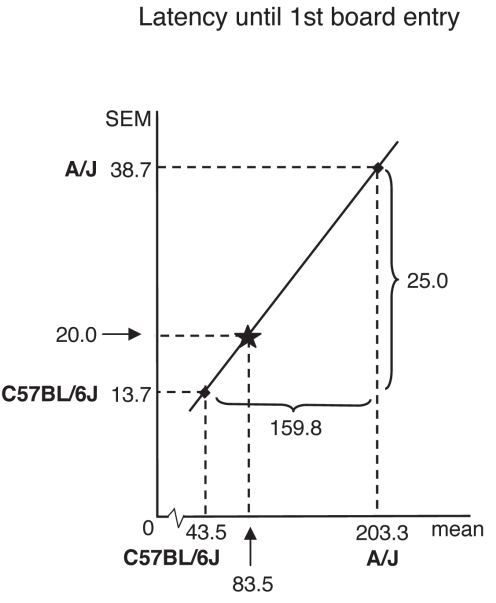

Figure 3 *Calculation of the standard error of the mean for a consomic line with a QTL using the behavioural data obtained for the parameter 'latency until the first board entry'.*

Table 1 *The number of board entries made by A/J and C57BL/6J male mice.*

A/J (n = 9, ♂♂)	C57BL/6J (n = 9, ♂♂)
0	1
0	6
0	10
0	12
0	14
4	15
5	16
6	17
13	20
median 0	*14*
IQR 5.5	*8.5*

Table 2 *The number of board entries of consomic mice (C57BL/6J-Chr19A/NaJ) and C57BL/6J male mice.*

	C57BL/6J-1^{A}/NaJ (n = 9)	C57BL/6J (n = 9)
	0	1
	2	6
	3	10
	3	12
	4	14
	4	15
	6	16
	12	17
	15	20
median	*4*	*14*
IQR	*6.5*	*8.5*

Although it is not correct for discrete data, the mean and SEM for the parameter 'number of board entries' were calculated to guarantee comparability of the shape of the distribution between the A/J, consomic and C57BL/6J strain: for the A/J strain, mean ± SEM = 3.1 ± 1.5 and for the C57BL/6J 12.3 ± 2.0; a chromosome substitution strain could be 5.4 ± 1.6.

The mean for a consomic strain containing one QTL (Figure 4) was calculated as follows:

$$3.1 + \left(\frac{12.3 - 3.1}{4} \right) = 5.4$$

The SEM for a consomic strain containing one QTL (linear interpolation; Figure 5) was calculated as follows:

$$1.5 + \left(\left[\frac{2.0 - 1.5}{12.3 - 3.1} \right] (5.4 - 3.1) \right) \approx 1.6$$

It appears that with the values presented in Table 2 for the C57BL/6J–Chr1A/NaJ (n = 9) consomic strain, the preferred results were obtained; Mann-Whitney U test, U = 15.5, P = 0.0269. Using 9 consomic animals and 27 host strain animals (median = 14; IQR = 6.0), P = 0.0060. If 27 consomic animals (median = 14, IQR = 3.0) and 27 host strain animals (median = 14, IQR = 6.0) were tested (see section 'Latency until the first board entry'), P = 0.0001 (U = 139.5). For this parameter (data were analysed using the Mann-Whitney U test), α was also adjusted because of the increased probability of a Type 1 error. Here as well, the criteria of Belknap

(2003) can be used ($P < 0.004$). Applying these rules, and using 27 consomic and 27 host strain animals for this parameter would satisfy the criteria. The criteria would not be satisfied using 9 consomic and 27 host strain animals ($U = 46.5$, $P = 0.006$); therefore, our suggestion is to use 12 consomic mice for this parameter.

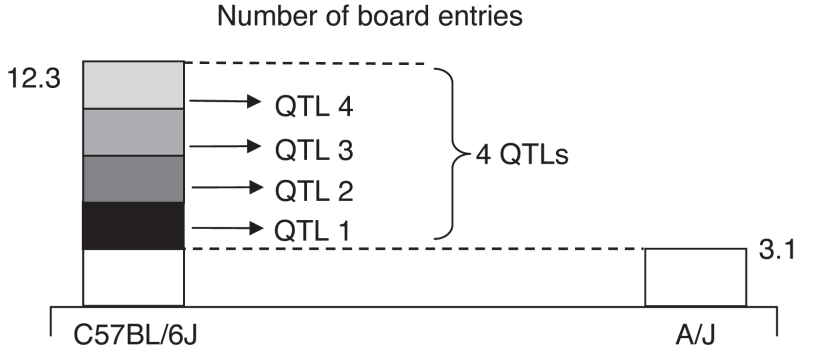

Figure 4 *Calculation of the mean value for a consomic line containing a QTL using the behavioural data obtained for the parameter 'number of board entries'.*

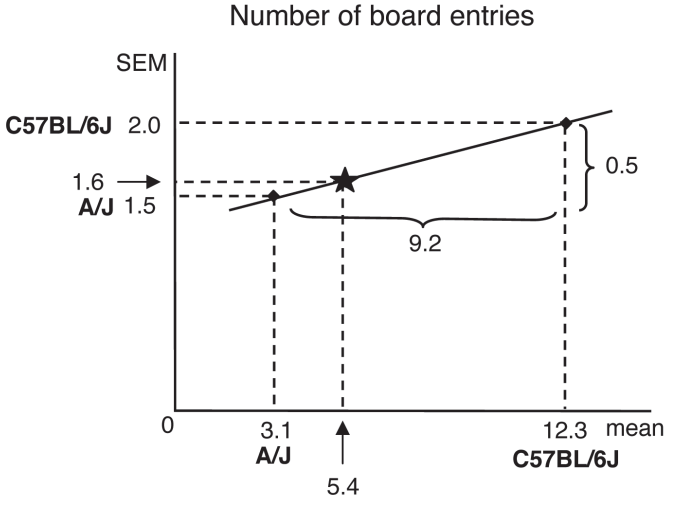

Figure 5 *Calculation of the standard error of the mean for a consomic line with a QTL using the behavioural data obtained for the parameter 'number of board entries'.*

Discussion

When more than one statistical test is performed when analysing data from a study, statisticians demand that a more stringent criterion is used for statistical significance than the conventional $P < 0.05$. As previously explained in the section 'Latency until the first board entry', multiple comparisons (comparing consomic strains with the host strain) were carried out, therefore there was an increased probability of a Type 1 error. A method to adjust for multiple tests is known as the Bonferroni adjustment: alpha is divided by the number of comparisons that are carried out, and for example, Singer et al. 2004 and Singer et al. 2005 use this method. A complete set of chromosome substitution strains of the mouse consists of 21 different strains (19 autosomes, the X and Y chromosome). Because we plan to compare all 21 available consomic strains with the host strain, the adapted α of Singer's method would give a value of ≈ 0.0024 (Singer et al. 2004; Singer et al. 2005).

Belknap's method (2003) is actually based on the method developed by Dunnett (1955) and applies to the situation where different test groups (in this case the consomic strains) are compared to only one control group (in this case the host strain). For example, the continuous parameter 'latency until the first board entry', an α of 0.004 (suggested by Belknap 2003) when compared to $\alpha = 0.0024$ (suggested by Singer et al. 2004; Singer et al. 2005) implies a reduction in the total number of animals that have to be tested (726 versus 594 animals). A further reduction in the number of laboratory animals used in such an experiment would be possible if the behavioural tests were started with 27 C57BL/6J host strain animals and 6 animals per consomic strain (according to Belknap, 2003 a 4.5:1, or 27:6, ratio is the most efficient) and extra animals (21 in case of the parameter 'latency until the first board entry' and 6 in case of the parameter 'board entry') of the appropriate consomic strains were only tested if $P < 0.05$. Table 3 shows an overview of the number of animals used for testing the parameter 'latency until the first board entry'. The reduction in the number of laboratory animals can be derived from this.

The two parameters 'latency until the first board entry' and 'number of board entries' are related to each other (Spearman's coefficient of rank correlation, $Rs = -0.9215$, $n = 18$, $P = 0.000$); therefore, a multivariate method, such as Hotelling's T2 test, could have been used. Furthermore, Turri et al. (2004) demonstrated that multivariate analysis, when compared to univariate analysis, has an increased power to detect QTLs when the genetic effects are correlated. However, several assumptions are necessary for proper application of the Hotelling's T2 test. One of the assumptions is that dependent variables should have a multivariate normal distribution. Because 'latency until the first board entry' is a continuous variable and 'number of board entries' is a discrete variable, the joint distribution can never be bivariate normal; therefore we have not attempted to analyse these two parameters jointly.

Based on the results obtained from the behavioural tests using the two parental strains, the minimum number of animals from the host and consomic strains that are needed for a successful behavioural genetic analysis can be estimated. By adjusting α to 0.004 (Belknap 2003), and performing a multiple phase approach (our suggestion), a reduction in the number of animals used in these experiments can be obtained. The multiple phase approach that we suggest is in fact a form of sequential analysis, which Russell and Burch (1959, reprinted 1992) had already suggested in 1959 as one method of reducing the number of animals used in experiments.

Table 3 *Overview of the number of laboratory animals for the parameter 'latency' until the first board entry' using different statistical methods*

α = 0.0024	α = 0.004	α = 0.004 + multiple phase approach
(Nadeau)	(Belknap)	(This article)
726 (33 x 22)	594 (27 x 22)	237 (27 + 6 x 21 + 21 x 4)

Acknowledgements

The authors would like to thank two anonymous referees for their useful comments on the manuscript.

Box 1

Number of mice needed for a breeding experiment for later QTL analysis using chromosome substitution strains

F_2-population size to generate a 5cM map

Using a backcross population of 380 animals, DNA-marker can be classified as being linked, if the genetic distance is 40 cM or less. The LOD-score is then still bigger than 3.0 (= threshold value for linkage) namely 3,022. An F_2-population gives (with respect to mouse chromosome 19) twice as much informative meioses compared to a backcross population of the same size (Lander & Botstein 1989). This means that 380 backcross animals correspond to 190 F_2 animals.
A distance of 5 cM between markers (i.e. DNA-markers M and N) means that when an F_2-population will be used, the following genotypic proportions have to be found (M_1M_1 / M_2M_2 = genotype C57BL/6J; N_1N_1 / N_2N_2 = genotype C57BL/6J-Chr19A/NaJ):

M_1M_1 / M_2M_2:	0,225625 (43)
M_1M_1 / M_2N_2:	0,02375 (4)
M_1M_1 / N_2N_2:	0,000625 (0)
M_1M_1 / M_2M_2:	0,02375 (5)
M_1N_1 / M_2N_2:	0,4525 (86)
M_1N_1 / N_2N_2:	0,02375 (4)
N_1N_1 / M_2M_2:	0,000625 (0)
N_1N_1 / M_2N_2:	0,02375 (5)
N_1N_1 / N_2N_2:	0,225625 (43)

The number of animals that can theoretically be found in a F_2-population of 190 animals is shown in brackets. It has to be noticed that the genotypes M_1M_1 / N_2N_2 and N_1N_1 / M_2M_2 are not found. When the calculation of the distance between the two markers using the computer program JoinMap® is based on these numbers, the genetic distance between M and N is about 5 cM. Concluding, a F_2-population of 190 animals is large enough to create a 5 cM genetic-map of mouse chromosome 19.

Phenotypic difference between host- and consomic strain

Male animals of the C57BL/6J (n = 6) mouse inbred strain and the C57BL/6J-Chr19A/NaJ consomic strain were recently tested in the modified hole board. Using this test set-up, among others the parameter 'relative duration on the board' was determined. In the modified hole board test, this parameter is indicative for the phenomenon 'anxiety'. The phenotypic difference D for the parameter 'relative duration on the board' between host strain and consomic strain was 1.2 (expressed in environmental standard-deviations).

F2-population size to detect a QTL

Using the article of Lander & Botstein (1989) and based on the assumption that the genetic map of mouse chromosome 19 has a marker density of 5 cM, it can be calculated that (with 90% chance that a QTL is detected) a minimum of 185 F_2 animals are necessary (see also figures 4 and 7b Lander & Botstein 1989).

LOD threshold:	1,68
D = difference between strains (in SDs):	1,2

Number of backcross progeny:	31,18
Recombination fraction between QTL and either marker (= u):	0,0256584
Recombination fraction between the two markers (= w:	0,05 (= 5 cM)
Power:	90%
Transfer from backcross to F_2-intercross (based on MMU19):	0,5

Number of F_1-animals

C57BL/6J mice usually have nests with offspring of 3-11 animals. An average of six mice are born per nest. If the F_2-population counts 192 animals, 192/6= 32 nests are necessary. This means that 32 male and 32 female F_1 animals are needed since F_1 brother-sister mating forms the F2-generation. If there were 11 nests of F_1 animals produced we would probably have enough F_1 male and female mice to create the F_2 generation. However, to correct for epigenetic effects, a reciprocal cross between the C57BL/6J host strain and the C57BL/6J-Chr19A/NaJ consomic strain has to be performed. That's why our proposal is (based on an even number of nests per group) to produce 12 nests with F_1 animals:

C57BL/6J-♂ x C57BL/6J-Chr19^A/NaJ-♀
(n = 6) (n = 6)

C57BL/6J-♀ x C57BL/6J-Chr19^A/NaJ-♂
(n = 6) (n = 6)

The expectation is that the cross, described above, will generate the following numbers of F1 animals:

36 animals of the type (C57BL/6J x C57BL/6J-Chr19^A/NaJ)F_1
36 animals of the type (C57BL/6J-Chr19A/NaJ x C57BL/6J)F_1

It is also our intention to phenotype this F1-generation in the modified hole board test.

In summery

To localize a QTL on mouse chromosome 19, the following numbers of animals are needed:

C57BL/6J-♂	6
C57BL/6J-♀	6
C57BL/6J-Chr19A/NaJ-♂	6
C57BL/6J-Chr19A/NaJ-♀	6
(C57BL/6J x C57BL/6J-Chr19A/NaJ)F_1	36
(C57BL/6J-Chr19A/NaJ x C57BL/6J)F_1	36

1st breeding round F_2:

(C57BL/6J x C57BL/6J-Chr19^A/NaJ)F_1 x (C57BL/6J x C57BL/6J-Chr19^A/NaJ)F_1	48
(C57BL/6J-Chr19^A/NaJ x C57BL/6J)F_1 x (C57BL/6J-Chr19^A/NaJ x C57BL/6J)F_1	48

2nd breeding round F_2:

(C57BL/6J x C57BL/6J-Chr19^A/NaJ)F_1 x (C57BL/6J x C57BL/6J-Chr19^A/NaJ)F_1	48
(C57BL/6J-Chr19A/NaJ x C57BL/6J)F_1 x (C57BL/6J-Chr19^A/NaJ x C57BL/6J)F_1	48

This ends up in a total of **288** mice.

Chapter 3

Chromosomal assignment of quantitative trait loci influencing modified hole board behaviour in laboratory mice using consomic strains, with special reference to anxiety related behaviour on mouse chromosome 19

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Frauke Ohl
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Behavior Genetics 2008, 38(2):159-184

Abstract

Male mice from a panel of chromosome substitution strains (CSS, also called consomic strains or lines) – in which a single full-length chromosome from the A/J inbred strain has been transferred onto the genetic background of the C57BL/6J inbred strain – and the parental strains were examined in the modified hole board test. This behavioural test allows assessing for a variety of different motivational systems in parallel (i.e. anxiety, risk assessment, exploration, memory, locomotion, and arousal). Such an approach is essential for behavioural characterization since the motivational system of interest is strongly influenced by other behavioural systems. Both, univariate and bivariate analyses, as well as a factor analysis, were performed. The C57BL/6J and A/J mouse parental inbred strains differed in almost all motivational systems. The chromosome substitution strain survey indicated that nearly all mouse chromosomes (with the exception of chromosome 2) each contain at least one quantitative trait locus (QTL) that is involved in modified hole board behaviour. The results agreed well with previous reports of QTLs for anxiety-related behaviour using the A/J and C57BL/6J as parental strains. The present study confirmed that mouse chromosomes 5, 8, 10, 15, 18 and 19 likely contain at least one anxiety QTL. There was also evidence for a novel anxiety QTL on the Y chromosome. With respect to anxiety-related avoidance behaviour towards an unprotected area, we have special interest for mouse chromosome 19. CSS-19 (C57BL/6J-Chr19^{A}/NaJ) differed in avoidance behaviour from the C57BL/6J, but not in locomotion. Thus pleiotropic contribution of locomotion could be excluded.

Keywords

Anxiety, A/J, C57BL/6J, chromosome substitution strain, consomic strain, mice, modified hole board test, factor analysis, bivariate– and univariate analysis.

Introduction

Anxiety and other psychiatric disorders are one of the most common diseases in humans. Kessler et al. (2005) found that about 29% of the U.S. population develops an anxiety disorder sometime during their life. Anxiety is a multidimensional phenomenon presumed to have a complex inheritance, involving the interaction of multiple genes in combination with epigenetic and environmental factors. Family, linkage and twin studies have consistently indicated that genes indeed play a role in the etiology of anxiety disorders; the heritability has been estimated to be 30-50% (Gordon & Hen 2004). Unfortunately, attempts to find these human genes have been largely unsuccessful. Therefore, animal models of anxiety were developed to facilitate the discovery of the genetic and neurobiological substrates of anxiety and test putative anxiolytic drugs (Ohl 2004).

Over the past decade, methods for genome analysis of animal models have been developed to identify and locate QTLs (Flint et al. 2005). Chromosome substitution strains (CSS, also called consomic strains or lines) (Sansom 2005; http://www.informatics.jax.org/ mgihome/nomen/strains.shtml#consomic; http://www.rgd.mcw.edu/ nomen/ rules-for-nomen. sht ml#consomic), represents a relatively new strategy and can accelerate the identification and mapping of QTLs. Chromosome substitution strains are produced by transferring a single, full-length chromosome from one inbred strain – the donor strain – onto the genetic background of a second strain – the host strain – by repeated backcrossing (Singer et al. 2004). Because the host and donor strain are genetically very diverse, the consomic panels can be used as a general genetic discovery tool. Therefore, panels of chromosome substitution strains are an advantage to researchers studying the genes affecting developmental, physiological and behavioural processes.

The first complete mouse CSS set, created from A/J and C57BL/6J strains, was produced in 2004 (Singer et al. 2004). The two parental inbred strains from this consomic panel are frequently used in anxiety research (Bouwknecht & Paylor 2002) and they also differ in terms of sensitivity for benzodiazepines (Mathis et al. 1995). Trullas & Skolnick (1993) ranked sixteen inbred strains of mice on anxiety-related phenotypes; the A/J and C57BL/6J strains were found to be at opposite ends of the phenotypic spectrum. The A/J strain has been identified as one of the most anxiogenic-like strain across a number of paradigms.

The Division of Laboratory Animal Science, Utrecht University is specifically interested in the identification of genetic factors underlying the development of (pathological) anxiety. Here we report that anxiety-related behaviour, when the mice were tested in the modified hole board test (Ohl 2004), differs between two inbred strains of mice (C57BL/6J and A/J), and the use of CSS generated from these two strains to identify chromosomes that harbor QTL that influence anxiety-related behaviour. (Singer et al. 2004; Singer et al. 2005) already examined this panel of CSS using other ethological tests of anxiety-like behaviour (open field and light-dark box). However in contrast to the open field, the light-dark box as well as the elevated plus maze, the modified hole board test is a complex behavioural test for rodents, that allows for the assessment of a variety of different

motivational systems in parallel (Ohl 2004; Ohl et al. 2001a). Turri et al. (2004) demonstrated that multivariate analysis when compared to univariate analysis, has an increased power to detect QTLs when the genetic effects are correlated. Since the anxiety-related behavioural parameters of the modified hole board were related to each other (Laarakker et al. 2006) we performed both univariate and bivariate statistical analyses. Previous QTL analyses, using A/J and C57BL/6J as parental strains and different mapping populations, suggest that mouse chromosome 19 plays a significant role in anxiety-related behaviour (Chesler et al. 2005; Gershenfeld et al. 1997; Gershenfeld & Paul 1998; Gill & Boyle 2005; Singer et al. 2004; Singer et al. 2005; Zhang et al. 2005). Therefore this study focused on mouse chromosome 19.

Materials & Methods

Ethical note

The protocols of the experiments were peer-reviewed by the scientific committee of the Department of Animals, Science & Society, Utrecht University, the Netherlands, and approved by the Animal Experiments Committee of the Academic Biomedical Centre, Utrecht-The Netherlands. The Animal Experiments Committee based its decision on 'De Wet op de Dierproeven' (The Dutch 'Experiments on Animals Act', 1996) and on the 'Dierproevenbesluit' (The Dutch 'Experiments on Animals Decision', 1996); both are available online (http://www.vet.uu.nl/nca_nl/legislation or http://wetten.overheid.nl/). Further, all animal experiments followed the 'Principles of laboratory animal care' and refer to the 'Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research' (National Research Council, 2003).

Animals and housing

This study was performed using naive male mice from the following commercially available inbred strains: A/J (the donor strain; n = 30), C57BL/6J (the host strain; n = 27), and the complete set of chromosome substitution strains between these parental strains (n = 6 per consomic line), obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All the strains were sampled on several litters in homogenized environmental conditions; the observed phenotypes were highly stable through litters and generations. The chromosome substitution strains, whose nomenclature is C57BL/6J-Chr#^{A}/NaJ, are simplified to CSS-#. For CSS-19 twenty-one extra male mice were tested. We tested more host strain animals when compared to consomic mice (except for CSS-19) to improve power to detect a chromosome that contains a QTL. According to Belknap (2003) a 4.5:1 ratio, or twenty-seven C57BL/6J host strain animals and six animals per consomic strain, is the most efficient for selecting chromosome substitution strains that contain a QTL.

The mice were 4-6 weeks old at arrival, and were housed for two weeks (pre-experimental period) for habituation in an animal room of the laboratory animal facility at the Department of Animals, Science & Society (Utrecht University) before the behavioural testing started. The animal room was sound-attenuated. Relative humidity was kept at a constant level of 50%, the ambient temperature was maintained at 21.0 ± 2.0oC and the ventilation rate was 15-20 air changes per hour. To reduce stress in the laboratory animal facility, during the whole day (24 hours) radio-sound (60 ± 3 dB) was provided. In addition conversational radio-sound (e.g. talk-shows) may accustom the animal to the human voice. Behavioural testing (modified hole board test; see below) was carried out in the same room. All mice were housed individually directly after arrival in enriched, wire topped Macrolon® Type II L (prolonged) cages (size: 365 x 207 x 140 mm, floor area 530 cm2; Techniplast, Milan, Italy). Enrichment, besides standard bedding material, included a shelter, a tissue (Kleenex®: Kimberly-Clark Professional BV, Ede, The Netherlands) and a small amount (less than a hand full) of paper shreds (EnviroDri®: Technilab-BMI BV, Someren, The Netherlands). Drinking water and standard laboratory food pellets were provided ad libitum. The light:dark cycle was reversed (white light: 1900h – 0700h, maximal 150 lux; red light: 0700h – 1900h, maximal 5 lux).

During the habituation period all mice were handled at least three times per week for a few minutes by the person who performed the behavioural testing (MC Laarakker), this included picking up the animal at the tail base and placing it on the hand or arm, and restraining it by hand for a few seconds at random times of the day.

Modified hole board testing

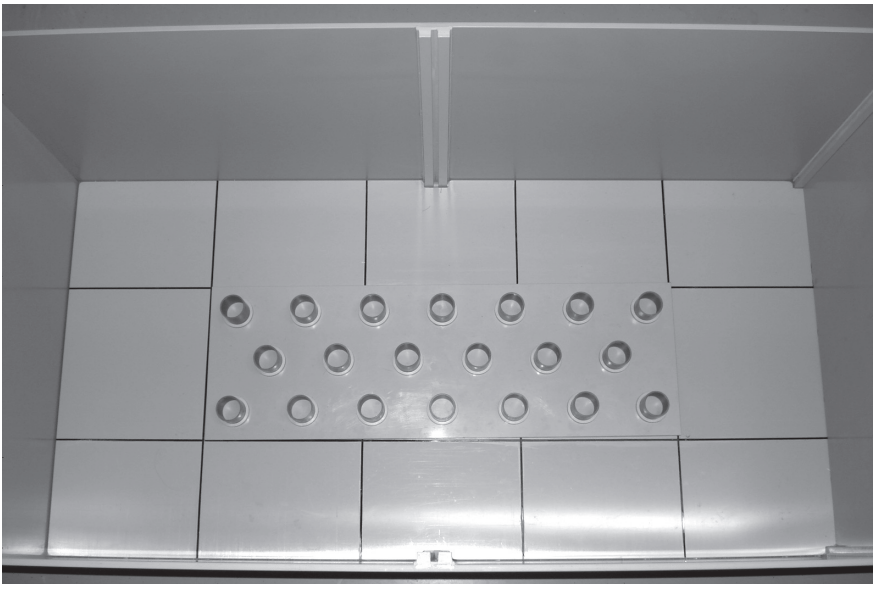
The behavioural testing was performed using the modified hole board test (Figure 1). This 5-min test combines the features of an open field and a hole board. It allows for testing a variety of motivational systems in parallel (Ohl 2003). The modified hole board basically consists of an opaque grey polyvinylchloride (PVC) box (100 x 50 x 50 cm, length x width x height) which consists of two areas, one protected area - the box - which is surrounded by the protective walls of the set-up, and an unprotected area - the board. Black lines divide the box into 10 rectangles (20 x 15 cm, length x width) and 2 squares (20 x 20 cm, length x width). The board (60 x 20 x 0.5 cm, length x width x height) is placed in the centre of the box, and contains 20 PVC cylinders (3 x 3 cm, diameter x height), positioned across the board in three intended rows. The board is lit with an additional red light lamp (80 W), in such way that the board is illuminated with approximately 35 lux, whereas the box is only illuminated with 1-3 lux. The familiar and an unfamiliar object (either a die or a screw nut, depending on what was used as familiar object) are placed in one corner of the box of the modified hole board set-up; this is done in a way that both have the same distance to the wall and that the mouse can still pass along freely.

Behavioural testing was performed between 1000h and 1400h (i.e. during the activity phase of the animals) under red-light conditions; all behavioural tests were videotaped (for raw data storage) from above the box. The behaviour was scored by hand using the program Observer 4.1 (Noldus, Wageningen, The Netherlands). The test set-up was cleaned with water and a damp towel between each mouse. Several parameters (Table 1) for anxiety-related behaviour, risk assessment, (undirected and directed) exploration, memory, locomotion, arousal and other behaviour (e.g. urination) were measured/calculated.

Table 1 *Behavioural parameters measured in the modified hole board*

Behavioural dimension	Behavioural parameter
Anxiety	Total number of board entries
	Latency until the first board entry
	Percentage of time on the board
	Average duration of a board entry
Risk assessment	Total number of risk assessments
	Latency until the first risk assessment
Undirected exploration	Total number of rearing in the box
	Latency until the first rearing in the box
	Total number of rearing on the board
	Latency until the first rearing on the board
	Total number of hole explorations
	Latency until the first hole exploration
Directed exploration	Total number of holes visited
	Latency until the first hole visited
	Total number of unfamiliar object explorations
	Latency until the first unfamiliar object exploration

Figure 1 *The modified hole board test.*



Description of the mouse behaviour
Mouse on the board
Stretched body posture, including hind-paws
Rearing on hind-paws in the box
Rearing on hind-paws on the board
Exploration of a cylinder (hole) on the board
Nose-poking into a cylinder (hole) on the board
Exploration of the unfamiliar (new) object

[Table 1 continued]

Behavioural dimension	Behavioural parameter
	Percentage of time being busy with unfamiliar object
Memory	Total number of familiar object explorations
	Latency until the first familiar object exploration
	Percentage of time being busy with familiar object exploration
	Average duration of a familiar object exploration
Locomotion	Total number of line crossings
	Latency until the first line crossing
Arousal	Total number of groomings
	Latency until the first grooming
	Percentage of time being busy with grooming
	Average duration of a grooming
	Total number of boli
	Latency until the first bolus is produced
Other behaviour	Number of times the mouse is in the box
	Percentage of time in the box
	Average duration of a stay in the box
	Total number of urinations
	Latency until the first time urine is produced

Statistical analyses

All statistical analyses were carried out according to Petrie and Watson (1999) and/or Quinn & Keough (2002), using a SPSS® for Windows (version 12.0.1) computer program (SPSS Inc., IL, USA). Two-sided, exact (i.e. for the non-parametric tests) probabilities were estimated throughout. Continuous data (latency, percentage of time, and average duration of the behavioural parameters) were summarized (both in tables and in Figures) as means with standard error of the mean (SEM), whereas discrete data on the ordinal scale (total number of the behavioural parameters) were presented in tables as medians with the interquartile range (IQR) and in Figures as box plots (also known as box-and-whisker plots). Box plots show median values with interquartile range, highest and lowest non-outlying values (i.e. values up to 1.5 box lengths from the upper or lower edge of the box). In the Figures with box plots (mild) outliers (i.e. cases with values

Description of the mouse behaviour
Exploration of the familiar object
Line crossing with all its paws in the box
Self-grooming
Defecation
Mouse is in the box
Urination

between 1.5 and 3 box lengths from the upper or lower edge of the box) and extreme cases (i.e. cases with values more than 3 box lengths from the upper or lower edge of the box) are also indicated. Latency of the behavioural parameters is a time to an event parameter and therefore it was also analyzed as survival data; the results were then plotted as Kaplan-Meier curves. The Kolmogorov-Smirnov one sample test was used to check Gaussianity of the continuous data. Group (= strain) analyses using the Kolmogorov-Smirnov test revealed a non-parametric distribution of several continuous parameters for some strains.

Significant differences in the normally distributed continuous data between C57BL/6J and A/J or each consomic strain was calculated using the unpaired Student's t test (univariate analysis) or Hotelling's T2 test (bivariate analysis). The unpaired Student's t-tests were performed using pooled (for equal variances) or separate (for unequal variances) variance estimates. Homoscedasticity was tested

using the Levene's test, which is a powerful and robust test based on the F statistic (Lim and Loh 1996). For the unpaired Student's t-test with separate variance estimates, SPSS® uses the Welch-Satterthwaite correction. The significance of differences for the ordinal data (= total number of the behavioural parameters) as well as the non-normally distributed continuous behavioural data were calculated using the Mann-Whitney U test. The Kaplan-Meier plots for latency of the behavioural parameters were compared using the Log-Rank test. Between behavioural parameters Spearman's coefficients of rank correlation (Rs) were calculated; significance was assessed by a two-tailed test based on the t statistic.

In addition, data were also analyzed by factor analysis using a principal components solution with orthogonal rotation (varimax) of the factor matrix. This method ensures that the extracted factors are independent of one other and should, therefore, reflect separate processes. The varimax algorithm was chosen, because this algorithm attempts to minimize the number of variables that have high loadings (see hereafter) on a factor. This should enhance the interpretability of the factors. The sampling adequacy was measured with the Kaiser-Meyer-Olkin measure (should be greater than 0.5). The Bartlett's test of sphericity was used for testing whether the correlation was appropriate for factor analysis. Factor pattern matrices were identified using a combination of the Kaiser criterion (factors must have eigenvalues ≥ 1) and the Scree test (on a simple line plot, the point of inflection of a plot of the eigenvalues from largest to smallest). The factor loading of each behavioural item indicates how well that item correlates with the factor; thus a loading of ±1.0 indicates a perfect (positive/negative) correlation, whereas a loading of less then 0.6 would suggest that the item is rather weakly linked to the factor. In the next step, via regression, factor scores were calculated for each mouse. The extracted, orthogonal factors were compared by the unpaired Student's t test (normally distributed data) or the Mann-Whitney U test (non-normally distributed data). A one-way analysis of variance with strain as main factor was carried out for each extracted, orthogonal factor across all 20 consomic strains and the host strain. R2, the sum of squares between strains divided by the total sum of squares, gives an estimate of the heritability of these factors (h2), or the proportion of the trait variance due to additive genetic influences (narrow sense heritability) (Belknap 2003).

Recently we suggested, for behavioural genetic experiments using chromosome substitution strains, a limited type of sequential design – the two-stage approach (Laarakker et al. 2006). Briefly, we propose to start the behavioural tests with twenty-seven C57BL/6J host strain animals and six animals per consomic strain. If the P value < 0.05 for the host versus consomic mice comparison, then it makes sense to test extra animals (n = 21) of the appropriate consomic strains. To take into account the greater probability of a Type I error (= erroneously conclude the presence of a significant strain difference) due to the multiple strain comparisons (i.e. host strain versus donor strain or consomic lines), the level of significance for the Student's t-tests, Hotelling's T2 tests, the Mann-Whitney U tests, and Log-Rank tests was pre-set at P < 0.004 (as suggested by Belknap, 2003). By adjusting

α to 0.004 and performing a two-stage approach, a reduction in the number of animals used in these experiments can be obtained (Laarakker et al. 2006).

However, it is well recognized that when one tests multiple hypotheses, all bearing on a single issue (e.g. a behavioural dimension), a more stringent criterion should be used for statistical significance. We approached this problem by calculating for each behavioural dimension separate so-called Dunn-Šidák corrections ($\alpha = 1 - [1 - 0.004]^{1/\gamma}$; γ = number of parameters per behavioural dimension). We did not use a highly conservative overall Bonferroni correction ($\alpha = 0.004/1171 \approx 0.000003$), because of the large numbers of tests (1171). This implies that for the comparisons (i.e. for the behavioural measures) with twenty-seven mice of the C57BL/6J host strain versus six mice per chromosome substitution strain $1 - [1 - 0.004]^{1/\gamma} \leq P < 1 - [1 - 0.05]^{1/\gamma}$ means suggestive evidence for a chromosome harbouring a QTL, whereas $P < 1 - [1 - 0.004]^{1/\gamma}$ means significant evidence for a QTL on a chromosome (Belknap 2003). For the extracted, orthogonal factors $0.004 \leq P < 0.05$ and $P < 0.004$ means suggestive and significant evidence, respectively. Table 2 gives an overview of the (corrected) thresholds used in the multiple strain comparisons.

Calculating numerous correlations also increases the risk of a Type I error. To avoid this, the level of statistical significance of Spearman correlation coefficients were adjusted by using also the Dunn-Šidák method ($\alpha = 1 - [1 - 0.05]^{1/35} \approx 0.001464$; 35 = total number of behavioural parameters). Again we did not use the highly conservative overall Bonferroni correction ($\alpha = 0.05/595 \approx 0.000084$), because of the large numbers of correlations (595). In all other cases (i.e. the Kolmogorov-Smirnov one sample test, Levene's test and the Bartlett's test of sphericity), the probability of a Type I error < 0.05 was taken as the criterion of significance.

Table 2 *Overview of the (corrected) thresholds for the multiple strain comparisons*

Table 2 Overview of the (corrected) thresholds for the multiple

Behavioural dimension / factor analysis	C57BL/6J (n = 27) *versus* A/J (n = 30) Significant difference
Anxiety	P < 0.001002
Risk assessment	P < 0.002002
Undirected exploration	P < 0.000668
Directed exploration	P < 0.000668
Memory	P < 0.001002
Locomotion	P < 0.002002
Arousal	P < 0.000668
Other behaviour	P < 0.000801
Factor analysis	P < 0.004

Results

Associations

For individual mice (i.e. consomic, host and donor strain mice; n = 204) we studied the association between the behavioural parameters of the modified hole board. Table 3 summarizes the calculated coefficients of Spearman's rank correlation. In this table the behavioural parameters are sorted by motivational system. As would be expected for measures within a behavioural test apparatus, there were many (in total 159) significant correlations.

Parental strain analyses

Mice from the strains A/J and C57BL/6J were used as donor and host strains for the consomic panel, respectively. The results obtained for the parental strains with the modified hole board are summarized in Figure 2 (anxiety), and Tables 4 (anxiety) & 5 (risk assessment, undirected exploration, directed exploration, memory, locomotion, arousal, and other behaviour). From these tables and Figure it is clear that the A/J and C57BL/6J inbred strains are contrasting: the strains differ in all behavioural dimensions. Highly significant differences between the

strain comparisons

C57BL/6J (n = 27) *versus* consomic lines (n = 6)		C57BL/6J (n = 27) *versus* CSS-19 (n = 27)
Evidence that a specific chromosome harbours a QTL		
Suggestive	**Significant**	**Significant difference**
0.001002 ≤ P < 0.012741	P < 0.001002	P < 0.001002
0.002002 ≤ P < 0.025321	P < 0.002002	P < 0.002002
0.000668 ≤ P < 0.008512	P < 0.000668	P < 0.000668
0.000668 ≤ P < 0.008512	P < 0.000668	P < 0.000668
0.001002 ≤ P < 0.012741	P < 0.001002	P < 0.001002
0.002002 ≤ P < 0.025321	P < 0.002002	P < 0.002002
0.000668 ≤ P < 0.008512	P < 0.000668	P < 0.000668
0.000801 ≤ P < 0.010206	P < 0.000801	P < 0.000801
0.004 ≤ P < 0.05	P < 0.004	P < 0.004

two parental strains were found for anxiety-related avoidance behaviour towards an unprotected area; indicated by the total number of board entries and latency until the first board entry (Table 4, Figure 2). These results corroborate earlier work (Laarakker et al. 2006). In contrast, there was no significant difference in percentage of time spent on the board and average duration of a board entry (Table 4).

Anxiety-related behaviour may significantly be confounded by the overall activity of an animal. The mouse may either avoid a certain area, because it is anxious to explore it or because it is not active enough to reach it. The number of line crossings and the latency until the first line crossing in the box indicates the level of overall activity in the modified hole board test. There were marked strain differences as to locomotor activity: C57BL/6J when compared with A/J mice are more active (Table 5, total number of line crossings).

Furthermore, mice of the A/J strain showed significantly more risk assessments (total number) than the C57BL/6J mice, and the strains differ also significantly in undirected (i.e. rearing, exploration of the holes) as well as in directed exploratory

behaviour (exploration of the unfamiliar object): mice of the A/J strain showed a significantly longer latency time until performing the first exploratory behaviour and a lower number of explorations and rearing in the box during behavioural testing than C57BL/6J mice (Table 5).

The A/J and C57BL/6J strain differ for memory performance and arousal behaviour. C57BL/6J mice performed significantly more familiar object explorations (total number and latency) when compared to A/J mice. The percentage of time spent grooming and the average duration of grooming was significantly higher for the A/J strain than for the C57BL/6J strain. In contrast, there was no difference in the number of boli and the latency until the first bolus was produced. C57BL/6J mice stay more frequently and longer in the box than A/J mice (Table 5).

Within the same behavioural dimension some of the parameters are related to each other (see the boxes in table 3). For example the parameters total number of board entries, latency until the first board entry, percentage of time spent on the board, and average duration of a board entry are significantly associated (table 3). Therefore - i.e. for the significantly associated parameters within the same behavioural dimension - a multivariate method, such as the Hotelling's T2 test, may be used. However, several assumptions are necessary for proper application of the Hotelling's T2 test. One of the assumptions is that dependent variables should have a multivariate normal distribution. Because total number is a discrete variable, the joint distribution can never be multivariate normal. To take the total number parameters into account we performed bivariate analyses with latency and average duration as the dependent variables (the parameter average duration is based on the parameters percentage of time and total number). In addition we also performed bivariate analyses with the dependent variables latency and percentage of time. These additional bivariate analyses also resulted in significant parental strain differences for anxiety-related behaviour and arousal (Tables 4 and 5). The obtained parental strain differences prompted the investigation into the chromosomal location of the QTLs involved by testing a set of chromosome substitution strains between the A/J and C57BL/6J strains.

Table 3 *Associations (Spearman's RS) between behavioural parameters measured in the modified hole board*

Behavioural dimension / parameter
Anxiety
Total number of board entries (1)
Latency until the first board entry (2)
Percentage of time on the board (3)
Average duration of a board entry (4)
Risk assessment
Total number of risk assessments (5)

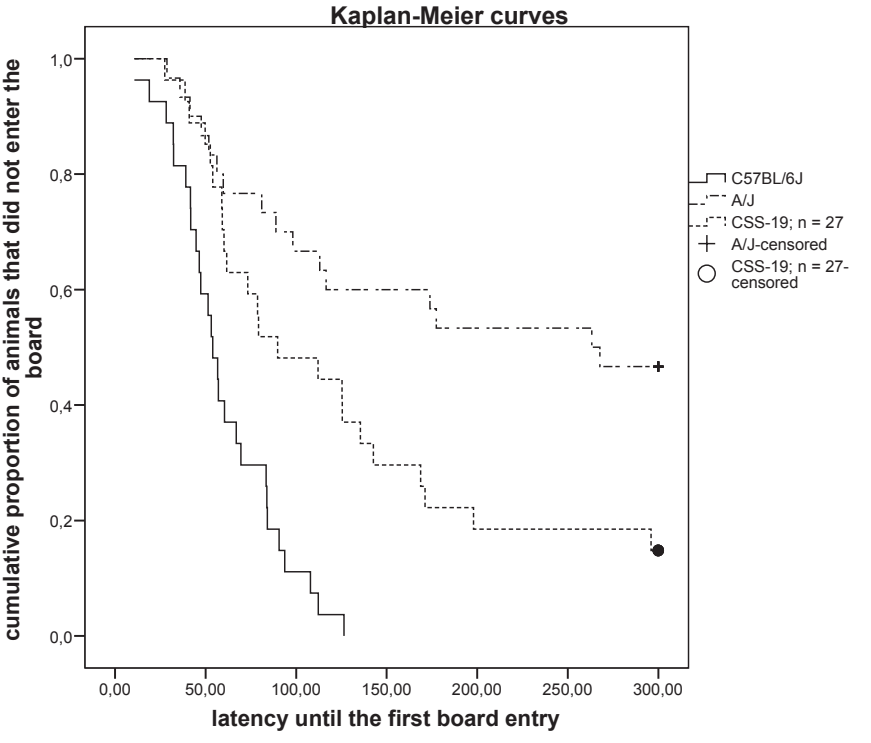


Figure 2 *Kaplan-Meier plots for the parameter latency until the first board entry. Results for male mice from the C57BL/6J host strain (n = 27), the A/J donor strain (n = 30) and CSS-19 (n = 27). For some mice from the CSS-19 and A/J strain latency until the first board entry is said to be censored, indicating that the testing period (300 seconds) was cut off before the event (= board entry) occurred. We do not know when (or indeed, whether) these mice will experience the event, only that these mice have not done so by the end of the testing period*

		Anxiety			***Risk assessment***	
(1)	(2)	(3)	(4)	(5)	(6)	
-	-	-	-	-	-	
-0.650	-	-	-	-	-	
0.785	**-0.552**	-	-	-	-	
0.448	**-0.398**	**0.836**	-	-	-	
-0.472	**0.393**	**-0.361**	**-0.243**	-	-	

[Table 3 continued]

Behavioural dimension / parameter
Latency until the first risk assessment (6)
Undirected exploration
Total number of rearing in the box (7)
Latency until the first rearing in the box (8)
Total number of rearing on the board (9)
Latency until the first rearing on the board (10)
Total number of hole explorations (11)
Latency until the first hole exploration (12)
Directed exploration
Total number of holes visited (13)
Latency until the first hole visited (14)
Total number of unfamiliar object explorations (15)
Latency until the first unfamiliar object exploration (16)
Percentage of time being busy with unfamiliar object explorations (17)
Average duration of an unfamiliar object exploration (18)
Memory
Total number of familiar object explorations (19)
Latency until the first familiar object exploration (20)
Percentage of time being busy with familiar object exploration (21)
Average duration of a familiar object exploration (22)
Locomotion
Total number of line crossings (23)
Latency until the first line crossing (24)
Arousal
Total number of groomings (25)
Latency until the first grooming (26)
Percentage of time being busy with grooming (27)
Average duration of a grooming (28)
Total number of boli (29)
Latency until the first bolus is produced (30)
Other behaviour
Number of times the mouse is in the box (31)
Percentage of time in the box (32)

Anxiety				*Risk assessment*	
(1)	(2)	(3)	(4)	(5)	(6)
0.094	-0.058	0.079	0.049	**-0.576**	-
0.418	**-0.441**	**0.244**	0.160	**-0.508**	0.137
-0.016	0.042	0.040	0.100	0.202	-0.135
0.096	-0.140	0.144	0.162	-0.069	-0.008
-0.090	0.134	-0.139	-0.162	0.069	0.003
0.940	**-0.630**	**0.845**	**0.577**	**-0.477**	0.094
-0.677	**0.952**	**-0.564**	**-0.379**	**0.413**	-0.069
0.154	-0.220	0.214	0.207	-0.080	0.040
-0.154	**0.223**	-0.217	-0.211	0.077	-0.036
0.357	**-0.315**	0.139	0.031	**-0.284**	-0.017
-0.342	**0.371**	**-0.303**	**-0.266**	**0.462**	-0.109
0.150	-0.162	0.087	0.012	-0.104	-0.061
-0.005	-0.064	0.066	0.128	0.046	-0.086
0.238	-0.182	0.042	-0.069	-0.186	-0.033
-0.290	**0.270**	**-0.337**	**-0.313**	**0.361**	-0.082
0.028	0.007	0.019	0.049	-0.016	-0.096
-0.117	0.112	0.020	0.184	0.099	-0.070
0.418	**-0.249**	0.141	-0.045	**-0.400**	0.086
-0.300	**0.229**	-0.217	-0.099	**0.376**	0.057
-0.013	-0.051	0.062	0.118	0.045	0.028
0.035	0.017	-0.021	-0.090	0.038	-0.028
-0.252	0.162	-0.143	-0.045	0.139	0.074
-0.135	0.128	-0.055	-0.011	0.056	0.105
-0.119	0.149	-0.066	-0.008	0.165	-0.039
0.066	-0.130	0.046	0.025	-0.107	0.054
0.842	**-0.587**	**0.588**	**0.265**	**-0.458**	0.080
-0.306	0.182	**-0.584**	**-0.502**	0.096	-0.001

[Table 3 continued]

Behavioural dimension / parameter
Average duration of a stay in the box (33)
Total number of urinations (34)
Latency until the first time urine is produced (35)

Association based on 204 animals. Significant ($P < 0.001464$) Spearman's RS are indicated in bold characters. Associations between the parameters of the same behavioural dimension are indicated with a box.

Behavioural dimension / parameter
Anxiety
Total number of board entries (1)
Latency until the first board entry (2)
Percentage of time on the board (3)
Average duration of a board entry (4)
Risk asssessment
Total number of risk assessments (5)
Latency until the first risk assessment (6)
Undirected exploration
Total number of rearing in the box (7)
Latency until the first rearing in the box (8)
Total number of rearing on the board (9)
Latency until the first rearing on the board (10)
Total number of hole explorations (11)
Latency until the first hole exploration (12)
Directed exploration
Total number of holes visited (13)
Latency until the first hole visited (14)
Total number of unfamiliar object explorations (15)
Latency until the first unfamiliar object exploration (16)
Percentage of time being busy with unfamiliar object explorations (17)
Average duration of an unfamiliar object exploration (18)
Memory
Total number of familiar object explorations (19)
Latency until the first familiar object exploration (20)

Anxiety				*Risk assessment*	
(1)	(2)	(3)	(4)	(5)	(6)
-0.853	**0.595**	**-0.686**	**-0.368**	**0.456**	-0.082
0.011	0.048	-0.035	-0.020	0.147	-0.131
-0.009	-0.052	0.034	0.019	-0.144	0.123

Undirected exploration					
(7)	(8)	(9)	(10)	(11)	(12)
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-0.296	-	-	-	-	-
-0.067	-0.212	-	-	-	-
0.067	0.216	**-0.999**	-	-	-
0.402	0.033	0.110	-0.104	-	-
-0.493	0.034	-0.073	0.064	**-0.669**	-
0.120	-0.115	0.047	-0.051	0.199	-0.167
-0.120	0.110	-0.044	0.048	-0.199	0.171
0.513	0.009	-0.158	0.160	**0.322**	**-0.353**
-0.372	0.125	-0.052	0.050	**-0.335**	**0.387**
0.167	0.110	-0.143	0.143	0.129	-0.208
-0.087	0.190	-0.085	0.084	0.018	-0.079
0.519	0.034	-0.205	0.208	0.209	**-0.226**
-0.391	0.114	-0.049	0.045	**-0.312**	**0.283**

[Table 3 continued]

Behavioural dimension / parameter
Percentage of time being busy with familiar object exploration (21)
Average duration of a familiar object exploration (22)
Locomotion
Total number of line crossings (23)
Latency until the first line crossing (24)
Arousal
Total number of groomings (25)
Latency until the first grooming (26)
Percentage of time being busy with grooming (27)
Average duration of a grooming (28)
Total number of boli (29)
Latency until the first bolus is produced (30)
Other behaviour
Number of times the mouse is in the box (31)
Percentage of time in the box (32)
Average duration of a stay in the box (33)
Total number of urinations (34)
Latency until the first time urine is produced (35)

Behavioural dimension / parameter
Anxiety
Total number of board entries (1)
Latency until the first board entry (2)
Percentage of time on the board (3)
Average duration of a board entry (4)
Risk assessment
Total number of risk assessments (5)
Latency until the first risk assessment (6)
Undirected exploration
Total number of rearing in the box (7)
Latency until the first rearing in the box (8)

Undirected exploration					
(7)	(8)	(9)	(10)	(11)	(12)
0.085	0.084	-0.095	0.099	0.029	-0.007
-0.273	0.059	0.122	-0.121	-0.091	0.120
0.666	-0.063	-0.151	0.154	**0.363**	**-0.314**
-0.335	0.179	-0.132	0.130	**-0.270**	**0.237**
-0.269	-0.008	0.070	-0.074	-0.057	0.010
0.209	0.033	-0.026	0.028	0.051	-0.030
-0.413	-0.108	0.052	-0.054	**-0.282**	0.217
-0.306	-0.163	0.083	-0.084	-0.171	0.170
-0.136	0.084	0.002	-0.007	-0.106	0.134
0.067	-0.051	-0.010	0.014	0.059	-0.117
0.528	-0.067	-0.020	0.024	**0.772**	**-0.614**
0.177	0.046	-0.168	0.167	**-0.365**	0.128
-0.465	0.071	-0.010	0.006	**-0.798**	**0.613**
0.100	0.052	-0.067	0.064	0.028	0.006
-0.104	-0.044	0.070	-0.068	-0.027	-0.011

Directed exploration					
(13)	(14)	(15)	(16)	(17)	(18)
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-

[Table 3 continued]

Behavioural dimension / parameter
Total number of rearing on the board (9)
Latency until the first rearing on the board (10)
Total number of hole explorations (11)
Latency until the first hole exploration (12)
Directed exploration
Total number of holes visited (13)
Latency until the first hole visited (14)
Total number of unfamiliar object explorations (15)
Latency until the first unfamiliar object exploration (16)
Percentage of time being busy with unfamiliar object explorations (17)
Average duration of an unfamiliar object exploration (18)
Memory
Total number of familiar object explorations (19)
Latency until the first familiar object exploration (20)
Percentage of time being busy with familiar object exploration (21)
Average duration of a familiar object exploration (22)
Locomotion
Total number of line crossings (23)
Latency until the first line crossing (24)
Arousal
Total number of groomings (25)
Latency until the first grooming (26)
Percentage of time being busy with grooming (27)
Average duration of a grooming (28)
Total number of boli (29)
Latency until the first bolus is produced (30)
Other behaviour
Number of times the mouse is in the box (31)
Percentage of time in the box (32)
Average duration of a stay in the box (33)
Total number of urinations (34)
Latency until the first time urine is produced (35)

Directed exploration					
(13)	(14)	(15)	(16)	(17)	(18)
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-0.999	-	-	-	-	-
-0.059	0.058	-	-	-	-
-0.111	0.107	**-0.429**	-	-	-
-0.076	0.075	**0.424**	-0.084	-	-
-0.026	0.024	-0.155	0.093	**0.664**	-
-0.076	0.070	**0.628**	-0.211	**0.304**	-0.011
-0.171	0.175	**-0.227**	**0.532**	-0.055	-0.004
-0.036	0.033	**0.276**	-0.136	**0.378**	0.206
0.035	-0.034	-0.149	-0.028	0.109	**0.314**
-0.051	0.052	**0.593**	**-0.284**	0.158	-0.151
0.044	-0.048	**-0.240**	**0.427**	0.031	0.137
-0.038	0.039	-0.053	-0.077	-0.017	-0.033
0.009	-0.011	0.045	0.097	0.038	0.043
-0.009	0.010	**-0.325**	0.037	-0.101	0.052
0.060	-0.060	**-0.261**	-0.012	-0.092	0.023
-0.001	-0.001	-0.207	0.156	-0.052	0.018
0.009	-0.006	0.179	-0.158	0.053	0.008
0.046	-0.048	**0.667**	**-0.441**	**0.264**	-0.119
-0.166	0.166	0.169	0.124	-0.105	-0.185
-0.102	0.103	**-0.588**	**0.462**	**-0.263**	0.070
0.041	-0.042	0.106	-0.002	0.026	-0.020
-0.049	0.051	-0.103	-0.004	-0.021	0.023

[Table 3 continued]

Behavioural dimension / parameter
Anxiety
Total number of board entries (1)
Latency until the first board entry (2)
Percentage of time on the board (3)
Average duration of a board entry (4)
Risk assessment
Total number of risk assessments (5)
Latency until the first risk assessment (6)
Undirected exploration
Total number of rearing in the box (7)
Latency until the first rearing in the box (8)
Total number of rearing on the board (9)
Latency until the first rearing on the board (10)
Total number of hole explorations (11)
Latency until the first hole exploration (12)
Directed exploration
Total number of holes visited (13)
Latency until the first hole visited (14)
Total number of unfamiliar object explorations (15)
Latency until the first unfamiliar object exploration (16)
Percentage of time being busy with unfamiliar object explorations (17)
Average duration of an unfamiliar object exploration (18)
Memory
Total number of familiar object explorations (19)
Latency until the first familiar object exploration (20)
Percentage of time being busy with familiar object exploration (21)
Average duration of a familiar object exploration (22)
Locomotion
Total number of line crossings (23)
Latency until the first line crossing (24)
Arousal
Total number of groomings (25)
Latency until the first grooming (26)

		Memory		***Locomotion***	
(19)	(20)	(21)	(22)	(23)	(24)
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-0.333	-	-	-	-	-
0.498	-0.105	-	-	-	-
-0.229	0.072	**0.567**	-	-	-
0.683	**-0.316**	0.183	**-0.300**	-	-
-0.177	**0.338**	-0.036	0.106	**-0.402**	-
-0.196	0.012	0.068	0.204	**-0.300**	0.047
0.232	0.002	0.066	-0.069	**0.281**	-0.040

[Table 3 continued]

Behavioural dimension / parameter
Percentage of time being busy with grooming (27)
Average duration of a grooming (28)
Total number of boli (29)
Latency until the first bolus is produced (30)
Other behaviour
Number of times the mouse is in the box (31)
Percentage of time in the box (32)
Average duration of a stay in the box (33)
Total number of urinations (34)
Latency until the first time urine is produced (35)

Behavioural dimension / parameter
Anxiety
Total number of board entries (1)
Latency until the first board entry (2)
Percentage of time on the board (3)
Average duration of a board entry (4)
Risk assessment
Total number of risk assessments (5)
Latency until the first risk assessment (6)
Undirected exploration
Total number of rearing in the box (7)
Latency until the first rearing in the box (8)
Total number of rearing on the board (9)
Latency until the first rearing on the board (10)
Total number of hole explorations (11)
Latency until the first hole exploration (12)
Directed exploration
Total number of holes visited (13)
Latency until the first hole visited (14)
Total number of unfamiliar object explorations (15)

Memory				Locomotion	
(19)	(20)	(21)	(22)	(23)	(24)
-0.420	0.156	-0.109	0.137	**-0.524**	0.160
-0.287	0.078	-0.097	0.089	**-0.387**	0.072
-0.012	0.007	-0.014	-0.038	-0.150	0.192
-0.015	0.012	0.059	0.107	0.068	-0.126
0.548	**-0.353**	0.168	**-0.249**	**0.562**	**-0.349**
0.211	0.145	-0.058	-0.099	**0.295**	0.040
-0.476	**0.381**	-0.163	0.202	**-0.469**	**0.344**
0.191	-0.045	-0.027	-0.153	0.136	0.078
-0.187	0.044	0.034	0.157	-0.137	-0.087

Arousal					
(25)	(26)	(27)	(28)	(29)	(30)
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-

[Table 3 continued]

Behavioural dimension / parameter
Latency until the first unfamiliar object exploration (16)
Percentage of time being busy with unfamiliar object explorations (17)
Average duration of an unfamiliar object exploration (18)
Memory
Total number of familiar object explorations (19)
Latency until the first familiar object exploration (20)
Percentage of time being busy with familiar object exploration (21)
Average duration of a familiar object exploration (22)
Locomotion
Total number of line crossings (23)
Latency until the first line crossing (24)
Arousal
Total number of groomings (25)
Latency until the first grooming (26)
Percentage of time being busy with grooming (27)
Average duration of a grooming (28)
Total number of boli (29)
Latency until the first bolus is produced (30)
Other behaviour
Number of times the mouse is in the box (31)
Percentage of time in the box (32)
Average duration of a stay in the box (33)
Total number of urinations (34)
Latency until the first time urine is produced (35)

Behavioural dimension / parameter
Anxiety
Total number of board entries (1)
Latency until the first board entry (2)
Percentage of time on the board (3)
Average duration of a board entry (4)

Arousal					
(25)	(26)	(27)	(28)	(29)	(30)
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
-0.711	-	-	-	-	-
0.666	**-0.575**	-	-	-	-
0.426	**-0.497**	**0.829**	-	-	-
0.011	0.018	-0.004	0.016	-	-
0.044	-0.034	0.038	0.000	**-0.924**	-
0.033	0.005	**-0.258**	-0.177	-0.151	0.096
-0.219	0.213	**-0.308**	**-0.270**	0.036	-0.051
-0.070	0.034	0.183	0.106	0.147	-0.096
-0.170	0.178	-0.168	-0.076	**0.321**	**-0.308**
0.172	-0.179	0.168	0.075	**-0.318**	**0.306**

Other behaviour				
(31)	(32)	(33)	(34)	(35)
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-

[Table 3 continued]

Behavioural dimension / parameter	
Risk assessment	
Total number of risk assessments (5)	
Latency until the first risk assessment (6)	
Undirected exploration	
Total number of rearing in the box (7)	
Latency until the first rearing in the box (8)	
Total number of rearing on the board (9)	
Latency until the first rearing on the board (10)	
Total number of hole explorations (11)	
Latency until the first hole exploration (12)	
Directed exploration	
Total number of holes visited (13)	
Latency until the first hole visited (14)	
Total number of unfamiliar object explorations (15)	
Latency until the first unfamiliar object exploration (16)	
Percentage of time being busy with unfamiliar object explorations (17)	
Average duration of an unfamiliar object exploration (18)	-
Memory	
Total number of familiar object explorations (19)	-
Latency until the first familiar object exploration (20)	-
Percentage of time being busy with familiar object exploration (21)	-
Average duration of a familiar object exploration (22)	-
Locomotion	
Total number of line crossings (23)	-
Latency until the first line crossing (24)	-
Arousal	
Total number of groomings (25)	-
Latency until the first grooming (26)	-
Percentage of time being busy with grooming (27)	-
Average duration of a grooming (28)	-
Total number of boli (29)	-
Latency until the first bolus is produced (30)	-

Other behaviour				
(31)	(32)	(33)	(34)	(35)
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-
-	-	-	-	-

[Table 3 continued]

Behavioural dimension / parameter
Other behaviour
Number of times the mouse is in the box (31)
Percentage of time in the box (32)
Average duration of a stay in the box (33)
Total number of urinations (34)
Latency until the first time urine is produced (35)

Other behaviour				
(31)	(32)	(33)	(34)	(35)
-	-	-	-	-
-0.212	-	-	-	-
-0.975	**0.392**	-	-	-
0.049	0.199	-0.013	-	-
-0.045	-0.200	0.010	**-0.996**	-

Chromosome substitution strain survey

Six males for each strain of the consomic panel were tested for modified hole board behaviour. Figure 3 and Table 6 give an overview of the results for anxiety-related behaviour. When compared to the host strain (n = 27) the consomic panel shows in the univariate analysis significant evidence for an anxiety QTL on chromosome 10. With this type of analysis there is suggestive evidence for anxiety QTLs on chromosomes 5, 8, and Y (table 6). Bivariate analysis results in two significant (on chromosomes 15 and Y) and three suggestive anxiety QTLs (on chromosomes 10, 18 and 19) (Table 6).

Table 4 *Anxiety-related behaviour in the modified hole board in C57BL/6J, A/J and CSS-19 male mice*

Parameter	C57BL/6J (host strain)	A/J (donor strain)	CSS-19 (consomic line)
Univariate analysis			
Total number of board entries			
(frequency)	11.0 (7.0)	1.5 (5.0)	5.0 (9.0)
Latency until the first board entry			
(seconds)	60.6 ± 5.6	196.7 ± 20.6	129.6 ± 17.9
Percentage of time spent on the board			
(%)	6.6 ± 0.9	4.0 ± 1.1	2.8 ± 0.7
Average duration of a board entry			
(seconds)	1.6 ± 0.2	3.6 ± 0.8	0.8 ± 0.1
Bivariate analysis			
Latency until the first board entry + percentage of time spent on the board			
Latency until the first board entry + average duration of a board entry			

Values are means ± SEM (latency, percentage and average duration) or medians with, in parenthese, the interquartile range (frequency) for 27 (C57BL/6J and CSS-19) or 30 (A/J) animals per strain. Note that a P value of 0.000000 (Student's t tests and Mann-Whitney U tests) or 0.0000 (Log-Rank tests) does not mean that it is zero, only that it is less than 0.0000005 or 0.00005, respectively. Significant

Table 7 summarizes suggestive and significant evidence for a QTL on a chromosome for the remaining parameters of modified hole board behaviour. The present chromosome substitution strain survey indicates that nearly all mouse chromosomes (with the exception of chromosomes 2 and 17) each contain at least one QTL that is involved in modified hole board behaviour. Further, there is evidence that chromosome 10 contains QTLs for all behavioural dimensions, whereas chromosomes 4, 7, 8, 9, 11, and 14 contain a QTL for only one behavioural dimension. On chromosome 19, besides evidence for an anxiety QTL (Table 6), there is also evidence for a risk assessment and direct exploration QTL (Table 7).

	P value	
	Host *vs.* donor	Host *vs.* consomic
	***0.000000**^{M}	***0.000890**^{M}
	***0.000000**^{W} and ***0.0000**^{L}	***0.000898**^{W} and ***0.0002**^{L}
	0.552282^{W}	0.001437^{S}
	0.023648^{w}	0.001134^{S}
	***0.000000**^{H}	***0.000445**^{H}
	***0.000000**^{H}	***0.000425**^{H}

(*, P < 0.001002) strain differences are indicated in bold characters. H = Hotelling's T2 test, M = Mann-Whitney U test, S = Student's t test, W = Student's t test with Welch-Satterthwaite correction, L = Log-Rank test.

Two-stage approach

As a proof of principle for the approach (two-phase procedure) that we suggest for behavioural genetic experiments using chromosome substitution strains (i.e. for the consomic survey), twenty-one additional male CSS-19 animals were tested. We selected CSS-19 because there was no evidence that chromosome 19 contains a locomotion QTL and thus a pleiotropic contribution of locomotion with respect to anxiety could be excluded. After behavioural testing the statistical analysis was repeated, but with 27 animals for both the chromosome substitution strain and the host strain (C57BL/6J). The suggestive evidence for an anxiety QTL (or QTLs) on chromosome 19 (detected with 27 host strain versus 6 CSS-19 animals; Table 6,

Table 5 *Modified hole board behaviour (excluding anxiety) in C57BL/6J, A/J and CSS-19 male mice*

Behavioural dimension / parameter	C57BL/6J (host strain)	A/J (donor strain)	CSS-19 (consomic line)
Risk assessment (significant strain difference: P < 0.002002)			
Total number of risk assessments			
(frequency)	2.0 (2.0)	5.0 (6.0)	4.0 (5.0)
Latency until the first risked assessment			
(seconds)	93.2 ± 24.2	44.1 ± 13.8	55.4 ± 19.1
Undirected explorations (significant strain difference: P < 0.000668)			
Total number of rearing in the box			
(frequency)	42.0 (15.0)	12.5 (11.0)	38.0 (13.0)
Latency until the first rearing in the box			
(seconds)	37.9 ± 3.0	42.9 ± 10.9	42.3 ± 3.7
Total number of rearing on the board			
(frequency)	0.0 (0.0)	0.0 (0.3)	0.0 (0.0)
Latency until the first rearing on the board			
(seconds)	299.9 ± 0.1	273.4 ± 12.1	300.0 ± 0.0
Total number of hole explorations			
(frequency)	8.0 (19.0)	0.0 (4.5)	5.0 (18.0)
Latency until the first hole exploration			
(seconds)	60.8 ± 5.6	228.0 ± 18.0	126.4 ± 18.4

bivariate analysis) now turns into a significant evidence (table 4). The evidence for a risk assessment QTL on chromosome 19 remains significant, whereas there was no longer evidence for a direct exploration QTL (Table 5). In addition, significant evidence for a QTL for percentage of time in the box on this chromosome turned up (Table 5).

P value	
Host *vs.* donor	Host *vs.* consomic
***0.000000**^{M}	***0.000278**^{M}
0.296661^{M} and 0.1241^{L}	0.115805^{WM} and 0.0975^{L}
***0.000000**^{M}	0.006278^{M}
0.658465^{W} and 0.9902^{L}	0.365358^{W} and 0.2980^{L}
0.050114^{M}	1.000000^{M}
0.025166^{W} and 0.0311^{L}	1.000000^{W} and 0.3173^{L}
***0.000000**^{M}	0.001852^{M}
***0.000000**^{M} and ***0.0000**^{L}	0.001776^{W} and 0.0008^{L}

[Table 5 continued]

Behavioural dimension / parameter	C57BL/6J (host strain)	A/J (donor strain)	CSS-19 (consomic line)
Directed explorations (significant strain difference: P < 0.000668)			
Total number of holes visited			
(frequency)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Latency until the first hole visited			
(seconds)	299.9 ± 0.0	289.4 ± 6.6	300.0 ± 0.0
Total number of unfamiliar object explorations			
(frequency)	8.0 (3.0)	2.0 (2.0)	7.0 (4.0)
Latency until the first unfamiliar object exploration			
(seconds)	42.3 ± 2.9	121.1 ± 19.3	69.9 ± 9.8
Percentage of time being busy with unfamiliar object explorations			
(%)	2.5 ± 0.5	1.3 ± 0.2	2.0 ± 0.3
Average duration of an unfamiliar object exploration			
(seconds)	1.0 ± 0.2	1.2 ± 0.2	0.9 ± 0.2
Memory (significant strain difference: P < 0.001002)			
Total number of familiar object explorations			
(frequency)	8.0 (4.0)	3.0 (2.3)	10.0 (5.0)
Latency until the first familiar object exploration			
(seconds)	47.7 ± 4.3	129.5 ± 21.4	59.4 ± 7.1
Percentage of time being busy with familiar object exploration			
(%)	1.4 ± 0.1	1.0 ± 0.1	2.0 ± 0.2
Average duration of a familiar object exploration			
(seconds)	0.5 ± 0.0	1.4 ± 0.4	0.6 ± 0.1
Locomotion (significant strain difference: P < 0.002002)			
Total number of line crossings			
(frequency)	151.0 (25.0)	26.0 (36.0)	153.0 (18.0)
Latency until the first line crossing			
(seconds)	10.0 ± 1.2	32.8 ± 11.5	9.4 ± 1.6
Arousal (significant strain difference: P < 0.000668)			
Total number of groomings			
(frequency)	1.0 (0.0)	1.0 (1.3)	1.0 (1.0)

P value	
Host *vs.* donor	Host *vs.* consomic
0.460684^{M}	0.490566^{M}
0.425341^{M} and 0.4348^{L}	0.496566^{M} and 0.1534^{L}
***0.000000**^{M}	0.581983^{M}
***0.000337**^{W} and ***0.0001**^{L}	0.011443^{W} and 0.0011^{L}
0.003837^{M}	0.387650^{W}
0.665362^{M}	0.346404^{M}
***0.000000**^{M}	0.200291^{M}
***0.000752**^{W} and 0.0011^{L}	0.165098^{W} and 0.1164^{L}
0.035999^{S}	0.054209^{W}
0.016086^{w}	0.171121^{S}
***0.000000**^{M}	0.670813^{M}
0.101683^{M} and 0.0208^{L}	0.739813^{W} and 0.8496^{L}
0.002225^{M}	0.362890^{M}

[Table 5 continued]

Behavioural dimension / parameter	C57BL/6J (host strain)	A/J (donor strain)	CSS-19 (consomic line)
Latency until the first grooming			
(seconds)	211.8 ± 14.9	156.5 ± 14.6	235.4 ± 13.1
Percentage of time being busy with grooming			
(%)	1.0 ± 0.1	6.1 ± 1.0	0.8 ± 0.2
Average duration of a grooming			
(seconds)	2.6 ± 0.4	12.6 ± 2.9	2.1 ± 0.5
Total number of boli			
(frequency)	0.0 (1.0)	0.0 (1.0)	0.0 (1.0)
Latency until the first bolus is produced			
(seconds)	198.0 ± 26.3	205.0 ± 21.0	227.0 ± 22.9
(bivariate analysis)			
Latency until the first grooming + percentage of time being busy with grooming			
Latency until the first grooming + average duration of a grooming			
Other behaviour (significant strain difference: P < 0.000801)			
Number of times the mouse is in the box			
(frequency)	28.0 (9.0)	14.5 (8.3)	23.0 (13.0)
Percentage of time in the box			
(%)	88.5 ± 1.0	79.4 ± 1.7	92.4 ± 0.9
Average duration of a stay in the box			
(seconds)	9.9 ± 0.5	19.1 ± 1.6	13.6 ± 1.0
Total number of urinations			
(frequency)	0.0 (0.0)	0.0 (0.0)	0.0 (1.0)
Latency until the first time urine is produced			
(seconds)	245.1 ± 20.7	300.0 ± 0.0	202.2 ± 25.1

Values are means ± SEM (latency, percentage and average duration) or medians with, in parenthese, the interquartile range (frequency) for 27 (C57BL/6J and CSS-19) or 30 (A/J) animals per strain. Note that a P value of 0.000000 (Student's t tests and Mann-Whitney U tests) or 0.0000 (Log-Rank tests) does not mean that it is zero, only that it is less than 0.0000005 or 0.00005, respectively.

P value	
Host *vs.* donor	Host *vs.* consomic
0.010409^{S} and 0.0135^{L}	0.238194^{S} and 0.2234^{L}
***0.000010**^{W}	0.643112^{S}
***0.000001**^{M}	0.482561^{S}
0.635285^{M}	0.779869^{M}
0.852796^{M} and 0.7893^{L}	0.386284^{M} and 0.4648^{L}
***0.000002**^{H}	0.494703^{H}
***0.000170**^{H}	0.495809^{H}
***0.000000**^{M}	0.012238^{M}
***0.000029**^{W}	***0.000000**^{S}
***0.000005**^{w}	0.001972^{W}
0.008157^{M}	0.223778^{M}
0.008157^{M} and 0.2244^{L}	0.298815^{M} and 0.2537^{L}

Significant (*) strain differences are indicated in bold characters. H = Hotelling's T^2 test, M = Mann-Whitney U test, S = Student's t test, W = Student's t test with Welch-Satterthwaite correction, L = Log-Rank test.

Table 6 *Suggestive and significant evidence for QTLs influencing the difference in anxiety-related behaviour between C57BL/6J (n = 27) and consomic (n = 6) male mice*

	Univariate analysis (*P* value)				
Consomic line	Latency until the first board entry		Total number of board entries	Percentage of time spent on the board	Average duration of a board entry
CSS-1	0.449123^{W}	0.2429^{L}	0.154187^{M}	0.934940^{S}	0.308524^{S}
CSS-2	0.795155^{W}	0.7160^{L}	0.972744^{M}	0.418021^{W}	0.624447^{W}
CSS-3	0.486352^{S}	0.4439^{L}	0.546447^{M}	0.424047^{S}	0.027449^{S}
CSS-4	0.677603^{W}	0.2520^{L}	0.168883^{M}	0.178726^{S}	0.437896^{W}
CSS-5	0.052928^{S}	*$^{\#}$0.0029*^{L}	0.416687^{M}	0.441484^{S}	0.050840^{S}
CSS-6	0.250479^{W}	0.0971^{L}	0.559561^{M}	0.326912^{S}	0.013329^{S}
CSS-7	0.522299^{S}	0.3902^{L}	0.217356^{M}	0.084428^{S}	0.135360^{S}
CSS-8	0.788546^{S}	0.9922^{L}	0.954535^{M}	0.096054^{S}	*#0.011709*^{S}
CSS-9	0.167417^{S}	0.1041^{L}	0.690026^{M}	0.686224^{S}	0.862264^{S}
CSS-10	0.362698^{S}	0.4938^{L}	0.485679^{M}	0.211063^{S}	***0.000704***^{S}
CSS-11	0.946635^{W}	0.4959^{L}	0.829091^{M}	0.155609^{S}	0.042604^{S}
CSS-12	0.599561^{W}	0.2407^{L}	0.882397^{M}	0.937677^{S}	0.777740^{S}
CSS-13	0.137512^{S}	0.2549^{L}	0.390433^{M}	0.795626^{S}	0.679103^{S}
CSS-14	0.344671^{W}	0.0969^{L}	0.134129^{M}	0.251965^{S}	0.950503^{S}
CSS-15	0.076108^{W}	0.0171^{L}	0.013522^{M}	0.383342^{S}	0.293972^{W}
CSS-16	0.943756^{W}	0.9258^{L}	0.810311^{M}	0.384557^{S}	0.085790^{S}
CSS-17	0.446468^{S}	0.1971^{L}	0.515115^{M}	0.835273^{W}	0.694079^{W}
CSS-18	0.636484^{S}	0.7798^{L}	0.133892^{M}	0.336169^{S}	0.087818^{W}
CSS-19	0.173463^{W}	0.0803^{L}	0.038287^{M}	0.146554^{S}	0.134412^{S}
CSS-X	0.421212^{W}	0.3573^{L}	0.576378^{M}	0.763177^{S}	0.697363^{S}
CSS-Y	0.040699^{W}	*#0.0012*^{L}	0.065561^{M}	0.318997^{S}	0.611008^{W}

Significant evidence (*, P < 0.001002) for a QTL on a chromosome is indicated in bold characters, whereas suggestive evidence (#, 0.001002 ≤ P < 0.012741) is in italics. H = Hotelling's T2 test, M = Mann-Whitney U test, S = Student's t test, W = Student's t test with Welch correction, L = Log-Rank test.

Bivariate analyses (*P* value)	
Latency until the first board entry *plus*	
Percentage of time spent on the board	Average duration of of a board entry
0.258690^{H}	0.100812^{H}
0.214112^{H}	0.608261^{H}
0.589949^{H}	0.090978^{H}
0.362718^{H}	0.098401^{H}
0.134911^{H}	0.016677^{H}
0.077632^{H}	0.017935^{H}
0.150948^{H}	0.301522^{H}
0.254198^{H}	0.042008^{H}
0.371587^{H}	0.383723^{H}
0.228876^{H}	*#0.003026*^{H}
0.312431^{H}	0.127455^{H}
0.717798^{H}	0.713713^{H}
0.337925^{H}	0.327658^{H}
0.189808^{H}	0.251590^{H}
#0.005532^{H}	***0.000423**^{H}
0.642054^{H}	0.233626^{H}
0.380317^{H}	0.389096^{H}
0.601366^{H}	*#0.010215*^{H}
0.012783^{H}	*#0.011458*^{H}
0.335827^{H}	0.316004^{H}
***0.000201**^{H}	***0.000187**^{H}

Factor analysis

This analysis included the total numbers, latencies and relative durations of all behavioural parameters. A specific assumption for a factor analysis is that a parameter should not be fully derived from one or more of the other included parameters. Therefore, average durations of the behavioural parameters were not included, because these parameters are based on the parameters percentage of time and total number. In contrast to the Hotelling's T2 test for factor analysis multivariate normality is not required, thus it is allowed to include the total numbers of the behavioural parameters. Nine clear factors emerged accounting for 74.5% of the total variance (Table 8). Factor 2 explained 11.4% of the total variance and appeared to reflect mainly anxiety, since the parameters total number of board entries, latency until the first board entry, and percentage of time on the board loaded highly on this factor. However, it should be noted that total number of hole explorations, latency until the first hole exploration and number of times the mouse is in the box also loaded highly on this factor.

Table 7 *Suggestive and significant evidence for QTLs influencing the difference in modified hole board behaviour (excluding anxiety) between C57BL/6J (n = 27) and consomic (n = 6) male mice*

Behavioural dimension / parameter	CSS1	CSS2	CSS3	CSS4
Risk assessment (significant strain difference: P < 0.002002;				
Total number of risk assessments				
(frequency)	*#0.004374*^{M}	0.659943^{M}	0.429205^{M}	0.547444^{M}
Latency until the first risked assessment				
(seconds)	0.220272^{M}	0.565111^{M}	0.375436^{M}	0.722032^{M}
	0.0593^{L}	0.6700^{L}	0.3846^{L}	0.5738^{L}
Undirected explorations (significant strain difference: P < 0.000668;				
Total number of rearing in the box				
(frequency)	*#0.005047*^{M}	0.499661^{M}	0.017058^{M}	0.970324^{M}
Latency until the first rearing in the box				
(seconds)	***0.000067***^{S}	0.584917^{S}	0.608470^{S}	0.148155^{S}
	0.0003^{L}	0.4560^{L}	0.5174^{L}	0.0624^{L}
Total number of rearing on the board				
(frequency)	1.000000^{M}	0.335227^{M}	0.077896^{M}	1.000000^{M}
Latency until the first rearing on the board				
(seconds)	1.000000^{M}	0.181818^{M}	0.053152^{M}	1.000000^{M}
	0.6374^{L}	0.2114^{L}	0.0200^{L}	0.6670^{L}

Table 9 summarizes suggestive and significant evidence for a QTL on a chromosome for the extracted, orthogonal factors. Factor analysis greatly reduced the total number of significant and suggestive QTLs as well as the number of chromosomes harbouring a QTL, but in general these results are in line with those obtained with the individual parameters (compare Table 9 to Tables 4 – 7). Interestingly, for chromosome 17 there is now evidence that this chromosome also contains QTL(s) for modified hole board behaviour. With respect to anxiety there was now only evidence for an anxiety QTL on chromosome 19. The narrow sense heritability of each factor was found to be within the expected range of behavioural phenotypes in mice (Valdar et al. 2006): 0.12 – 0.27 (Table 9).

CSS5	CSS6	CSS7	CSS8	CSS9	CSS10
suggestive strain difference: 0.002002 ≤ P < 0.025321)					
0.181870^{M}	0.234108^{M}	0.561755^{M}	0.043108^{M}	0.927322^{M}	***0.000001**^{M}
0.302361^{M}	0.827333^{M}	0.908612^{M}	0.059062^{M}	0.668902^{M}	0.070327^{M}
0.2984^{L}	0.6465^{L}	0.8290^{L}	0.0736^{L}	0.4436^{L}	*#0.0187*^{L}
suggestive strain difference: 0.000668 ≤ P < 0.008512)					
0.514526^{M}	***0.000050**^{M}	***0.000102**^{M}	0.284650^{M}	0.021124^{M}	***0.000005**^{M}
0.798117^{S}	***0.000606**^{S}	0.585184^{S}	0.711696^{S}	0.702265^{S}	***0.000191**^{S}
0.9394^{L}	*#0.0018*^{L}	0.6358^{L}	0.7781^{L}	0.5954^{L}	*#0.0019*^{L}
1.000000^{M}	1.000000^{M}	1.000000^{M}	0.077896^{M}	0.335227^{M}	1.000000^{M}
1.000000^{M}	1.000000^{M}	1.000000^{M}	0.028409^{M}	0.181818^{M}	1.000000^{M}
0.6374^{L}	0.6374^{L}	0.6374^{L}	0.0145^{L}	0.2114^{L}	0.6374^{L}

[Table 7 continued]

Behavioural dimension / parameter	CSS1	CSS2	CSS3	CSS4
Total number of hole explorations				
(frequency)	0.592030^{M}	0.954502^{M}	0.775893^{M}	0.186715^{M}
Latency until the first hole exploration				
(seconds)	0.448468^{W}	0.657486^{W}	0.484168^{S}	0.968310^{W}
	0.2429^{L}	0.5850^{L}	0.4439^{L}	0.2797^{L}
Directed explorations (significant strain difference: P < 0.000668;				
Total number of holes visited				
(frequency)	0.142287^{M}	0.742669^{M}	1.000000^{M}	1.000000^{M}
Latency until the first hole visited				
(seconds)	0.035007^{M}	0.742669^{M}	1.000000^{M}	1.000000^{M}
	0.0497^{L}	0.4376^{L}	0.5009^{L}	0.5389^{L}
Total number of unfamiliar object explorations				
(frequency)	***0.000330**^{M}	0.462197^{M}	*#0.005668*^{M}	0.346282^{M}
Latency until the first unfamiliar object exploration				
(seconds)	0.139324^{W}	0.033217^{S}	0.227776^{W}	0.982556^{W}
	#0.0015^{L}	0.0767^{L}	0.0916^{L}	0.6179^{L}
Percentage of time being busy with unfamiliar object explorations				
(%)	0.627392^{M}	0.096953^{M}	0.320936^{M}	0.874449^{M}
Average duration of an unfamiliar object exploration				
(seconds)	0.045260^{M}	0.423895^{M}	0.945636^{M}	0.417984^{M}
Memory (significant strain difference: P < 0.001002; suggestive strain				
Total number of familiar object explorations				
(frequency)	0.019659^{M}	0.606274^{M}	*#0.001233*^{M}	0.010617^{M}
Latency until the first familiar object exploration				
(seconds)	0.291556^{W}	0.284958^{S}	0.163486^{S}	0.163148^{W}
	0.1704^{L}	0.1654^{L}	0.2036^{L}	0.0341^{L}
Percentage of time being busy with familiar object exploration				
(%)	0.380165^{W}	0.382420^{W}	0.341390^{S}	0.827134^{S}
Average duration of a familiar object exploration				
(seconds)	0.290866^{W}	0.049854^{S}	0.041615^{W}	0.213040^{W}

CSS5	CSS6	CSS7	CSS8	CSS9	CSS10
0.882156^{M}	0.723900^{M}	0.200101^{M}	0.485503^{M}	0.724090^{M}	0.954499^{M}
0.053082^{S}	0.250025^{W}	0.518547^{S}	0.790842^{S}	0.165792^{S}	0.362571^{S}
#0.0029^{L}	0.0971^{L}	0.3902^{L}	0.9922^{L}	0.1041^{L}	0.4905^{L}

suggestive strain difference: 0.000668 ≤ P < 0.008512)

1.000000^{M}	1.000000^{M}	1.000000^{M}	1.000000^{M}	1.000000^{M}	1.000000^{M}
0.742669^{M}	1.000000^{M}	1.000000^{M}	0.742669^{M}	1.000000^{M}	1.000000^{M}
0.4376^{L}	0.5009^{L}	0.5009^{L}	0.4376^{L}	0.5009^{L}	0.5009^{L}
0.050171^{M}	0.041571^{M}	0.064722^{M}	0.080922^{M}	0.342122^{M}	*#0.002357*^{M}
0.029293^{S}	0.211134^{W}	0.209753^{W}	0.331141^{S}	0.196537^{S}	0.167044^{W}
0.0676^{L}	0.0749^{L}	0.0459^{L}	0.2496^{L}	0.1179^{L}	0.0479^{L}
0.245003^{M}	0.976090^{M}	0.446814^{M}	0.903769^{M}	0.141458^{M}	0.832223^{M}
0.665420^{M}	0.131878^{M}	1.000000^{M}	0.837892^{M}	0.600377^{M}	0.071736^{M}

difference: 0.001002 ≤ P < 0.012741)

0.020260^{M}	*#0.007601*^{M}	0.153383^{M}	0.020395^{M}	0.216909^{M}	***0.000465**^{M}
0.950785^{S}	0.040864^{S}	0.525313^{S}	0.052102^{S}	0.889463^{S}	0.165748^{W}
0.8682^{L}	0.0711^{L}	0.5408^{L}	0.1740^{L}	0.9905^{L}	0.0344^{L}
0.440489^{S}	0.195559^{S}	0.839321^{S}	0.275757^{S}	0.533249^{S}	0.628705^{S}
0.582442^{S}	0.025114^{W}	0.190343^{S}	0.292882^{W}	0.690575^{S}	***0.000481**^{S}

[Table 7 continued]

Behavioural dimension / parameter	CSS1	CSS2	CSS3	CSS4
Locomotion (significant strain difference: P < 0.002002; suggestive				
Total number of line crossings				
(frequency)	***0.000005**^{M}	0.086345^{M}	***0.000006**^{M}	***0.000646**^{M}
Latency until the first line crossing				
(seconds)	*#0.001542*^{S}	0.304349^{S}	0.371500^{S}	0.723090^{S}
	0.0259^{L}	0.2791^{L}	0.1043^{L}	0.6233^{L}
Arousal (significant strain difference: P < 0.000668; suggestive strain				
Total number of groomings				
(frequency)	0.332503^{M}	0.161156^{M}	0.887508^{M}	0.986860^{M}
Latency until the first grooming				
(seconds)	0.552246^{S}	0.553021^{S}	0.735835^{S}	0.516490^{S}
	0.9691^{L}	0.9556^{L}	0.6643^{L}	0.7334^{L}
Percentage of time being busy with grooming				
(%)	0.108307^{S}	0.677399^{S}	0.722608^{S}	0.585164^{S}
Average duration of a grooming				
(seconds)	0.429787^{S}	0.478581^{S}	0.799386^{S}	0.371266^{S}
Total number of boli				
(frequency)	0.449849^{M}	0.141109^{M}	0.967578^{M}	0.517867^{M}
Latency until the first bolus is produced				
(seconds)	0.910452^{M}	0.142022^{M}	0.612113^{M}	0.768840^{M}
	0.7445^{L}	0.0998^{L}	0.7270^{L}	0.5665^{L}
(bivariate analysis)				
Latency until the first grooming + Percentage of time being busy with grooming				
	0.083975^{H}	0.645978^{H}	0.756116^{H}	0.786282^{H}
Latency until the first grooming + Average duration of a grooming				
	0.490755^{H}	0.732988^{H}	0.938139^{H}	0.922515^{H}
Other behaviour (significant strain difference: P < 0.000801; suggestive strain				
Number of times the mouse is in the box				
(frequency)	*#0.001051*^{M}	0.864518^{M}	*#0.006469*^{M}	0.018086^{M}

CSS5	CSS6	CSS7	CSS8	CSS9	CSS10
strain difference: 0.002002 ≤ P < 0.025321)					
#0.003161^{M}	***0.000006***^{M}	0.608225^{M}	0.090956^{M}	0.776161^{M}	***0.000106***^{M}
0.414200^{S}	***0.000961***^{S}	0.561355^{S}	0.479130^{S}	*#0.002912*^{W}	0.085539^{W}
0.3121^{L}	*#0.0096*^{L}	0.8583^{L}	0.3093^{L}	*#0.0081*^{L}	*#0.0117*^{L}
difference: 0.000668 ≤ P < 0.008512)					
0.853019^{M}	0.114188^{M}	0.690363^{M}	0.549582^{M}	0.125289^{M}	0.690363^{M}
0.697220^{S}	*#0.001587*^{W}	0.460603^{S}	0.916765^{S}	0.147718^{S}	0.775575^{S}
0.8221^{L}	*#0.0017*^{L}	0.8690^{L}	0.8028^{L}	0.0693^{L}	0.8558^{L}
0.617240^{W}	0.069422^{S}	0.406281^{S}	0.541840^{S}	0.115538^{S}	0.455311^{S}
0.810318^{S}	0.211316^{S}	0.511193^{S}	0.470053^{S}	0.064807^{S}	0.486907^{S}
0.902733^{M}	0.141109^{M}	0.962174^{M}	0.376182^{M}	0.376182^{M}	*#0.005105*^{M}
0.743023^{M}	0.142023^{M}	1.000000^{M}	0.211833^{M}	0.370342^{M}	0.173486^{M}
0.8144^{L}	0.0998^{L}	0.9346^{L}	0.3092^{L}	0.3578^{L}	0.0657^{L}
0.747662^{H}	0.042310^{H}	0.676818^{H}	0.814441^{H}	0.238846^{H}	0.600268^{H}
0.857492^{H}	0.052235^{H}	0.720967^{H}	0.760277^{H}	0.146037^{H}	0.670635^{H}
difference: 0.000801 ≤ P < 0.010206)					
0.010496^{M}	0.043325^{M}	0.605742^{M}	0.470777^{M}	0.575199^{M}	0.029614^{M}

[Table 7 continued]

Behavioural dimension / parameter	CSS1	CSS2	CSS3	CSS4
Percentage of time in the box				
(%)	0.217758^{S}	0.426010^{W}	0.719308^{S}	0.169083^{S}
Average duration of a stay in the box				
(seconds)	*#0.002541*^{S}	0.805359^{S}	*#0.005891*^{S}	0.150521^{W}
Total number of urinations				
(frequency)	1.000000^{M}	0.324980^{M}	0.324980^{M}	0.554565^{M}
Latency until the first time urine is produced				
(seconds)	0.630154^{M}	0.356672^{M}	0.356672^{M}	0.528742^{M}
	0.7014^{L}	0.2244^{L}	0.2244^{L}	0.2244^{L}

Significant (*) evidence for a QTL on a chromosome is indicated in bold characters, whereas suggestive (#) evidence is in italics. M = Mann-Whitney U test, S = Student's t test, W =

Behavioural dimension / parameter	CSS11	CSS12	CSS13	CSS14	CSS15
Risk assessment (significant strain difference: P < 0.002002;					
Total number of risk assessments					
(frequency)	0.794504^{M}	0.321743^{M}	0.133061^{M}	0.203339^{M}	***0.000014**^{M}
Latency until the first risked assessment					
(seconds)	0.915491^{M}	0.882011^{M}	0.606179^{M}	0.375436^{M}	0.170970^{M}
	0.5368^{L}	0.8269^{L}	0.5361^{L}	0.3496^{L}	0.0452^{L}
Undirected explorations (significant strain difference: P < 0.000668;					
Total number of rearing in the box					
(frequency)	0.244602^{M}	***0.000006**^{M}	0.142049^{M}	0.608456^{M}	***0.000550**^{M}
Latency until the first rearing in the box					
(seconds)	0.681718^{S}	0.182403^{W}	0.511698^{W}	0.201930^{S}	0.193600^{W}
	0.5591^{L}	0.0277^{L}	0.6853^{L}	0.5170^{L}	0.0947^{L}
Total number of rearing on the board					
(frequency)	1.000000^{M}	1.000000^{M}	0.335227^{M}	1.000000^{M}	1.000000^{M}
Latency until the first rearing on the board					
(seconds)	1.000000^{M}	1.000000^{M}	0.181818^{M}	1.000000^{M}	1.000000^{M}
	0.6374^{L}	0.6374^{L}	0.2114^{L}	0.6374^{L}	0.6374^{L}

CSS5	CSS6	CSS7	CSS8	CSS9	CSS10
0.195699^{S}	0.172767^{S}	0.277575^{S}	0.199238^{S}	0.912770^{S}	0.368333^{S}
0.259517^{S}	0.232213^{W}	0.887658^{S}	0.433008^{W}	0.540893^{W}	0.118022^{W}
0.324980^{M}	0.324980^{M}	0.324980^{M}	1.000000^{M}	0.324980^{M}	*#0.009594*^{M}
0.356672^{M}	0.356672^{M}	0.356672^{M}	0.630154^{M}	0.356672^{M}	0.014848^{M}
0.2244^{L}	0.2244^{L}	0.2244^{L}	0.7014^{L}	0.2244^{L}	*#0.0069*^{L}

Student's t test with Welch-Satterthwaite correction, L = Log-Rank test.

Behavioural dimension / parameter	CSS11	CSS12	CSS13	CSS14	CSS15
suggestive strain difference: 0.002002 ≤ P < 0.025321)					
0.941818^{M}	0.538301^{M}	*#0.012235*^{M}	***0.000952**^{M}	*#0.020353*^{M}	0.175983^{M}
0.765212^{M}	0.543379^{M}	0.598768^{M}	0.056046^{M}	0.096766^{M}	0.319638^{M}
0.9317^{L}	0.4593^{L}	0.2553^{L}	***0.0009***^{L}	*#0.0241*^{L}	0.4853^{L}
suggestive strain difference: 0.000668 ≤ P < 0.008512)					
#0.001361^{M}	0.029658^{M}	*#0.002290*^{M}	0.014653^{M}	*#.000826*^{M}	0.110433^{M}
0.180630^{S}	0.031782^{S}	0.038726^{S}	0.829403^{S}	0.104527^{S}	0.352154^{W}
0.0819^{L}	0.0094^{L}	0.0270^{L}	0.5003^{L}	0.1821^{L}	0.1923^{L}
0.181818^{M}	0.335227^{M}	1.000000^{M}	1.000000^{M}	1.000000^{M}	1.000000^{M}
0.181818^{M}	0.181818^{M}	1.000000^{M}	1.000000^{M}	1.000000^{M}	1.000000^{M}
0.2114^{L}	0.2114^{L}	0.6374^{L}	0.6374^{L}	0.6374^{L}	0.6374^{L}

[Table 7 continued]

Behavioural dimension / parameter	CSS11	CSS12	CSS13	CSS14	CSS15
Total number of hole explorations					
(frequency)	0.776048^{M}	0.828742^{M}	0.864538^{M}	0.296003^{M}	0.038474^{M}
Latency until the first hole exploration					
(seconds)	0.944770^{W}	0.620009^{W}	0.147454^{S}	0.344834^{W}	0.076025^{W}
	0.4959^{L}	0.2407^{L}	0.2719^{L}	0.1058^{L}	0.0171^{L}
Directed explorations (significant strain difference: P < 0.000668; suggestive					
Total number of holes visited					
(frequency)	1.000000^{M}	1.000000^{M}	1.000000^{M}	1.000000^{M}	0.742669^{M}
Latency until the first hole visited					
(seconds)	0.742669^{M}	1.000000^{M}	1.000000^{M}	0.742669^{M}	0.742669^{M}
	0.4376^{L}	0.5009^{L}	0.5009^{L}	0.4376^{L}	0.4376^{L}
Total number of unfamiliar object explorations					
(frequency)	0.063434^{M}	0.080374^{M}	0.101925^{M}	0.058019^{M}	0.388746^{M}
Latency until the first unfamiliar object exploration					
(seconds)	0.804521^{W}	0.078318^{W}	0.057072^{W}	0.942159^{S}	0.094741^{W}
	0.5386^{L}	*#0.0071*^{L}	*#0.0028*^{L}	0.6728^{L}	*#0.0036*^{L}
Percentage of time being busy with unfamiliar object explorations					
(%)	0.595330^{M}	0.277471^{M}	0.627392^{M}	0.170748^{M}	0.306885^{M}
Average duration of an unfamiliar object exploration					
(seconds)	0.423895^{M}	0.981874^{M}	0.508346^{M}	0.837892^{M}	0.172995^{M}
Memory (significant strain difference: P < 0.001002; suggestive strain					
Total number of familiar object explorations					
(frequency)	0.040258^{M}	0.011579^{M}	0.031729^{M}	0.106287^{M}	0.043290^{M}
Latency until the first familiar object exploration					
(seconds)	0.780785^{W}	0.330886^{W}	0.427344^{W}	0.885978^{W}	0.043929^{W}
	0.6300^{L}	0.2161^{L}	0.4669^{L}	0.9896^{L}	*#0.0061*^{L}
Percentage of time being busy with familiar object exploration					
(%)	0.328510^{S}	0.578226^{W}	0.319027^{W}	0.422789^{W}	0.237147^{W}
Average duration of a familiar object exploration					
(seconds)	0.092306^{S}	0.251356^{W}	0.114603^{W}	0.293594^{W}	0.129576^{W}

Behavioural dimension / parameter	CSS11	CSS12	CSS13	CSS14	CSS15
0.544976^{M}	0.515290^{M}	0.353104^{M}	0.100641^{M}	0.545408^{M}	0.208766^{M}
0.943438^{W}	0.447514^{W}	0.404675^{S}	0.176963^{W}	0.428979^{W}	0.040564^{W}
0.9386^{L}	0.1971^{L}	0.5893^{L}	0.0824^{L}	0.3838^{L}	*#0.0012*^{L}
strain difference: 0.000668 ≤ P < 0.008512)					
1.000000^{M}	1.000000^{M}	1.000000^{M}	1.000000^{M}	1.000000^{M}	0.742669^{M}
1.000000^{M}	1.000000^{M}	1.000000^{M}	1.000000^{M}	0.742669^{M}	0.742669^{M}
0.5009^{L}	0.5009^{L}	0.5009^{L}	0.5009^{L}	0.4376^{L}	0.4376^{L}
0.556721^{M}	0.439493^{M}	0.079387^{M}	0.500474^{M}	1.000000^{M}	*#0.006094*^{M}
0.119603^{S}	0.336176^{S}	0.082817^{W}	0.070841^{W}	0.010137^{S}	0.040573^{W}
0.2751^{L}	0.5609^{L}	*#0.0032*^{L}	*#0.0018*^{L}	0.0385^{L}	*#0.0024*^{L}
0.446819^{M}	0.202453^{M}	0.867851^{M}	0.457297^{M}	0.832223^{M}	0.017552
0.698912^{M}	0.158399^{M}	0.172995^{M}	0.451151^{M}	0.632573^{M}	0.909530^{M}
difference: 0.001002 ≤ P < 0.012741)					
0.215187^{M}	0.849386^{M}	#0.008604^{M}	0.339929^{M}	0.584296^{M}	0.028926^{M}
0.256593^{S}	0.635672^{S}	0.121401^{W}	0.619239^{S}	0.667817^{S}	0.125233^{W}
0.1652^{L}	0.6189^{L}	0.0180^{L}	0.4518^{L}	0.8285^{L}	0.0164^{L}
0.495375^{W}	0.114626^{W}	0.646259^{S}	0.067782^{W}	0.306590^{W}	0.123279^{W}
0.302818^{W}	0.130686^{W}	0.075529^{W}	0.149422^{W}	0.235046^{W}	0.016261^{W}

[Table 7 continued]

Behavioural dimension / parameter	CSS11	CSS12	CSS13	CSS14	CSS15
Locomotion (significant strain difference: P < 0.002002; suggestive strain					
Total number of line crossings					
(frequency)	***0.000030**^{M}	***0.000023**^{M}	***0.000030**^{M}	0.758450^{M}	***0.000006**^{M}
Latency until the first line crossing					
(seconds)	0.909049^{S}	0.232423^{W}	0.985184^{S}	0.089507^{S}	0.082197^{W}
	0.9316^{L}	*#0.0121*^{L}	0.8869^{L}	0.1282^{L}	*#0.0057*^{L}
Arousal (significant strain difference: P < 0.000668; suggestive strain					
Total number of groomings					
(frequency)	0.549582^{M}	0.114188^{M}	0.513576^{M}	0.513576^{M}	0.080700^{M}
Latency until the first grooming					
(seconds)	0.243765^{S}	0.401689^{S}	0.977526^{S}	0.900063^{S}	0.409676^{S}
	0.2774^{L}	0.2442^{L}	0.7729^{L}	0.6837^{L}	0.4056^{L}
Percentage of time being busy with grooming					
(%)	0.071317^{S}	0.038476^{S}	0.525074^{S}	0.426324^{W}	0.051245^{S}
Average duration of a grooming					
(seconds)	0.133094^{S}	0.128245^{S}	0.714858^{S}	0.405628^{W}	0.951158^{S}
Total number of boli					
(frequency)	0.608940^{M}	0.076288^{M}	0.962174^{M}	0.723638^{M}	0.141109^{M}
Latency until the first bolus is produced					
(seconds)	0.604769^{M}	0.550564^{M}	0.829482^{M}	0.521984^{M}	0.142023^{M}
	0.4264^{L}	0.3521^{L}	0.8373^{L}	0.5144^{L}	0.0998^{L}
(bivariate analysis)					
Latency until the first grooming + Percentage of time being busy with grooming					
	0.192996^{H}	0.113885^{H}	0.754585^{H}	0.152250^{H}	0.152696^{H}
Latency until the first grooming + Average duration of a grooming + Latency until the first bolus is produced					
	0.277804^{H}	0.304003^{H}	0.918049^{H}	0.132075^{H}	0.638651^{H}

Behavioural dimension / parameter	CSS11	CSS12	CSS13	CSS14	CSS15
difference: 0.002002 ≤ P < 0.025321)					
***0.000010**^{M}	0.069602^{M}	***0.000002**^{M}	0.864789^{M}	*#0.001354*^{M}	0.073556^{M}
0.171209^{S}	0.464090^{S}	***0.000022**^{S}	0.524978^{S}	0.056733^{S}	0.078890^{S}
0.2798^{L}	0.3319^{L}	***0.0008**^{L}	0.5358^{L}	0.1055^{L}	0.1050^{L}
difference: 0.000668 ≤ P < 0.008512)					
0.549582^{M}	0.887508^{M}	0.017381^{M}	0.874016^{M}	1.000000^{M}	0.027331^{M}
0.975673^{S}	0.184783^{S}	0.101742^{S}	0.296223^{S}	0.771761^{W}	0.578976^{S}
0.7398^{L}	0.3534^{L}	0.2278^{L}	0.2372^{L}	0.8098^{L}	0.6471^{L}
0.415849^{S}	0.696515^{S}	0.073476^{W}	0.557029^{S}	0.805894^{S}	0.167084^{S}
0.559191^{S}	0.499445^{S}	0.316622^{S}	0.153270^{S}	0.536720^{S}	0.443317^{S}
0.608940^{M}	0.399585^{M}	0.493079^{M}	0.585164^{M}	0.048865^{M}	0.962174^{M}
0.569869^{M}	0.946992^{M}	0.910452^{M}	0.211832^{M}	0.406711^{M}	0.786853^{M}
0.4153^{L}	0.7586^{L}	0.7313^{L}	0.3092^{L}	0.1678^{L}	0.8361^{L}
0.641374^{H}	0.407967^{H}	0.001856^{H}	0.584531^{H}	0.899780^{H}	0.021537^{H}
0.827343^{H}	0.410657^{H}	0.258713^{H}	0.338599^{H}	0.725866^{H}	0.723597^{H}

[Table 7 continued]

Behavioural dimension / parameter	CSS11	CSS12	CSS13	CSS14	CSS15
Other behaviour (significant strain difference: P < 0.000668; suggestive					
Number of times the mouse is in the box					
(frequency)	0.104988^{M}	0.296458^{M}	0.069157^{M}	*#0.008960*^{M}	0.029546^{M}
Percentage of time in the box					
(%)	0.209691^{S}	0.992510^{S}	0.793332^{S}	0.689351^{S}	0.650914^{S}
Average duration of a stay in the box					
(seconds)	0.114749^{S}	0.300819^{W}	0.018117^{S}	0.011155^{S}	0.116645^{W}
Total number of urinations					
(frequency)	0.324980^{M}	1.000000^{M}	0.324980^{M}	1.000000^{M}	0.324980^{M}
Latency until the first time urine is produced					
(seconds)	0.630154^{M}	0.356672^{M}	0.630154^{M}	0.356672^{M}	0.630154^{M}
	0.2244^{L}	0.7014^{L}	0.2244^{L}	0.7014^{L}	0.2244^{L}

Table 8 *Orthogonal factor loadings for modified hole board behavioural parameters*

Behavioural dimension/ parameter	*Eigenvalue:* *% of the total variance:*	***DI/ME/LO*** Factor 1 7.58 25.3	***AN/UN/OT*** Factor 2 3.42 11.4	***AR*** Factor 3 2.53 8.4
Anxiety (AN)				
Total number of board entries		0.237	**0.910**	0.088
Latency until the first board entry		-0.480	**-0.657**	0.037
Percentage of time on the board		-0.019	**0.864**	-0.065
Risk assessment (RI)				
Total number of risk assessments		-0.443	-0.347	0.081
Latency until the first risk assessment		0.121	0.137	0.079
Undirected exploration (UN)				
Total number of rearing in the box		0.575	0.262	0.276
Latency until the first rearing in the box		-0.029	0.012	-0.036
Total number of rearing on the board		0.013	0.061	-0.057
Latency until the first rearing on the board		-0.046	0.031	0.037
Total number of hole explorations		0.141	**0.923**	0.102
Latency until the first hole exploration		-0.521	**-0.664**	-0.069

Behavioural dimension / parameter	CSS11	CSS12	CSS13	CSS14	CSS15
strain difference: 0.000668 ≤ P < 0.008512)					
0.542156^{M}	0.217306^{M}	0.038257^{M}	0.161021^{M}	0.414953^{M}	*#0.003455*^{M}
0.132872^{S}	0.082549^{S}	0.066143^{S}	0.927937^{S}	0.762005^{S}	0.774146^{S}
0.780299^{S}	0.504834^{S}	0.115963^{S}	0.182100^{W}	0.241037^{S}	0.066758^{W}
1.000000^{M}	0.324980^{M}	0.324980^{M}	0.324980^{M}	1.000000^{M}	0.324980^{M}
0.356672^{M}	0.356672^{M}	0.356672^{M}	0.356672^{M}	0.881217^{M}	0.356672^{M}
0.7014^{L}	0.2244^{L}	0.2244^{L}	0.2244^{L}	0.7941^{L}	0.2244^{L}

OT Factor 4	***DI*** Factor 5	***AR*** Factor 6	***RI/UN*** Factor 7	***UN*** Factor 8	***DI*** Factor 9
1.82	1.79	1.59	1.31	1.21	1.11
6.1	6.0	5.3	4.4	4.0	3.7
-0.004	0.015	0.039	-0.088	-0.017	-0.036
-0.009	0.096	-0.112	0.130	0.015	0.126
-0.084	-0.264	-0.094	-0.007	-0.055	0.080
0.205	-0.026	0.032	0.576	0.026	-0.039
-0.206	-0.002	-0.060	**-0.656**	-0.113	-0.065
0.110	-0.011	0.058	-0.480	0.190	-0.036
-0.141	0.049	-0.125	**0.726**	0.069	-0.009
-0.022	0.060	0.049	-0.047	**-0.848**	0.060
-0.017	0.071	-0.005	0.099	**0.821**	0.033
-0.023	-0.034	-0.012	-0.073	-0.014	-0.018
0.000	0.047	-0.067	0.137	-0.061	0.143

[Table 8 continued]

Behavioural dimension/ parameter	*Eigenvalue:* *% of the total variance:*	***DI/ME/LO*** Factor 1 7.58 25.3	***AN/UN/OT*** Factor 2 3.42 11.4	***AR*** Factor 3 2.53 8.4
Directed exploration (DI)				
Total number of holes visited		0.012	0.096	-0.004
Latency until the first hole visited		-0.023	-0.088	-0.053
Total number of unfamiliar object explorations		**0.694**	0.224	0.081
Latency until the first unfamiliar object exploration		**-0.751**	-0.228	0.124
Percentage of time being busy with unfamiliar object explorations		0.113	0.039	0.037
Memory (ME)				
Total number of familiar object explorations		**0.670**	0.093	0.291
Latency until the first familiar object exploration		**-0.721**	-0.274	0.026
Percentage of time being busy with familiar object exploration		0.260	-0.200	0.168
Locomotion (LO)				
Total number of line crossings		**0.702**	0.231	0.366
Latency until the first line crossing		**-0.667**	-0.065	0.115
Arousal (AR)				
Total number of groomings		0.078	0.026	**-0.811**
Latency until the first grooming		-0.008	0.029	**0.824**
Percentage of time being busy with grooming		-0.361	-0.196	-0.584
Total number of boli		-0.076	-0.016	-0.015
Latency until the first bolus is produced		0.104	0.031	-0.033
Other behaviour (OT)				
Number of times the mouse is in the box		0.459	**0.749**	0.040
Percentage of time in the box		0.463	-0.434	0.349
Total number of urinations		0.096	-0.019	0.103
Latency until the first time urine is produced		-0.069	0.037	-0.111

The data from all mice of this study (n = 204) were subject to factor analysis. The Kaiser-Meyer-Olkin measure is 0.742, indicating a high sampling adequacy for the factor analysis. Bartlett's test of sphericity indicates that the factor model is appropriate (P < 0.0005). Factor loadings > 0.6 are considered to be high and are indicated in bold. The nine factors account

OT	***DI***	***AR***	***RI/UN***	***UN***	***DI***
Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9
1.82	1.79	1.59	1.31	1.21	1.11
6.1	6.0	5.3	4.4	4.0	3.7
0.007	**-0.906**	0.007	-0.060	-0.036	-0.039
-0.003	**0.897**	-0.018	-0.017	-0.023	0.034
0.150	0.175	0.200	0.027	0.169	0.194
-0.008	0.075	-0.051	0.171	0.142	-0.128
0.045	0.119	0.174	-0.056	0.157	**0.679**
0.138	0.128	-0.015	-0.001	0.226	0.339
-0.018	0.161	0.070	0.167	0.106	-0.173
-0.197	-0.033	-0.166	0.212	-0.075	**0.617**
0.089	0.147	0.092	-0.195	0.227	0.006
0.050	0.078	-0.042	-0.020	0.094	0.103
-0.078	0.067	-0.022	0.120	-0.048	-0.076
0.111	-0.065	0.020	-0.026	-0.014	0.099
0.027	-0.168	0.154	-0.080	-0.126	0.161
0.191	0.025	**-0.895**	0.104	0.018	-0.009
-0.209	-0.001	**0.879**	0.026	-0.044	0.061
0.073	0.134	0.088	-0.099	0.078	0.149
0.121	0.273	0.050	0.053	0.094	-0.508
0.913	0.004	-0.231	0.038	-0.006	-0.007
-0.921	0.010	0.192	-0.038	-0.012	0.064

for 74.5% of the total variance. AN = anxiety, RI = risk assessment, UN = undirected exploration, DI = directed exploration, ME = memory, LO = locomotion, AR = arousal, OT = other behaviour.

Table 9 *Suggestive and significant evidence for QTLs influencing the difference in orthogonal factors between C57BL/6J (n = 27), A/J (n = 30) and consomic (n = 6 or n= 27) male mice and heritability (h2) of each orthogonal factor*

		DI/ME/LO	***AN/UN/OT***	***AR***
	h^2	*0.23*	*0.19*	*0.16*
Consomic line		Factor 1	Factor 2	Factor 3
CSS-1		***0.002869**^{S}	0.385539^{S}	0.638828^{S}
CSS-2		0.467514^{W}	0.758224^{W}	0.764877^{S}
CSS-3		*#0.006080*^{S}	0.959452^{S}	0.849246^{S}
CSS-4		0.140365^{S}	0.106764^{S}	0.704278^{S}
CSS-5		0.410666^{S}	0.865571^{S}	0.597058^{W}
CSS-6		***0.000892**^{S}	0.800449^{S}	***0.001890**^{W}
CSS-7		*#0.008106*^{S}	0.218616^{S}	0.498230^{S}
CSS-8		0.055153^{S}	0.597808^{S}	0.485749^{S}
CSS-9		0.186902^{S}	0.609744^{S}	0.079279^{S}
CSS-10		***0.000028**^{S}	0.477049^{S}	0.656405^{S}
CSS-11		*#0.026001*^{S}	0.803801^{S}	0.195953^{S}
CSS-12		*#0.004699*^{S}	0.817362^{S}	0.354524^{S}
CSS-13		*#0.018059*^{S}	0.603338^{S}	0.339801^{S}
CSS-14		0.431757^{S}	0.065601^{S}	0.999431^{S}
CSS-15		0.051778^{S}	0.054616^{S}	0.266905^{S}
CSS-16		0.054761^{S}	0.612627^{S}	0.562601^{S}
CSS-17		0.859001^{S}	0.598247^{S}	0.360360^{S}
CSS-18		***0.001968**^{S}	0.862802^{S}	0.141458^{W}
CSS-19 (n = 6)		0.578773^{S}	0.077985^{S}	0.177143^{S}
CSS-X		0.641326^{S}	0.489276^{S}	0.581028^{S}
CSS-Y		0.140447^{W}	0.016268^{S}	0.579818^{S}
A/J		***0.000000**^{W}	***0.000005**^{S}	***0.000034**^{S}
CSS19 (n = 27)		0.900181^{S}	***0.000821**^{S}	0.109817^{S}

Significant evidence (*, P <0.004) for a QTL on a chromosome is indicated in bold characters, whereas suggestive evidence (#, 0.004 ≤ P < 0.05) is in italics. M = Mann-Whitney U test, S = Student's t test, W = Student's t test with Welch correction.

OT	***DI***	***AR***	***RI/UN***	***UN***	***DI***
0.28	*0.16*	*0.15*	*0.27*	*0.19*	*0.12*
Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9
0.188532^{M}	0.120364^{W}	0.372230^{M}	***0.000775**^{S}	0.193268^{S}	0.880718^{S}
0.260266^{M}	0.348328^{W}	0.945636^{M}	0.585118^{S}	0.326374^{W}	0.814133^{S}
#0.035301^{M}	0.342586^{S}	0.423895^{M}	0.270202^{S}	0.142016^{W}	0.235089^{S}
0.240913^{M}	0.665321^{S}	0.762782^{M}	0.384352^{S}	0.625712^{S}	0.166404^{S}
#0.045260^{M}	0.342086^{W}	0.280658^{M}	0.604197^{S}	0.629578^{S}	0.223293^{S}
0.079910^{M}	0.789961^{S}	1.000000^{M}	*#0.022981*^{S}	0.219425^{S}	0.945246^{S}
0.119897^{M}	0.958739^{S}	0.568924^{M}	0.248440^{S}	0.117562^{S}	0.558485^{S}
0.479295^{M}	0.319623^{S}	0.945636^{M}	0.317295^{S}	0.166314^{W}	0.557963^{S}
0.051004^{M}	0.794201^{S}	0.909530^{M}	0.736204^{S}	*#0.013222*^{S}	0.435485^{S}
0.088791^{M}	0.231005^{S}	0.057315^{M}	***0.000139**^{S}	*#0.009035*^{S}	0.478910^{S}
0.108754^{M}	0.281451^{W}	0.665420^{M}	0.835975^{W}	0.500369^{S}	0.484871^{S}
0.108754^{M}	0.568399^{S}	0.071736^{M}	*#0.012775*^{S}	0.318477^{S}	0.374022^{S}
0.098387^{M}	0.643895^{S}	0.837892^{M}	0.077346^{S}	0.182564^{W}	0.400532^{S}
0.423895^{M}	0.160641^{W}	0.347861^{M}	0.632126^{S}	0.583669^{S}	0.712059^{S}
0.222446^{M}	0.338418^{W}	0.451151^{M}	***0.000279**^{S}	0.722159^{S}	0.613346^{S}
0.372230^{M}	0.565102^{S}	0.538219^{M}	0.258155^{S}	0.272934^{W}	0.342402^{S}
#0.035301^{M}	0.567539^{S}	0.204997^{M}	0.082386^{S}	*#0.006181*^{S}	*#0.020760*^{S}
0.347861^{M}	0.825900^{S}	0.423895^{M}	*#0.030589*^{S}	0.545806^{S}	0.614157^{S}
0.665420^{M}	0.667780^{S}	0.397594^{M}	*#0.009561*^{S}	0.264264^{S}	*#0.043440*^{S}
0.538219^{M}	0.292938^{W}	0.222446^{M}	***0.000042**^{W}	0.147080^{S}	0.552526^{S}
***0.001439**^{M}	0.318307^{W}	0.108754^{M}	0.670873^{S}	0.624064^{S}	0.324276^{S}
0.674238^{M}	***0.001930**^{M}	0.697581^{M}	0.341429^{S}	***0.002429**^{M}	0.746125^{S}
0.371306^{M}	0.600606^{W}	0.449646^{M}	0.011880^{S}	0.170484^{S}	0.798753^{S}

AN = anxiety, RI = risk assessment, UN = undirected exploration, DI = directed exploration, ME = memory, LO = locomotion, AR = arousal, OT = other behaviour.

Discussion

We examined a commercially available set of mouse chromosome substitution strains for which the parental strains differ in their anxiety-related behaviour in the modified hole board test (Table 4, Figures 2 and 3). In this study we implemented the Hotelling's T2 test for identifying chromosomes that bear QTLs for anxiety-related behaviour and compared the results obtained with this type of multivariate (bivariate) analysis with those from the univariate analyses (Student's t test, Mann Whitney U test and Log-Rank test). Bivariate when compared with univariate analyses resulted in a slightly higher number of chromosomes with (significant) evidence for an anxiety QTL (Tabel 7), and the identified chromosomes were partly different: univariate analysis, chromosomes 5, 8, 10 and Y; bivariate analysis, chromosomes 10, 15, 18, 19 and Y. Therefore, for the consomic survey we suggest to carry out both univariate and bivariate (genetic) analyses in parallel.

Table 10 gives an overview of the chromosomes which show evidence for a QTL influencing the difference in modified hole board behaviour between C57BL/6J and A/J mice. With respect to anxiety-related behaviour, the present results for anxiety agreed well with previous genetic studies using the A/J and C57BL/6J as parental strains (Table 11). However, in contrast with the other (consomic) studies and probably as a result of the behavioural test we used (modified hole

Table 10 *Overview of suggestive and significant evidence for QTLs influencing the difference in modified hole board behaviour between C57BL/6J and A/J mice*

Behavioural dimension	1	2	3	4	5	6	7	8	9
Anxiety	-	-	-	-	x	-	-	x	-
Risk assessment	x	-	-	-	-	-	-	-	-
Undirected exploration	**X**	-	-	-	x	**X**	**X**	-	-
Directed exploration	**X**	-	-	-	-	-	-	-	-
Memory	-	-	x	-	-	x	-	-	-
Locomotion	**X**	-	**X**	**X**	x	**X**	-	-	x
Arousal	-	-	-	-	-	x	-	-	-
Other behaviour	x	-	x	-	-	-	-	-	-
Factor 1: DI / ME / LO	**X**	-	x	-	-	**X**	x	-	-
Factor 2: AN / UN / OT	-	-	-	-	-	-	-	-	-
Factor 3: AR	-	-	-	-	-	X	-	-	-
Factor 4: OT	-	-	x	-	x	-	-	-	-
Factor 5: DI	-	-	-	-	-	-	-	-	-

board), our results suggest that the transfer of the Y chromosome from the A/J mouse onto the C57BL/6J background increased anxiety-related behaviour (Figure 3a and Table 6). Thus, genetic variation in genes on the Y chromosome may influence anxious behaviour in the mouse. To the best of our knowledge this has not been described previously. Up until now, associations of the Y chromosome with behavioural traits in mice have only been reported for inter-male aggression (e.g. Roubertoux et al. 1994). Intriguingly, Guillot and Chapouthier (1996) have found that males of more aggressive strains are also more anxious. Unfortunately, Guillot and Chapouthier (1996) didn't test males from the A/J strain and it is well known that A/J mice are markedly unaggressive (Brodkin et al. 2002). It can be concluded that if there is a relationship between inter-male aggression and anxiety-related behaviour it is certainly not a simple one. Sry (sex determining region of chromosome Y), a transcription factor, might be a good candidate gene. The 3'-end of this mouse gene (i.e. the CAG trinucleotide repeat) shows strain and natural variants (Albrecht & Eicher 1997; Coward et al. 1994). Furthermore, based on the light-dark box and an F2 mapping population, there is evidence that the X chromosome also harbours a QTL influencing anxiety-related behaviour (Table 11). Thus both X- and Y-linked QTLs might at least in part explain gender and strain differences in anxiety-related behaviour.

Chromosomes											
10	11	12	13	14	15	16	17	18	19[1]	X	Y
X	-	-	-	-	**X**	-	-	x	**X**	-	**X**
x	-	-	-	-	**X**	-	-	x	**X**	x	-
X	-	**X**	-	-	**X**	x	-	x	-	x	x
x	-	x	x	-	x	-	-	x	-	-	x
X	-	-	-	-	x	-	-	x	-	-	-
X	**X**	**X**	**X**	-	**X**	**X**	-	**X**	-	x	-
x	-	-	-	-	-	-	-	-	-	-	-
x	-	-	-	x	-	-	-	-	**X**	-	x
X	x	x	x	-	-	-	-	**X**	-	-	-
-	-	-	-	-	-	-	-	-	**X**	-	-
-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	x	-	-	-	**X**
-	-	-	-	-	-	-	-	-	-	-	-

[Table 10 continued]

Behavioural dimension	1	2	3	4	5	6	7	8	9
Factor 6: AR	-	-	-	-	-	-	-	-	-
Factor 7: RI / UN	**X**	-	-	-	-	x	-	-	-
Factor 8: UN	-	-	-	-	-	-	-	-	x
Factor 9: DI	-	-	-	-	-	-	-	-	-

X = significant, x = suggestive, and - = no evidence for a QTL on a particular chromosome. AN = anxiety, RI = risk assessment, UN = undirected exploration, DI = directed exploration, ME =

It seems that almost all chromosomes, with the exception of chromosomes 2, 7, 12 and 16, carry QTL(s) that influence the difference in anxiety-related behaviour between C57BL/6J and A/J mice (Table 11). Table 11 shows that significant evidence for anxiety QTLs is clustered mainly on chromosomes 1 (number of significant QTLs = 4), 10 (number of significant QTLs = 7) and 19 (number of significant QTLs = 4). Willis-Owen & Flint (2006) inspected the literature and found largest quantity of evidence pointing towards the presence of one or more anxiety QTL on chromosome 1. In the present study, using the modified hole board test and avoidance behaviour towards an unprotected area as anxiety parameter, we failed to detect an association with mouse chromosome 1 (Tables 6, 9 and 10, Figure 3). Combining the results from Table 11 with those from Willis-Owen and Flint (2006) we may conclude that all mouse chromosomes (including both sex chromosomes) harbour genes that influence anxiety-related behaviour in the laboratory mouse.

Chromosomes											
10	11	12	13	14	15	16	17	18	19[1]	X	Y
-	-	-	-	-	-	-	-	-	-	-	-
X	-	x	-	-	**X**	-	-	x	-	**X**	-
x	-	-	-	-	-	-	x	-	-	-	-
-	-	-	-	-	-	-	x	-	-	-	-

memory, LO = locomotion, AR = arousal, OT = other behaviour. [1]Based on 27 host and 27 consomic mice (see Tables IV, V and IX).

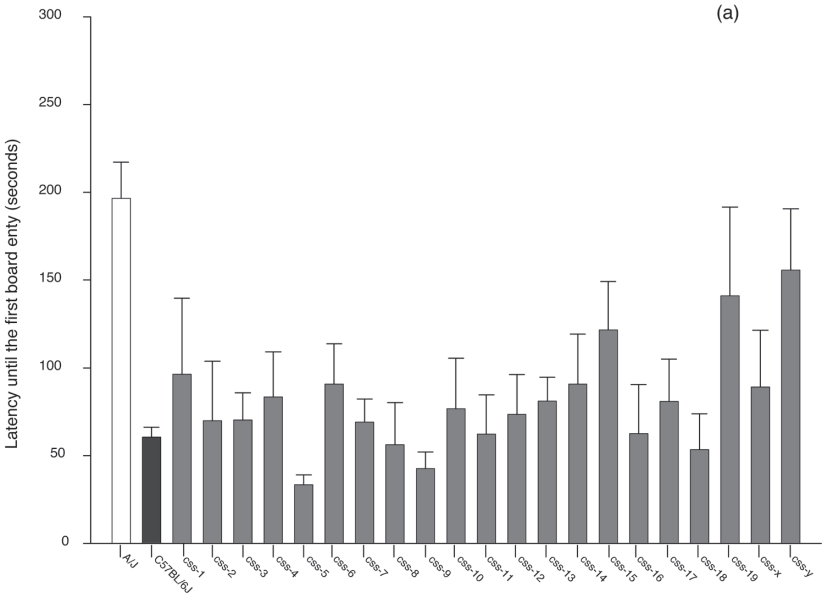
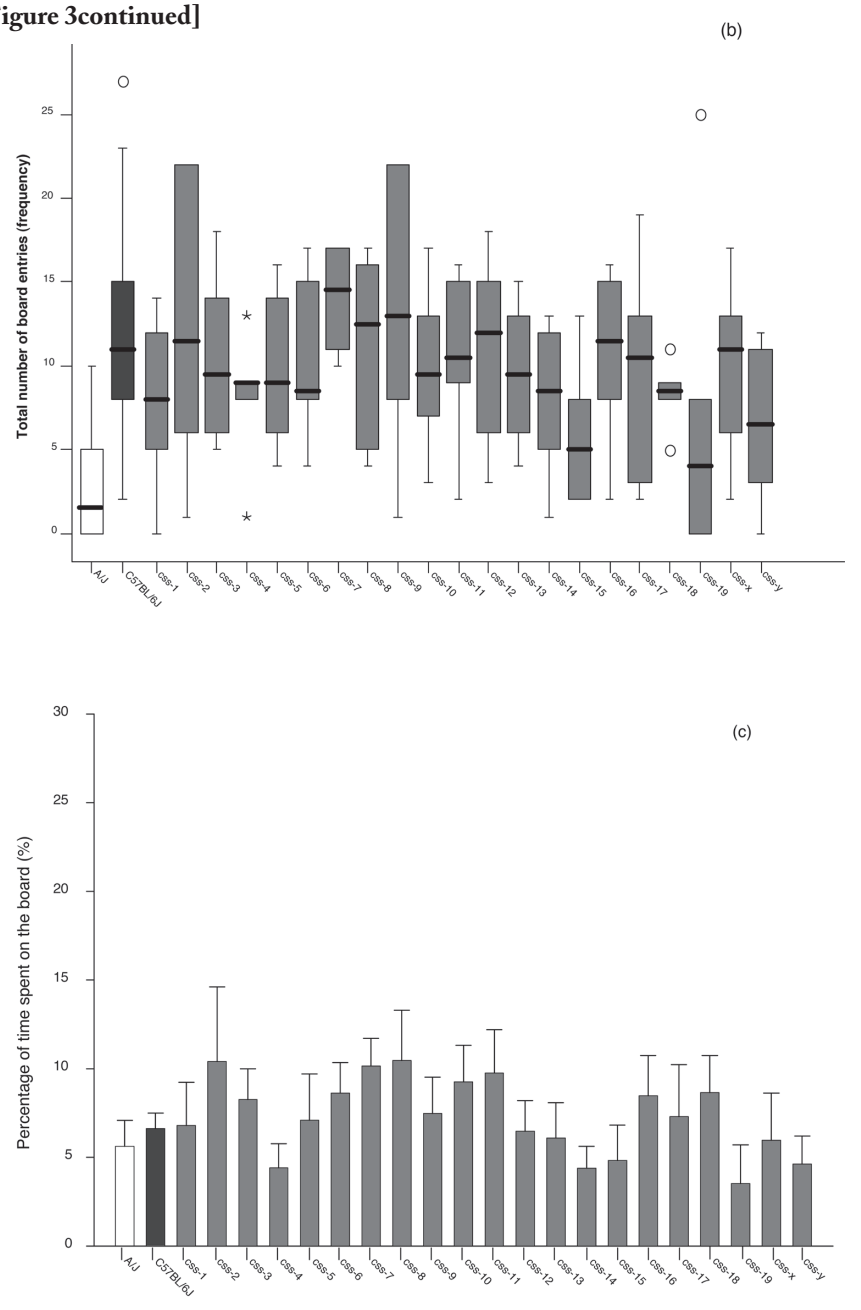
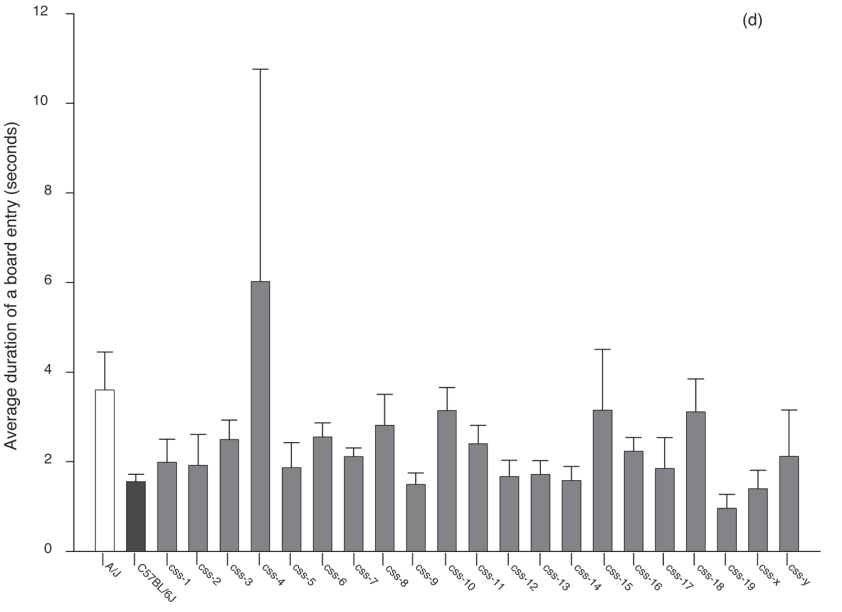


Figure 3 – *d Anxiety-related behaviour in the modified hole board. Results for 20 CSSs (n = 6/consomic strain), the C57BL/6J host strain (n = 27), and A/J donor strain (n = 30). (a) Latency until the first board entry; (b) Total number of board entries; (c) Percentage of time spent on the board; (d) Average duration of a board entry. Results are presented as means + SEM (diagrams a, c, and d) or box plots (diagram b). Significant (P < 0.001002) and suggestive (0.001002 ≤ P < 0.012741) evidence for an anxiety QTL on a particular chromosome is indicated by $ and #, respectively. In diagrams (b) and (d) outliers and extreme cases are indicated with o and *, respectively.*

[Figure 3continued]





The open field has been used as behavioural test for detecting QTLs influencing anxiety-related behaviour in six mapping populations with A/J and C57BL/6J as progenitors: an F2 intercross, a set of recombinant inbred strains (RIS), a set of recombinant congenic strains (RCS), an advanced intercross population (AIL), a panel of interval-specific congenic strains (ISCS) and a panel of chromosome substitution strains (Table 11). The open field QTLs on chromosome 4 was only detected with RIS and those on chromosomes 17 and 18 were exclusively mapped with the RCS method (Table 11). This clearly illustrates why the use of more than one mapping population derived from the same parental strains is often advantageous (Bergeson et al. 2001). From Table 11 it can be seen that the open field QTL on chromosome 5 was up until now only detected with RIS. However, Singer et al. (2005) did not test a complete CSS panel: CSS-5 was at the time of testing not complete. Another reason for using more than one mapping population is the identification of gene-gene interactions. Chromosome substitution strains are not suitable for studies on epistatic interactions between QTLs on different chromosomes (i.e. identifying the chromosomes that contain the QTLs that interact with each other), but are a sensitive method in the search for additive QTLs or interacting QTLs on the same chromosome with relative small effects. Other mapping populations (i.e. F2 intercross, RIS, RCS and AIL) are more ideal for detecting interchromosomal, interlocus interactions. For instance, using the computer programme Map Manager QTX and AIL as mapping population, found (suggestive) evidence for epistatic interactions with respect to anxiety-related behaviour between chromosomes 10 and 1, and chromosomes 19 and 1 (Zhang et al. 2005). To study this interaction it would be worthwhile to generate so-called double consomic lines.

Interestingly, based on three behavioural tests (light-dark box, open field and modified hole board) and five mapping populations (F2, RIS, RCS, AIL and CSS) there is strong evidence for QTL(s) influencing the difference in anxiety-related behaviour between C57BL/6J and A/J mice on chromosome 19 (Table 11). This prompted us – together with the finding that CSS-19 did not differ in locomotion from the C57BL/6J host strain (Table 10) – to focus on mouse chromosome 19. Other groups, e.g. the Wellcome Trust Centre for Human Genetics in Oxford, U.K. (Fullerton 2006; Willis-Owen & Flint 2006), have already focused on mouse chromosome 1. Rat chromosome 1 is homologous mainly to mouse chromosome 7. However, some rat chromosome 1 segments are syntenic to other mouse chromosomes (Pravenec et al. 1999). For example, there is strong conservation in genetic content between the entire mouse chromosome 19 and the distal part of

Table 11 *Suggestive and significant evidence for QTLs influencing the difference in anxiety-related behaviour between C57BL/6J and A/J mice*

Method	Behavioural test (apparatus)	Number of chromosomes with a QTL	1	2	3	4	5	6	7	8	9
F_2	Open field	*7*	**X**	-	**X**	-	-	-	-	x	-
F_2	Light-dark box	*5*	-	-	-	-	-	x	-	-	-
	Open field	*3*	**X**	-	-	-	-	x	-	-	-
RIS	Light-dark box	*2*	-	-	-	-	-	-	-	-	x
	Open-field	*3*	-	-	**X**	-	-	-	-	-	**X**
RIS	Open field	*8*	x	-	-	x	**X**	-	-	**X**	x
RCS	Open field	*8*	-	-	**X**	-	-	**X**	-	**X**	-
AIL	Light-dark box	*2*	-	-	-	-	-	-	-	-	-
	Open field	*1*	-	-	-	-	-	-	-	-	-
ISCS	Light-dark box	(*1*)	-	-	-	-	-	-	-	-	-
	Open field	(*1*)	-	-	-	-	-	-	-	-	-
CSS	Light-dark box	*5*	**X**	-	-	-	-[1]	**X**	-	-	**X**
	Open field	*4*	**X**	-	-	-	-[1]	**X**	-	-	-
CSS	Modified hole board	*7*	-	-	-	-	x	-	-	x	-

X = significant, x = suggestive, and - = no evidence for an anxiety QTL on a particular chromosome. [1]Singer et al. (2005) did not test a complete CSS panel: CSS-5 was at the time of testing not complete. [2]Based on 27 host and 27 consomic mice (see Tables IV and IX). Abbreviations:

the long arm of rat chromosome 1 (Yamasaki et al. 2001). Fernández-Teruel et al. (2002) have found a suggestive QTL influencing anxiety on rat chromosome 1. In addition, the whole genome search of Terenina-Rigaldie et al. (2003) also revealed a significant anxiety QTL on this rat chromosome. Unfortunately, the rat chromosome 1 segments indentified by Fernández-Teruel et al. (2002) and (Terenina-Rigaldie et al. 2003) are not syntenic to mouse chromosome 19. This is an example in which comparative genomics fails to narrow the murine QTL interval (DiPetrillo et al. 2005). Furthermore, linkage for an anxiety proneness phenotype (early onset susceptibility to anxiety disorders) was suggested on the q-arm of human chromosome 10 (Smoller et al. 2001), and mouse chromosome 19 has homologous regions on human chromosomes 9, 10 and 11 (Poirier & Guenet 1999).

Chromosomes												
10	11	12	13	14	15	16	17	18	19	X	Y	Reference
X	x	-	-	-	x	-	-	-	**X**	-	-	Gershenfeld *et al.*, 1997
X	-	-	-	-	x	-	-	-	x	x	-	Gershenfeld & Paul, 1997
-	-	-	-	x	-	-	-	-	-	-	-	
-	x	-	-	-	-	-	-	-	-	-	-	Mathis *et al.*, 1995
-	**X**	-	-	-	-	-	-	-	-	-	-	
-	-	-	x	**X**	-	-	-	-	x	-	-	Gill and Boyle, 2005
-	-	-	x	x	-	-	x	x	x	-	-	Gill and Boyle, 2005
X	-	-	-	-	-	-	-	-	**X**	-	-	Zhang *et al.*, 2005
X	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	
X	-	-	-	-	-	-	-	-	-	-	-	Zhang *et al.*, 2005
X	-	-	-	-	-	-	-	-	-	-	-	
-	-	-	-	-	-	-	**X**	-	**X**	-	-	Singer *et al.*, 2004 & 2005
-	**X**	-	-	-	**X**	-	-	-	-	-	-	
X	-	-	-	-	**X**	-	-	x	$\mathbf{X}^2$	-	**X**	This article

F2 = an F2 intercross population, RIS = a set of recombinant inbred strains, RCS = a set of recombinant congenic strains, AIL = an advanced intercross population, ISCS = a panel of interval-specific congenic strains, CSS = a panel of chromosome substitution strains.

In summary, the present study with chromosome substitution strains suggests that mouse chromosomes 5, 8, 10, 15, 18, 19, and Y each contain at least one QTL that is involved in anxiety-related behaviour in the modified hole board. We suggest to do the consomic survey with both univariate and multivariate (bivariate) analyses and to use a two-stage approach. While others focus on chromosome 1, we have special interest for mouse chromosome 19 because of its more specific association with anxiety-related behaviour. The mapping of the QTL(s) for anxiety on chromosome 19 by using an F2 intercross between CSS-19 and the C57BL/6J host strain is the subject of a subsequent study. Further experiments, including the development of (double) congenic strains or knockout strains after gene cloning, are necessary to precisely map the QTL(s) and to confirm the role of the suggested candidate genes.

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Chapter 4

The alpha 2A-adrenoceptor underlies arousal behaviour in C57BL/6J-Chr19^{A}/NaJ mice

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submitted

Abstract

Acute stress is known to impair memory functions in both men and laboratory rodents. In human the alpha 2A-adrenoceptor system is known to play a critical role in regulating acute neuropsychological stress responses and, ultimately, stress coping behaviour. In search for neurobiological and central nervous mechanisms behind these behaviours we investigated if the alpha 2A-adrenoceptor is involved in these mechanisms. Phenotypical differences between the A/J and C57BL/6J (B6) mouse inbred strains were evaluated in previous studies. These data showed significant strain differences in various motivational systems (anxiety, exploration, locomotion, arousal etc). From the literature it is known that chromosome 19 contains the gene for the adrenergic alpha 2A receptor that is thought to be involved in emotional behaviours, among others anxiety-related behaviour and arousal. We investigated if this pathway could possibly be involved in anxiety/arousal susceptibility by applying an agonist (dexmedetomidine) and an antagonist (atipamezole) of the alpha 2A-adrenoceptor to mice from a consomic strain (C57BL/6J-Chr 19^{A}/NaJ, abbreviated to CSS19 = anxious), and the corresponding donor (A/J = anxious) and host (B6 = non-anxious) strains. The mice were tested in the modified hole board (mHB) test which allows for the assessment of a variety of behavioural patterns by use of only one test. Besides the mHB-test, a forced swimming test (FST) was conducted to test for stress coping behaviour. Results of the behavioural testing in the mHB-test showed a wide variety of strain differences and also a treatment effect for some of the arousal parameters. The FST showed

[1] Both authors contributed equally to this paper

some effects of dexmedetomidine and atipamezole on stress coping behaviour. In conclusion, the involvement of the alpha 2A-adrenoceptor, located on mouse chromosome 19, on anxiety-related behaviour remains unclear and will possibly not play a main role in the development of anxiety related behaviour in mice. However, we could show involvement of the receptor in stress-coping behaviour by modulation of arousal related parameters.

Keywords

alpha 2A-adrenoceptor, arousal, atipamezole, consomic strain, dexmedetomidine, forced swim test, modified hole board, stress responds

Introduction

In human the alpha 2-adrenoceptor system is known to play a critical role in regulating acute neuropsychological stress responses and, ultimately, stress coping behaviour (Domes et al. 2004; Ruffolo et al. 1993; Stamatakis et al. 2008). Based on the different affinities on a series of agonists, antagonists, and the identification in different cell line cultures, the alpha 2-adrenoceptors were initially sub classified into two subtypes termed alpha 2A and alpha 2B (McGrath et al. 1989). By binding studies in opossum kidney epithelial cells, Murphy & Bylund (1988) identified a third alpha 2-adrenoceptor subtype, alpha 2C. Activating the alpha 2-adrenoceptors with a specific agonist can result in e.g. bradycardia, hypotension, and reduced anxiety (Sallinen et al. 1997). In search for the central nervous mechanisms behind anxiety related avoidance behaviour in mice, we investigated if the alpha 2A-adrenoceptor affects experimental behaviour in this species.

From the literature it is known that mouse chromosome 19 contains the gene for the adrenergic alpha 2A-adrenaceptor (Oakey et al. 1991), that is thought to be involved in emotional behaviours among such as anxiety-related behaviour. A/J and C57BL/6J (B6) mice show a variety of significant behavioural strain differences (Laarakker et al. 2008a). Based on these two inbred strains, 21 chromosome substitution strains (CSSs, also called consomic strains or lines) were created (Singer et al. 2004; Singer et al. 2005) for which the A/J served as donor and B6 as host strain. Being interested in the regulatory function of the alpha 2A-adrenoceptor with respect to anxiety-related avoidance behaviour, we included the C57BL/6J-Chr 19^{A}/NaJ (CSS19) consomic strain in our study. Thus, the effect of a highly specific agonist (dexmedetomidine) and antagonist (atipamezole) of the alpha 2A-adrenoceptor were tested on the CSS19, A/J and B6 mice. It has been shown that the A/J strain displays a high degree of anxiety-related behaviour, which is - in contrast to the B6 strain - showing intermediate levels of anxiety (Crawley & Davis 1982; Crawley et al. 1997; Laarakker et al. 2008a).

Mice were tested in the modified hole board (mHB) test, which allows for the assessment of a variety of behavioural patterns by use of only one test (Ohl et al. 2001a.). By use of this test, we aimed at evaluating the avoidance towards a novel,

unprotected area as well as potentially confounding motivational systems (Ohl et al. 2001a, Kas et al. 2004). Subsequently, a forced swimming test (FST) was performed to more specifically investigate the stress coping behaviour in the same animals, since it has often been shown that neuronal circuits regulating anxiety- and stress-coping are closely interacting. In the FST, struggling behaviour is understood as being indicative for active stress coping behaviour, while floating reflects passive coping behaviour (Armario et al. 1988; Liebsch et al. 1998). Besides behavioural parameters, we also looked at circulating corticosterone levels, which are known to be indicative for the hormonal stress response (Amico et al. 2004; Booker et al. 2007).

To prove a direct involvement of the alpha 2A-adrenoceptor in avoidance and stress-coping behaviour, it could be expected that atipamezole would act anxiogenic while dexmedetomidine should induce an anxiolytic effect.

Materials & Methods

Ethics

The protocol of the experiment was peer-reviewed by the scientific committee of the Department of Animals, Science & Society, Utrecht University, the Netherlands, and approved by the Animal Experiments Committee of the Academic Biomedical Centre, Utrecht-The Netherlands. The Animal Experiments Committee based its decision on 'De Wet op de Dierproeven' (1996) and on the 'Dierproevenbesluit' (1996); both are available online (http://www.nca-nl.org/). Further, all animal experiments followed the 'Principles of laboratory animal care' and refer to the 'Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research' (National Research Council 2003).

Mice

Studies were performed with naive, 6-8-week-old male B6 (n = 45), A/J (n = 45) and CSS19 (n = 45) mice, which were purchased from The Jackson Laboratory (Bar Harbor, USA). All animals were kept in the test room in the laboratory animal facilities of the Department of Animal Science & Society (Utrecht University) for 2 weeks under standard laboratory conditions (reversed light: dark cycle: white lights on at 1900h. (maximal 150 lux), red lights on at 0700h; ambient temperature, 21.0 ± 2.0°C; relative humidity, minimum 45%; radio music turned constantly on as background noise and ad libitum water and food). All mice were housed singly in Macrolon® Type II L (prolonged) cages (size: 365 x 207 x 140 mm, floor area 530 cm2; Techniplast, Milan, Italy), with standard bedding material, a carton shelter and two tissues. After a habituation period of two weeks in which the mice were handled at least three times per week for a few minutes the experimental procedures started. Two days before, and 30 minutes after the modified hole board test (mHB-test) experiment, blood was collected from each

animal by performing a tail cut. After a recuperation period of three weeks, mice were tested in a forced swimming test (FST). 30 minutes after the FST, mice were euthanized by decapitation. Trunk blood was collected.

Drugs

Dexmedetomidine (5μg/kg body weight; Orion Corporation, Orion Pharma, Turku, Finland), atipamezole (3mg/kg body weight; Veterinary Pharmacy, Faculty of Veterinary Medicine, Utrecht) or saline (0.9%) was injected intra peritoneal in a volume of 100 μl.

Modified hole board

The mHB-test (Figure 1) is a combination of the original hole board (Baldwin et al. 1989), and an open field test (Picciotto 1998). The mHB-test makes it possible to test rodents for behavioural and cognitive parameters. The (hole) board, consisted of an opaque gray PVC-board (60 x 20 x 0.5cm, length x width x height) with 20 cylinders (15x15 mm, diameter x height) placed in three lines. In order to create a central area of an open field, the board was positioned in the centre of a box (100 x 50 x 50 cm, length x width x height) that was made of the same material as the board. The board was moderately illuminated (red light, maximal 150 lux) with the help of a stage lamp. The area surrounding the hole board was divided by grey lines into 10 rectangles (20 x 15 cm, length x width) and 2 squares (20 x 20 cm, length x width). Each of the animals had an object (either a die or a hex nut) in their home cage for 48 hours. Objects were removed 24 hours prior to the experiment and placed in the mHB. Besides a familiar object there was also an unfamiliar object placed in the mHB. The animals were subsequently placed in the mHB for 5 minutes each, always starting in the same corner, facing the open area.

Experimental procedure mHB-test

Experiments were carried out between 10:00 a.m. and 2:00 p.m. throughout. This time frame represents the first part of the dark phase, when animals are most active. Per day nine randomly selected animals were tested for 5 min in the mHB. Dexmedetomidine, atipamezole or saline was intra peritoneal (i.p.) injected 30 min before testing. Animals were randomly assigned to treatment conditions. The experiments took place under red light conditions (maximal 5 lux).

From all individuals two blood samples were taken for circulating corticosterone determination. In order to measure the basal corticosterone concentration of the animals, a blood sample was taken 48 hours before the experiment. 30 minutes after a stress response, the blood plasma corticosterone concentration reaches its peak; therefore the second blood sampling took place 30 minutes after an individual was taken out of the mHB. Blood sampling was conducted by fixating the tail and making a small incision at the absis of the tail.

Each animal was transferred directly from its home cage to mHB, for 5 minutes of free exploration. All behavioural tests were videotaped (for raw data storage) from above the box. The behaviour was scored by hand using the program Observer 4.1 (Noldus, Wageningen, The Netherlands). The test set-up was cleaned with water and a damp towel between each mouse. Several parameters (Table 1) being indicative for anxiety-related behaviour, locomotion, exploration, risk assessment, memory, arousal and immobility (Ohl et al. 2001b) were measured.

Table 1 *Behavioural parameters measured in the modified hole board*

Behavioural dimension	***Behavioural parameter***
Anxiety	Total number of board entries
	Latency until the first board entry
	Percentage of time on the board
Risk assessment	Total number of risk assessments
	Latency until the first risk assessment
Undirected exploration	Total number of rearings in the box
	Latency until the first rearing in the box
	Total number of rearings on the board
	Latency until the first rearing on the board
	Total number of hole explorations
	Latency until the first hole exploration
Directed exploration	Total number of holes visited
	Latency until the first hole visited
	Total number of unfamiliar object explorations
	Latency until the first unfamiliar object exploration
	Percentage of time being busy with unfamiliar object explorations
Memory	Total number of familiar object explorations
	Latency until the first familiar object exploration
	Percentage of time being busy with familiar object exploration

Description of the mouse behaviour
Mouse on the board
Stretched body posture, including hind-paws
Rearing on hind-paws in the box
Rearing on hind-paws on the board
Exploration of a cylinder (hole) on the board
Nose-poking into a cylinder (hole) on the board
Exploration of the unfamiliar (new) object
Exploration of the familiar object

[Table 1 continued]

Behavioural dimension	*Behavioural parameter*
Locomotion	Total number of line crossings
	Latency until the first line crossing
Arousal	Total number of groomings
	Latency until the first grooming
	Percentage of time being busy with grooming
	Total number of boli
	Latency until the first bolus is produced
Other behaviour	Total number of being motionless
	Latency until the first time the mouse is motionless
	Percentage of time being motionless
	Number of times the mouse is in the box
	Percentage of time in the box

Forced swimming test

The FST, is a variant of the originally described Porsolt swim test by Porsolt et al. (1977), involves placing a rodent in a with water filled cylinder for a few minutes, and measure their behaviour. Here each mouse was placed in a transparent glass cylinder (20 x 40 cm, diameter x height), containing water at 20 ± 1.0°C to a depth of 14 cm for 5 min. The water was changed between every subject. All test sessions were recorded by a video camera positioned directly in front of the cylinder for subsequent analysis. The predominant behaviour in the total 5 minutes of each trial was scored (Observer 4.1; Noldus, Wageningen, The Netherlands). Swimming behaviour was assigned when a horizontal movement throughout the entire water surface was observed. Struggling behaviour was defined as upward-directed movement along the side of the cylinder, using all four paws. Floating behaviour consisted of no additional activity other than that required keeping the mouse's head above the water. Tests were conducted under white light conditions (maximal 150 lux), and with a radio constantly turned on as background noise. After each session, mice were dried with a cotton towel and put back into their home cage. The home cage was placed under a red lamp providing additional heat.

Description of the mouse behaviour
Line crossing with all its paws in the box
Self-grooming
Defecation
Being completely motionless
Mouse is in the box

5 minutes swim sessions were conducted by placing an individual by its tail in the middle of the glass cylinder. There was enough water in the cylinder to make sure the animals were not able to support themselves by placing its paws or tail on the bottom of the cylinder.

Experimental procedure FST

All experimental sessions were conducted in the same time frame as the mHB experiment (between 1000h and 1400h) The type of drug, volume and concentration that each animal got injected was equal to the mHB experiment. The FST was conducted 3 weeks after the mHB session. This wash-out period is long enough for returning the circulating corticosterone levels to the baseline values.

After their trial, mice were transferred to their home cage. 30 minutes after the mice were taken out of the FST, they were euthanized by decapitation. Trunk blood was collected and used for corticosterone determination.

Corticosterone determination

Blood plasma corticosterone levels were assayed using a solid-phase ^{125}I radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA).

Statistical analyses

All statistical analyses were carried out according to Petrie and Watson (1995) and/or Quinn and Keough (2002), using a SPSS® for Windows (version 15.0) computer program (SPSS Inc., IL, USA). Two-sided, exact (i.e. for the non-parametric tests; Mundry and Fischer 1998) probabilities were estimated throughout. Continuous data were summarized as means with standard deviation (SD), whereas discrete data on the ordinal scale were presented as medians with the inter quartile range (IQR).

The Kolmogorov-Smirnov one sample test was used to check Gaussianity of the continuous data. This revealed a non-parametric distribution of several continuous parameters for some treatment groups. For some non-normally distributed continuous parameters it was possible to logarithmically transform the data to a normal distribution. The remaining non-normally distributed continuous parameters were rank-transformed (Conover and Iman 1981). For the continuous behavioural data (except the latency parameters) that were normally distributed within groups as well as for the transformed continuous behavioural data, the significance of the differences between groups was calculated by a two-way analysis of variance (ANOVA) with strain and gender as main factors. Blood plasma corticosterone level data were subjected to multivariate repeated measurements ANOVA with strain and gender as main (between-subject) factors and time of blood sampling as within-subject factor. Homoscedasticity was tested using the Levene's test, which is a powerful and robust test based on the F statistic (Lim and Loh 1996). When necessary the variances were equalized by logarithmic transformation of the data. After transformation the variances were similar and the transformed within-group data were still normally distributed. If the (repeated measurements) ANOVA showed significant effects the group means were further compared with the unpaired Student's t-test. The unpaired Student's t-tests were performed using pooled (for equal variances) or separate (for unequal variances) variance estimates. Again, the equality of variances was tested using the Levene's test. For the unpaired Student's t-test with separate variance estimates, SPSS® uses the Welch-Satterthwaite correction. Since the Mann-Whitney U test assumes equal variances in the two groups (Kasuya 2001), the non-normally distributed continuous data were first log-transformed and then analyzed using the unpaired Student's t test, rather than calculating the significance of differences using the Mann-Whitney U test. Discrete data on the ordinal scale were first rank-transformed (Conover and Iman 1981).The statistical significance of differences for the ranked data between groups was calculated by a two-way ANOVA with strain and gender as main factors. For these data, the groups were further compared with the Mann-Whitney U test. Latency of the behavioural parameters is a time

to an event parameter; therefore the results for these behavioural parameters were compared using the Log-Rank test.

To take the greater probability of a Type I error due to multiple hypotheses into account, a more stringent criterion should be used for statistical significance (i.e. for the ANOVAs, Student's t-tests, Mann-Whitney U tests, and Log-Rank tests). We approached this problem by calculating for each behavioural dimension separate so-called Dunn-Šidák corrections (ANOVAs: $\alpha = 1 - [1 - 0.05]^{1/\gamma}$; γ = number of parameters per behavioural dimension; Student's t-tests, the Mann-Whitney U tests, and Log-Rank tests: $\alpha = 1 - [1 - 0.05]^{1/\gamma}$; γ = number of parameters per behavioural dimension multiplied by the number of comparisons between groups). We did not use a highly conservative overall Bonferroni correction because of the large numbers of tests. Table 2 gives an overview of the (corrected) thresholds. In all other cases (i.e. the Kolmogorov-Smirnov one sample test and Levene's test), the probability of a Type I error _ < 0.05 was taken as the criterion of significance.

Table 2 *Overview of thresholds of significance used in the statistical analyses*

Behavioural dimension/ parameter	(repeated measures) ANOVA	Multiple comparisons
Anxiety	$p < 0.016952$	$p < 0.000949$
Risk assessment	$p < 0.025321$	$p < 0.001424$
Undirected exploration	$p < 0.008512$	$p < 0.000475$
Directed exploration	$p < 0.010206$	$p < 0.000570$
Memory	$p < 0.016952$	$p < 0.000949$
Locomotion	$p < 0.025321$	$p < 0.001424$
Arousal	$p < 0.010206$	$p < 0.000570$
Other behaviour	$p < 0.010206$	$p < 0.000570$
FST behaviour		
Swimming	$p < 0.016952$	$p < 0.000949$
Struggling	$p < 0.016952$	$p < 0.000949$
Floating	$p < 0.016952$	$p < 0.000949$
Circulating corticosterone level	$p < 0.050000$	$p < 0.000633$

Results

Modified hole board

Table 3 summarizes the results obtained with the modified hole board, with the behavioural parameters in these tables being sorted by motivational system.

Significant strain differences were found for various parameters and usually found in the comparisons of A/J to B6 and A/J to CSS19. B6 and CSS19 did not differ significantly in behaviour. For the behavioural dimension risk assessment, indicated by the parameter 'stretch attends' A/J mice showed a lower latency time then the corresponding CSS19 mice, however this effect only became significant for the atipamezole treated animals. For all three treatment groups, A/J did significantly more stretch attends then the other two strains. Undirected exploration is measured by the behaviours 'rearing in the box' and 'hole exploration'. All treatment groups of the A/J strain showed significantly lower numbers of rears in the box compared to their counterparts from the other two strains. However, only the atipamezole treated A/J group also had a longer latency to show the first rear compared to the CSS19 atipamezole group. Latency until the first hole exploration was in A/J control animals higher then in B6 and CSS19 controls. This result was also seen when the A/J atipamezole group was compared to B6 atipamezole, as well as for A/J dexmedetomidine compared to CSS19 dexmedetomidine. Directed exploration, indicated by exploratory behaviour toward a new object

Table 3 *Modified hole board behaviour (latency (s), relative duration (%) and frequentie (#) - the animal performs a certain behaviour) of A/J, C57BL/6J and CSS19 mice with saline (control), dexmedetomidine (Dex) or atipamezole (Ati) intra peritoneal injection.*[1]

	A/J	
Measure (day 30)	**control**	**Dex**
Anxiety		
Total number of board entries	0.0 (2.0)	0.0 (1.0)
Latency until 1st board entry	242.60 (86.08)	265.07 (60.10)
Relative duration spend on the board	1.16(2.08)	6.20 (13.81)
Risk assessment		
Total number of stretched attends	12.0 (6.0)[ad3]	11.0 (9.0)[be]
Latency until 1st stretched attends	26.30 (24.35)	17.96 (7.45)
Undirected exploration		
Total number of rearing in box	3.0 (4.0)[ad]	3.0 (3.0)[be]
Latency until 1st rearing in box	169.85 (115.86)	169.82 (89.14)

showed that the A/J atipamezole and control group started exploring the new object significantly later then the corresponding B6 and CSS19 groups and at the same time also explored it less often. Atipamezole treated A/J mice explored the new object relatively shorter then CSS19 animals treated with this compound. Memory of the animals was tested by a recognition task using a familiar object. Control and atipamezole treated A/J mice explored this object significantly less often then CSS19 mice of these groups. Control and atipamezole treated A/J animals also showed less locomotor activity compared to the other two strains, which was indicated by the longer inhibition of the first line crossing and the lower total number of line crossings. Arousal is a behavioural dimension measured in defecation and grooming. Atipamezole treated A/J mice showed higher latency to first grooming in the mHB compared to B6 and CSS19 counterparts, and lower numbers of grooming compared to atipamezole treated B6 mice. Control and dexmedetomodine treated A/J animals had shorter latency to first defecation and higher numbers of boli compared to the corresponding B6 and CSS19 groups.

Treatment effects in the mHB were less represented. CSS19 control animals did significantly earlier the first stretch attend compared to atipamezole treated mice, indicating decreasing latency time for risk assessment after treatment. B6 animals treated with dexmedetomidine showed a longer latency time to the first time defecation compared to atipamezole treated B6 mice, indicating that the treatment affected arousal.

	C57BL/6J		
Ati	**control**	**Dex**	**Ati**
0.0 (1.0)	1.0 (3.0)	1.0 (4.0)	1.0 (7.0)
257.43 (75.05)	220.73 (100.08)	230.98 (92.38)	230.08(78.91)
2.97 (7.15)	1.51 (2.88)	2.21 (3.38)	3.55 (5.35)
8.0 (6.0)[cf]	1.0 (3.0)[a]	1.0 (2.0)[b]	1.0 (2.0)[c]
41.10 (72.62)[a]	110.26 (139.10)	128.22 (132.00)	132.70 (142.07)
5.0 (7.0)[cf]	29.0 (9.0)[a]	23.0 (5.0)[b]	23.0 (4.0)[c]
114.87 (80.92)[a]	51.62 (30.33)	75.90 (66.46)	56.60(31.83)

[Table 3 continued]

	A/J	
Measure (day 30)	**control**	**Dex**
Total number of hole explorations	2.0 (15.0)	1.0 (16.0)
Latency until 1st hole exploration	193.51 (108.88)[ab]	202.92 (108.29)[c]
Directed exploration		
Total number of holes visits	0.0 (0.0)	0.0 (0.0)
Latency until 1st hole visit	283.54 (47.77)	300.00 (0.00)
Total number of explorations of unfamiliar object	1.0 (2.0)[ac]	2.0 (4.0)
Latency until 1st exploration of unfamiliar object	225.84 (89.50)[ab]	178.33 (105.80)
Relative duration spend exploring the unfamiliar object	0.42 (0.54)	0.90 (1.10)
Memory		
Total number of explorations of familiar object	1.0 (2.0)[a]	1.0 (2.0)
Latency until 1st exploration of familiar object	172.64 (93.72)	171.84 (97.47)
Relative duration spend exploring the familiar object	0.56 (0.49)	0.47 (0.51)
Locomotor activity		
Total number of line crossings	34.0 (34.0)[ad]	32.0 (21.0)[be]
Latency until 1st line crossing	47.86 (46.64)[ab]	38.33 (23.54)
Arousal		
Total number of self-grooming	0.0 (1.0)	0.0 (1.0)
Latency until 1st self-grooming	266.75 (46.52)	273.38 (41.54)
Relative duration spend self-grooming	0.45 (0.68)	0.63 (0.90)
Total number of boli	6.0 (3.0)[ac]	4.0 (4.0)[bd]
Latency until 1st defecation	32.42 (15.86)[ab]	62.76 (37.72)[cd]
Other behavioural parameters		
Total number of box entries	6.0 (3.0)[b]	6.0 (6.0)
Relative duration spend in the box	95.42 (4.98)	91.49 (13.27)
Total number of immobility	0.0 (1.0)	0.0 (1.0)
Latency until 1st immobility	233.01 (101.23)	266.06 (71.17)
Relative duration spend immobile	1.99 (5.47)	0.30 (0.85)

	C57BL/6J		
Ati	**control**	**Dex**	**Ati**
1.0 (13.0)	15.0 (10.0)	12.0 (16.0)	13.0 (12.0)
183.10 (111.85)[d]	26.20 (16.58)[a]	89.32 (89.12)	48.33(29.31)
0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
280.16 (52.54)	300.00 (0.00)	300.00 (0.00)	284.55 (39.04)
1.0 (2.0)[bd]	4.0 (3.0)[a]	3.0 (4.0)	4.0 (3.0)[b]
217.50 (93.23)[cd]	63.15 (48.45)[b]	104.54 (99.82)	58.23 (47.80)[c]
0.33 (0.42)[a3]	0.76 (0.31)	0.80 (0.67)	1.07 (0.67)
1.0 (1.0)[b]	3.0 (2.0)	1.0 (4.0)	3.0 (3.0)
209.81 (90.08)	66.38 (63.73)	166.23 (119.73)	72.66 (65.05)
0.35 (0.44)	0.81 (0.70)	0.50 (0.54)	0.69 (0.51)
27.0 (24.0)[cf]	147.0 (50.0)[a]	120.0 (39.0)[b]	114.0 (41.0)[c]
55.55 (26.45) [cd]	7.06 (5.20)[a]	25.61 (76.00)	9.06 (5.84)[c]
1.0 (1.0)[c]	1.0 (1.0)	1.0 (1.0)	2.0 (2.0)[c]
244.91 (70.12) [ab]	191.78 (76.06)	185.17 (75.01)	117.71(58.52)[a]
0.80 (0.90)	1.07 (0.78)	1.46 (1.07)	1.07 (0.60)
3.0 (2.0)	0.0 (2.0)[a]	0.0 (0.0)[b]	2.0 (3.0)
32.51 (26.47)	216.75 (122.20)[a]	284.94 (58.31)[ce]	121.97(116.02)[e]
4.0 (3.0)[ab]	9.0 (4.0)	10.0 (7.0)	12.0 (10.0)[a]
95.47 (7.11)	95.85 (3.34)	93.15 (7.06)	93.43 (5.59)
0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
284.41 (56.85)	300.00 (0.00)	286.64 (51.46)	292.42 (26.56)
0.10 (0.25)	0.00 (0.00)	1.88 (7.19)	0.19 (0.59)

[Table 3 continued]

Measure (day 30)	CSS-19 control	CSS-19 Dex
Anxiety		
Total number of board entries	0.0 (3.0)	1.0 (2.0)
Latency until 1st board entry	228.87 (100.08)	259.90 (51.63)
Relative duration spend on the board	1.95 (4.37)	1.33 (2.27)
Risk assessment		
Total number of stretched attends	2.0 (2.0)[d]	1.0 (3.0)[e]
Latency until 1st stretched attend	35.28 (31.16)[b]	142.16 (143.03)
Undirected exploration		
Total number of rearing in box	22.0 (9.0)[d]	24.0 (8.0)[e]
Latency until 1st rearing in box	52.33 (25.88)	71.06 (6.26)
Total number of holes explored	13.0 (12.0)	10.0 (11.0)
Latency until 1st hole explored	58.40 (49.40)[b]	66.30 (82.54)[c]
Directed exploration		
Total number of holes visited	0.0 (0.0)	0.0 (0.0)
Latency until 1st holes visited	293.55 (24.98)	295.89 (12.28)
Total number of explorations of unfamiliar object	4.0 (2.0)[c]	2.0 (4.0)
Latency until 1st exploration of unfamiliar object	72.74 (46.23)[a]	64.09 (69.71)
Relative duration spend exploring the unfamiliar object	t1.47 (0.95)	1.06 (1.05)
Memory		
Total number of explorations of familiar object	3.0 (4.0)[a]	2.0 (5.0)
Latency until 1st exploration of familiar object	66.41 (41.41)	80.88 (91.36)
Relative duration spend exploring the familiar object	0.95 (0.49)	0.71 (0.63)
Locomotor activity		
Total number of line crossings	133.0 (22.0)[d]	128.0 (25.0)[e]
Latency until 1st line crossing	9.21 (6.31)[b]	26.00 (75.93)

Ati	Sign.[2]
0.0 (3.0)	
260.00 (63.51)	
1.37 (2.65)	[4]
1.0 (2.0)[f]	S, T
161.13 (115.11)[ab]	
23.0 (8.0)[f]	S
52.12 (19.28)[a]	
9.0 (7.0)	S
77.46 (51.94)	
0.0 (0.0)	
294.14 (16.46)	
4.0 (1.0)[d]	S
53.97 (32.01)[d]	
1.17 (0.58)[a]	S[4]
3.0 (2.0)[b]	S
57.08 (45.51)	
0.60 (0.36)	
125.0 (36.0)[f]	S
8.33 (5.31)[d]	

[Table 3 continued]

Measure (day 30)	CSS-19 control	CSS-19 Dex
Arousal		
Total number of self-grooming	1.0 (1.0)	1.0 (2.0)
Latency until 1st self-grooming	193.98 (71.10)	234.91 (72.71)
Relative duration spend self-grooming	1.26 (0.79)	1.00 (1.28)
Total number of boli	0.0 (3.0)[c]	0.0 (0.0)[d]
Latency until 1st defecation	230.75 (95.40)[b]	266.43 (89.69)[d]
Other behavioural parameters		
Total number of box entries	12.0 (7.0)[b]	9.0 (6.0)
Relative duration spend in the box	94.38 (4.47)	91.18 (16.31)
Total number of immobility	0.0 (0.0)	0.0 (0.0)
Latency until 1st immobility	300.00 (0.00)	279.90 (63.48)
Relative duration spend immobile	0.00 (0.00)	4.71 (17.19)

Frequencies

[1]Results (median and, in parenthese, the interquartile range) are for 15 animals per group. [2]Significance (for thresholds of significance, see Table 2) based on two-way ANOVA after ranking of the data with main between-subject factors strain and treatment. S indicates effect of strain; T, effect of treatment; SxT, interaction effect. [3]Post-hoc testing was done by Mann-Whitney U test. Values bearing the same superscript letters in the same row are significantly (for thresholds of significance, see Table 2) different.

Latency

[1]Results (mean and, in parenthese, the SD) are for 15 animals per group, where mice that didn't show the scored behaviour within 5 minutes got a value of 300 seconds (= duration of the mHB-test). Values bearing the same superscript letters in the same row are significantly (for thresholds of significance, see Table 2) different Log-Rank test).

Relative duration

[1]Results (mean and, in parenthese, the SD) are for 15 animals per group. [2]Significance (for thresholds of significance, see Table 2) based on two-way ANOVA with main between-subject factors strain and treatment. S indicates effect of strain; T, effect of treatment; SxT, interaction effect. [3]Post-hoc testing was done by unpaired Student's t test, Welch test or Mann-Whitney U test. Values bearing the same superscript letters in the same row are significantly (for thresholds of significance, see Table 2) different. [4]ANOVA after ranking of the data.

Ati	Sign.[2]
2.0 (1.0)	S
136.65 (63.95)[b]	
1.35 (0.86)	[4]
1.0 (3.0)	S,T, SxT
142.27 (122.26)	
10.0 (7.0)[b]	S
95.50 (2.79)	[4]
0.0 (0.0)	
300.00 (0.00)	
0.00 (0.00)	[4]

Forced swimming test

Table 4 summarizes the data obtained from the FST. In the FST both strain differences and treatment differences were found.

A/J control and atipamezole treated mice showed a significant lower latency time until the first attempt to escape the water by struggling compared to the corresponding B6 and CSS19 animals. At the same time the A/J dexmedetomidine group had a significantly higher latency time to the first floating behaviour compared to dexmedetomidine treated B6 mice. Significant treatment effects were mainly found for atipamezole treatment. CSS19 control mice showed shorter inhibition

Table 4 *Forced swimming test behaviour (latency (s), relative duration (%) and frequentie (#) – the animal performs a certain behaviour) of A/J, C57BL/6J and CSS19 mice with saline (control), dexmedetomidine (Dex) or atipamezole (Ati) intra peritoneal injection.1*

		A/J
Measure (day 30)	**control**	**Dex**
Total number of swimming	14,5 (6,5)	15,5 (6,25)[a3]
Latency until 1st swimming	5.26 (13.15)	0.64 (0.36)
Relative duration of swimming	42,41 (15,58)	53,44 (17,42)
Total number of struggling	4.5 (3.0)	3.0 (1.75)
Latency until 1st struggling	4.66 (1.82)[a]	34.98 (93.18)
Relative duration of struggling	21,69 (10,29)	18,54 (15,84)
Total number of floating	11.0 (5.5)	12.5 (5.75)
Latency until 1st floating	57.62 (23.13)	103.73 (46.36)[a]
Relative duration of floating	35,65 (16,35)	27,81 (10,15)
		CSS-19
Measure (day 30)	**control**	**Dex**
Total number of swimming	11.5 (7.0)	12.5 (5.5)
Latency until 1st swimming	0.47 (0.34)[a]	1.65 (3.74)
Relative duration of swimming	35,10 (15,03)[a3]	37,97 (15,98)
Total number of struggling	3.5 (4.5)	4.0 (4.5)
Latency until 1st struggling	53.23 (100.67)	81.55 (125.19)
Relative duration of struggling	16,94 (29,03)	10,19 (12,50)
Relative duration of floating	9.0 (4.5)	11.0 (6.0)
Latency until 1st floating	71.87 (44.28)[c]	56.93 (40.13)[d]
Relative duration of floating	47,81 (19,49)[a]	51,71 (19,14)

to the first time swimming behaviour compared to the corresponding atipamezole treated group. Control and dexmedetomidine treated CSS19 animals started floating the first time during testing significantly later then atipamezole treated CSS19 mice. The B6 control group also showed greater inhibition to first time floating compared to B6 mice injected with atipamezole. CSS19 control mice swam significantly longer then the corresponding atipamezole treated animals, whereas this control group floated significantly shorter then the corresponding atipamezole treated group. Dexmedetomidine treated A/J animals swam more often then atipamezole treated A/J mice.

	C57BL/6J		
Ati	**control**	**Dex**	**Ati**
7,0 (3,5)[a]	18,0 (10,0)	17,5 (4,0)	16,0 (11,5)
2.41 (1.92)	0.37 (0.24)	2.02 (3.27)	1.82 (2.80)
15,04 (10,23)	44,43 (14,93)	32,99 (13,39)	30,55 (18,08)
3.0 (2.5)	3.0 (4.0)	3.5 (5.75)	5.0 (5.0)
10.65 (7.69)[b]	95.94 (114.49)[a]	21.67 (18.47)	25.71(16.79)
23,47 (8,87)	8,20 (12,26)	11,38 (7,16)	8,84 (3,74)
7.0 (4.5)	15.0 (7.0)	14.5 (5.75)	13.0 (9.5)
43.79 (33.49)	52.40 (25.43)[b]	37.92 (19.06)[a]	12.92 (13.66)[b]
60,98 (7,93)	47,25 (11,54)	55,36 (13,96)	60,28 (19,91)

Ati	**Sign.[2]**
11.5 (4.75)	S,T, SxT[4]
3.44 (3.10)[a]	
20,00 (10,79)[a]	T[4]
6.0 (6.5)	
33.98 (20.06)[b]	
11,58 (6,72)	S,T[5]
12.0 (4.25)	S,T, SxT
10.79 (8.92)[cd]	
67,84 (15,44)[a]	S,T, SxT[5]

[Table 4 continued]

Frequencies

[1]Results (median and, in parenthese, the interquartile range) are for 8-14 animals per group (Due to video recording errors, for some animals behaviour in the FST could not be determined; A/J: control n = 8, Dex n = 10, Ati n = 9; B6: control n = 11, Dex n = 8, Ati n = 9; CSS19: control n = 8, Dex n = 10, Ati n = 14). [2]Significance (for thresholds of significance, see Table 2) based on two-way ANOVA with main between-subject factors strain and treatment. S indicates effect of strain; T, effect of treatment; SxT, interaction. [3]Post-hoc testing was done by Mann-Whitney U test. Values bearing the same superscript letters in the same row are significantly different (for thresholds of significance, see Table 2). [4]ANOVA after ranking of the data.

Latency

[1]Results (mean and, in parenthese, the SD) are for 8-14 animals per group (Due to video recording errors for some animals behaviour in the FST could not be determined; A/J: control n = 8, Dex n = 10, Ati n = 9; B6: control n = 11, Dex n = 8, Ati n = 9; CSS19: control n = 8, Dex n = 10, Ati n = 14). Values bearing the same superscript letters in the same row are significantly (for thresholds of significance, see Table 2) different (Log-Rank test).

Relative duration

[1]Results (mean and, in parenthese, the SD) are for 8-14 animals per group (Due to video recording errors for some animals behaviour in the FST could not be determined; A/J: control n = 8, Dex n = 10, Ati n = 9; B6: control n = 11, Dex n = 8, Ati n = 9; CSS19: control n = 8, Dex n = 10, Ati n = 14). [2]Significance (for thresholds of significance, see Table 2) based on two-way ANOVA with main between-subject factors strain and treatment. S indicates effect of strain; T, effect of treatment; SxT, interaction effect. [3]Post-hoc testing was done by unpaired Student's t test, Welch test or Mann-Whitney U test. Values bearing the same superscript letters in the same row are significantly different (for thresholds of significance, see Table 2). [4]ANOVA after logarithmic transformation. 5ANOVA after ranking of the data.

Corticosterone

Circulating corticosterone levels were measured on three different time points, namely at baseline level, after mHB testing and after FST. These data were analyzed by repeated measures ANOVA. Overall, a significant main effect was found for the factors strain, treatment and time point (Table 5). There were significant time point x strain and time point x treatment interaction effects.

Post hoc analysis showed that for all groups, circulating corticosterone levels significantly raised after FST compared to baseline levels. After the mHB test only A/J mice treated with atipamezole, B6 control mice, atipamezole treated B6 mice and the CSS19 atipamezole group had significantly higher blood plasma corticosterone levels compared to baseline measures. FST resulted in higher group mean corticosterone levels compared to mHB testing for all treatments. Another interesting feature is the fact that atipamezole treated B6 and CSS19 mice showed higher corticosterone levels after mHB and after FST compared to saline treated mice. In B6 mice after mHB testing and in CSS19 mice after FST also atipamezole treated groups have significantly higher blood plasma corticosterone levels compared to the corresponding control animals.

Further, for all treatment groups A/J mice showed significantly higher circulating corticosterone values after FST compared to the B6. A/J mice when compared to CSS19 mice showed significantly higher corticosterone levels after FST in the saline and atipamezole treated groups, but not after dexmedetomidine treatment.

Table 5 *Circulating corticosterone levels of A/J, C57BL/6J and CSS19 mice with saline (control), dexmedetomidine (Dex) or atipamezole (Ati) intra peritoneal injection before and after tested in the modified hole board and after the forced swimming test.*[1]

	A/J		
Measure	**control**	**dex**	**ati**
Plasma (Baseline)			
Corticosterone (nM)	61.79 (36.33)[a]	79.88 (56.78)[c]	75.25 (41.84)[e,f]
Plasma (after mHB-test)			
Corticosterone (nM)	120.33 (43.45)[b]	92.47 (62.38)[d]	180.68 (63.51)[e,g]
Plasma (after FS-test)			
Corticosterone (nM)	839.33(201.10)[a,b,G,H]	851.23(189.23)[c,d,J]	987.70(126.77)[f,g,K,L]
	CSS-19		
Measure	**control**	**dex**	**ati**
Plasma (Baseline)			
Corticosterone (nM)	42.97 (31.81)[o]	41.88 (29.62)[q]	57.30 (42.19)[r,s]
Plasma (after mHB-test)			
Corticosterone (nM)	94.76 (40.08)[p,D]	94.60 (49.54)[F]	234.06 (62.30)[r,D,F]
Plasma (after FS-test)			
Corticosterone (nM)	522.53(139.33)[o,p,E,H]	628.81(421.29)[q]	749.31(164.28)[s,E,L]

[1]Results (mean and, in parenthese, the SD) are for 15 animals per group. [2]Significance (for thresholds of significance, see Table 2) based on repeated measures ANOVA on rank transformed data with main between-subject factors strain , treatment and time. S indicates effect of strain, T effect of treatment, D effect of time, SxD interaction effect, TxD interaction effect.

Discussion

The three inbred strains (A/J, B6 and CSS19) revealed overall strain differences when tested in the mHB test as well as in the FST. Furthermore, results indicate that pharmacological treatment with atipamezole or dexmedetomidine did not modulate anxiety-related behaviour. However, in the mHB a weak effect on arousal was revealed after treatment with atipamezole, reflected by a modulation of grooming behaviour: a higher group mean in the total number of grooming events in B6 mice was observed (Table 5) as well as a lower group mean of the latency until the first grooming in the B6 and CSS19 strain (Table 3). In addition, higher plasma cort levels were measured after atipamezole treatment, confirming an arousing effect of this compound in B6 and CSS19 mice.

In the FST, latency until the first floating behaviour showed an increase on stress coping behaviour for atipamezole (Table 6). These effects correspond with

	C57BL/6J	
control	**dex**	**ati**
31.90 (19.98)[h,i]	45.60 (37.46)[k]	45.66 (59.66)[m,n]
76.61 (19.03)[h,j,*A*]	97.97 (45.82)[l]	235.97 (104.54)[m,*A*]
491.52(151.33)[l,j,B,G]	586.27(136.34)[k,l,C,J]	772.80(118.90)[n,B,C,K]
Sign.[2]		
S, T, D, SxD, TxD		

Post-hoc testing was done by paired Student's t test (lower case letters) or unpaired Student's t test, Welch test or Mann-Whitney U testt (capital letters). Values bearing the same letters in one row or column are significantly different (for thresholds of significance, see Table 2).

previous findings (Aho et al. 1993; Karaaslan et al. 2006; Sallinen et al. 1997). We therefore conclude that indeed atipamesole enhances arousal. With respect to plasma corticosterone levels it can be stated that A/J mice seem to be more susceptible to short term stress compared to the other two strains, indicated by the higher corticosterone levels in A/J animals when compared to CSS19 and B6 (Table 5) (Adamec et al. 2007; Flügge et al. 2001; Flügge et al. 2003; McNaughten et al. 2000; Vazquéz et al. 1998). Interestingly, atipamezole treatment seems to affect corticosterone levels only in B6 and CSS19 mice. A/J mice supposedly remain unaffected by treatment because in this strain control levels already are relatively high when compared to control levels of the other two strains and may therefore have reached a plateau value. In both B6 and CSS19 post-testing corticosterone levels were increased after treated with atipamezole. The fact that B6 and CSS19 respond the same way, while differing from A/J mice, which is the donor of chromosome 19 in CSS19 animals, underlines that the genes involved in the observed arousal effects are not entirely located on chromosome 19.

These results further underline our earlier conclusion that the alpha 2A-adrenoceptor is not primarily involved in anxiety related avoidance-behaviour. Otherwise it would have to be postulated that pharmacological manipulation of this receptor system would modulate anxiety related behavioural characteristics in both CSS19 and A/J mice. Further, treatment effects in B6 and CSS19 animals would differ from each other if they were depending on the alpha 2A- adrenoceptor, while in fact both strains are modulated in the same direction. Either a different single gene, or more then one gene on chromosome 19 regulate anxiety related avoidance behaviour in these mice, or epistatic interactions may play a role as well.

Turri et al. (2004) found several QTL regions containing genes that are hypothesized to be involved in anxiety-related behaviour, but these QTL regions did not contain the gene expressing the alpha 2A-adrenergic receptor. Interestingly, different potentially anxiety-related genes have been found after analyzing either open field behaviour or elevated plus maze test behaviour, suggesting that distinct behavioural test set-ups may mirror distinct aspects of anxiety-related behaviour (Valdar et al. 2006), probably modelling distinct anxiety endophenotypes. Following this line of thoughts, our results point towards the involvement of the alpha 2A-adrenoceptor in acute stress-coping behaviour, since treatment with two selective substances modulated behavioural parameters corresponding to the state of arousal in mice, while leaving parameters of avoidance behaviour unchanged. The arousing effect of atipamezole on the plasma cort concentration is additionally supporting this conclusion. Both dexmedetomidine and atipamezole are highly selective for the alpha 2A-adrenoceptor when compared to other drugs with the same anxiolytic / anxiogenic effect e.g. clondine and yohimbine (Cole et al. 1995; Idanpaan-Heikkila et al. 1995; Jaakola 1994; Newman-Tancredi et al. 1998). The observed results thus are highly unlikely to be due to unspecific drug effects.

Thus, it is not likely that the alpha 2A-adrenoceptor on mouse chromosome 19 is primarily involved in anxiety-related behaviour in mice. However, this gene might still be involved in an epistatic effect. Furthermore, we could show involvement of the receptor in arousal-like behaviour like grooming in the mHB and struggling in the FS test, indicating stress-coping behaviour in mice. This confirms a variety of studies in mice that show involvement of the alpha 2A adrenoceptor in stress-coping behaviour (Adamec et al. 2007; Flügge et al. 2001; Flügge et al. 2003; McNaughten et al. 2000; Vazquéz et al. 1998). Future research by QTL analysis, eventually gene expression and KO-mouse models is necessary to unravel the genetic background of avoidance behaviour in CSS19 mice.

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Chapter 5

Quantitative trait loci on mouse chromosome 19 play a key role in anxiety related avoidance behaviour

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Abstract

Anxiety and mood disorders are the most prevalent of all psychiatric disorders. We focus on the genetic dissection of anxiety related avoidance behaviour in mice using the modified hole board (mHB), which assesses a variety of different motivational systems in parallel. This approach is essential since the motivational system of interest is strongly influenced by others. In previous experiments the C57BL/6J and A/J mouse inbred strains showed differences in almost all motivational systems. To elucidate the genetic mechanisms underlying those differences, we performed further analyses with a set of chromosome substitution strains. For this set C57BL/6J (non-anxious) is the host strain and A/J (anxious) is the donor strain. We identified one consomic strain (C57BL/6J-Chr 19^{A}/NaJ) that differed in anxiety related avoidance behaviour from C57BL/6J, but not in locomotion. To identify which of the genomic regions - that this consomic strain inherited from the A/J - are responsible for this phenotype, an F2-intercross between C57BL/6J and the consomic strain was produced. Here we report on quantitative trait locus (QTL) analyses on mouse chromosome 19 with respect to anxiety related avoidance behaviour. Mouse chromosome 19 contains several regions that influence this phenotype. At least four chromosome 19 segments with significant evidence for additive QTLs could be identified. These QTLs explained

11.6 – 15.6% of the phenotypic variance. The QTLs showed either a dominant or a recessive mode of inheritance and the A/J chromosome 19 genome had either a plus or a minus contribution to the phenotype. Further, there was evidence for within-chromosomal (cis) epistatic interactions. The present findings clearly illustrate the complexity of the genetic architecture for anxiety related avoidance behaviour in the mHB.

Introduction

Millions of people worldwide suffer from anxiety and other psychiatric disorders. About 29% of the U.S. population are affected by an anxiety disorder sometime during their life and still little is known about their cause and development (Kessler et al. 2005). Anxiety is a multidimensional phenomenon presumed to have a complex inheritance, involving (the interaction of) multiple genes in combination with epigenetic and environmental factors. Family, linkage and twin studies have consistently indicated that genes indeed play a role in the etiology of anxiety disorders (Gordon & Hen 2004). However, attempts to find these human genes have been largely unsuccessful. Since the basic neuronal mechanisms are shared across mammalian species, the same set of genes may regulate critical aspects of anxiety in humans and in lower species. Therefore, animal models of anxiety were developed to facilitate the discovery of the genetic and neurobiological substrates of anxiety (Ohl 2005). Over the past decade, methods for genome analysis of animal models have been developed to identify and locate quantitative trait loci (QTLs) (Flint et al. 2005). Chromosome substitution strains (CSS, also called consomic strains) can accelerate the identification and mapping of QTLs (Singer et al. 2004). Male mice from a panel of consomic strains - in which a single full-length chromosome from the A/J inbred strain (donor strain) has been transferred onto the genetic background of the C57BL/6J inbred strain (host strain)- and the parental strains were examined in the modified hole board test. With this test several parameters for anxiety-related behaviour towards an unprotected area were measured (Ohl 2003). We identified one consomic strain (the strain in which chromosome 19 was substituted, CSS19) that differed in avoidance behaviour from the C57BL/6J, but not in locomotion (Laarakker et al. 2008a). The aim of the present study was to locate the QTLs on mouse chromosome 19 influencing anxiety related avoidance behaviour in the modified hole board. The results of the QTL analysis of male progeny from an F2-intercross between C57BL/6J and CSS19 will be presented. The identification of QTLs is a first step in the identification of genes underlying this trait. Possible candidate genes located in the vicinity of these QTLs will also be discussed.

Materials & Methods

Ethical note

The protocols of the experiments were peer-reviewed by the scientific committee of the Department of Animals in Science & Society, Utrecht University, the Netherlands and approved by the Animal Experiments Committee of the Academic Biomedical Centre, Utrecht, The Netherlands. The Animal Experiments Committee based its decision on 'De Wet op de Dierproeven' (The Dutch 'Experiments on Animals Act', 1996) and on the 'Dierproevenbesluit' (The Dutch 'Experiments on Animals Decision', 1996); both are available online (http://www.vet.uu.nl/nca_nl/legislation or http://wetten.overheid.nl/) and are the result of implementation of EC Directive 86/609/EEC (Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes). Further, all animal experiments followed the national 'Code on laboratory animal care and welfare' and the 'Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research' (National Research Council, 2003).

Animals and housing

The C57BL/6J and C57BL/6J-Chr 19^{A}/NaJ (simplified to CSS19) strains, both from The Jackson Laboratory (Bar Harbor, ME, USA), were used as progenitor strains. The F1- generation was derived by reciprocal matings of C57BL/6J and CSS19 animals. The F1-hybrids were intercrossed (brother x sister matings) producing 96 male F2 segregants.

The male F2 segregants, which were weaned at 4-6 weeks, were housed for two weeks (pre-experimental period) for habituation in an animal room of the laboratory animal facility at the Department of Neuroscience and Pharmacology (University Medical Centre Utrecht) before the behavioral testing started. The animal room was sound-attenuated. Relative humidity was kept at a constant level of 50%, the ambient temperature was maintained at 21.0 ± 2.0°C and the ventilation rate was 15-20 air changes per hour. To reduce stress in the laboratory animal facility, during the whole day (24 hours) radio-sound (60 ± 3 dB) was provided. In addition conversational radio-sound (e.g. talk shows) may accustom the animal to the human voice. Behavioural testing (modified hole board test; see below) was carried out in the same room. All mice were housed individually directly after weaning in enriched, wire topped Macrolon® Type II L (prolonged) cages (size: 365 x 207 x 140 mm, floor area 530 cm2; Techniplast, Milan, Italy). Enrichment, besides standard bedding material, included a shelter, a tissue (Kleenex®: Kimberly-Clark Professional BV, Ede, The Netherlands) and a small amount (less than a hand full) of paper shreds (EnviroDri®: Technilab-BMI BV, Someren, The Netherlands). Drinking water and standard laboratory food pellets were provided ad libitum. The light:dark cycle was reversed (white light: 1900h – 0700h maximal 150 lux; red light: 0700h – 1900h, maximal 5 lux).

During the habituation period all mice were handled at least three times per week for a few minutes by the person who performed the behavioural testing (MC Laarakker), this included picking up the animal at the tail base and placing it on the hand or arm, and restraining it by hand for a few seconds at random times of the day.

Modified hole board testing

The behavioural testing was performed using the modified hole board test. This 5-min test combines the features of an open field and a hole board. It allows for testing a variety of motivational systems in parallel (Ohl 2003). The modified hole board basically consists of an opaque grey polyvinylchloride (PVC) box (100 x 50 x 50 cm, length x width x height) which consists of two areas, one protected area - the box - which is surrounded by the protective walls of the set-up, and an unprotected area - the board. Black lines divide the box into 10 rectangles (20 x 15 cm, length x width) and 2 squares (20 x 20 cm, length x width). The board (60 x 20 x 0.5 cm, length x width x height) is placed in the centre of the box, and contains 20 PVC cylinders (3 x 3 cm, diameter x height), positioned across the board in three intended rows. The board is lit with an additional red light lamp (80 W), such that the board is illuminated with approximately 35 lux, whereas the box is only illuminated with 1-3 lux. The familiar and an unfamiliar object (either a die or a screw nut, depending on what was used as familiar object) are placed in one corner of the box of the modified hole board set-up; this is done in a way that both have the same distance to the wall and that the mouse can still pass along freely.

Behavioural testing was performed between 1000h and 1400h (i.e. during the activity phase of the animals) under red-light conditions; all behavioural tests were videotaped (for raw data storage) from above the box. The behaviour was scored by hand using the program Observer 4.1 (Noldus, Wageningen, The Netherlands). The test set-up was cleaned with water and a damp towel between each mouse. Several parameters for anxiety-related behaviour, risk assessment, (undirected and directed) exploration, memory, locomotion, arousal and other behaviour (e.g. urination) were measured/calculated (Laarakker et al. 2008a). However, only the results for the 'anxiety'-parameters (i.e. total number of board entries, latency until the first board entry, percentage of time on the board, and average duration of a board entry) have been presented here.

DNA samples

Spleens were dissected after decapitation, frozen on dry ice and kept in -80°C until used for DNA isolation. Total genome DNA was isolated using a PureGene DNA isolation kit (Gentra Systems, Venlo, The Netherlands) according to the accompanying protocol for frozen tissues. Isolated DNA was re-suspended in TE buffer (10 mM Tris, 0.2 mM EDTA, pH 8.0) at a concentration of 40 ng/_l. The DNA concentrations were determined by measuring the A260 with a PU8700

UV/Visible spectrophotometer (Unicam Analytical Systems B.V., Eindhoven, The Netherlands). DNA samples were stored at 4°C.

PCR amplification of mouse chromosome 19 microsatellite loci

For generating a genetic map of chromosome 19, twenty-one microsatellites were chosen from the mouse genome database (Mouse Genome Informatics, http://www.informatics.jax.org/). These markers were dispersed throughout mouse chromosome 19. Primers flanking these microsatellites (MIT mouse MapPair primers) were purchased from Invitrogen Life Sciences (Breda, The Netherlands). Thermal cycling was performed in a GeneAmp thermocycler (PCR System 9700, Applied Biosciences Inc., Foster City, CA, USA). For the following fifteen microsatellites: D19Mit59, D19Mit109, D19Mit61, D19Mit16, D19Mit106, D19Mit86, D19Mit46, D19Mit65, D19Mit119, D19Mit10, D19Mit123, D19Mit36, D19Mit1, D19Mit34 and D19Mit137, the genomic DNA samples were amplified according to the supplied protocol accompanying the microsatellite primers using Super Taq polymerase (SphaeroQ, Gorinchem, The Netherlands). PCR products were analyzed by electrophoresis on 3% (w/v) agarose gels (Hispanagar MS-8 panarose, SphaeroQ) and visualized by ethidium bromide staining (0.5 μg/ml PCR products).

For microsatellites D19Mit130, D19Mit40, D19Mit63, D19Mit19, D19Mit100 and D10Mit7, a phage M13-based tag (GTTTTCCCAGTCACGAC) was added at the 5' end of the forward primers. PCR amplification was carried out according to the protocol described by Temwichitr et al. (2007). The PCR reactions were diluted 10- to 30-fold with H2O and 2 μl of the dilution was mixed with 10 μl formamide and 0.2 μl GS500 LIZ or TAMRA size standard (Applied Biosystems). The products were analyzed after capillary electrophoresis and automatically detected using the Genetic Analyzer 3100 (Applied Biosystems). The DNA products were classified by size with Genemapper version 4.0 software (Applied Biosystems) and alleles were assigned (Temwichitr et al. 2007).

Map construction

Segregation ratio of the genotypes of individual markers was checked with the Chi-squared goodness-of-fit-test. None of the markers showed significant segregation distortion ($P > 0.05$). The genetic map distance for the markers was computed with the software package JoinMapTM, version 3.0 (Van Ooijen & Voorrips 2001). The critical LOD scores used to establish linkage groups and calculate map distances are called 'linklod' and 'maplod', respectively. Marker pairs with a recombination LOD score above a critical 'linklod' are considered to be linked. Only information for marker pairs with a LOD score above 'maplod' is used in the calculation of map distances. To be sure that all markers are placed in the genetic map, a low value for 'maplod' should be used. For the establishment of linkage groups, a critical minimal LOD score ('linklod') of 3.0 was used. For calculation of map distances and estimating most likely gene orders, a critical LOD score

('maplod') of 0.05 was used. Recombination frequencies were converted to map distances in centiMorgans (cM) using the Kosambi function (Kosambi 1944).

Statistical analyses

All statistical analyses were carried out according to Petrie and Watson (1995) and/or Quinn and Keough (2002), using a SPSS® for Windows (version 15.0.1) computer program (SPSS Inc., IL, USA). Two-sided, exact (i.e. for the non-parametric tests; Mundry & Fischer 1998) probabilities were estimated throughout. The probability of a Type I error < 0.05 was taken as the criterion of significance. The Kolmogorov-Smirnov one sample test was used to check Gaussianity of the continuous data (latency, percentage of time, and average duration). The non-normally distributed traits were transformed to a normal distribution using a logarithmic function ($y = {}^{10}\text{Log}[x + a]$, where y is the transformed trait value, x is the original trait and a is a constant).

QTL analyses

The location of the QTLs affecting the measured (transformed) quantitative traits and the variance explained by each locus were determined using the MapQTL® software package, version 4.0 (van Ooijen et al. 2002). QTL analysis was performed by MQM (multiple-QTL-model or marker-QTL-marker) mapping, which is more powerful than the traditional interval mapping approach (Jansen 1994). This method is essentially a combination of interval mapping and multiple regression. The model involves regression both on QTL within an interval and on marker loci outside that interval. Markers take over the role of nearby QTLs and are fitted as cofactors while testing for a single QTL elsewhere in the genome. This way, the cofactors function as a genetic background control and absorb most of the genetic effects of their nearby QTLs from the residual variance. As a result, the power of the QTL analyses is enhanced. Cofactors for the MQM analysis were identified by applying the 'automatic selection of cofactors' option of MapQTL. Results were expressed as LOD scores. Based on Lander & Botstein (1989), having an average distance between markers of 3.5 cM, and taking into account that a genetic scan was performed across a single, complete chromosome rather than the entire genome, an association was assumed significant when the LOD score was ≥ 1.72. Based on the paper of Lander & Kruglyak (1995) an association was assumed suggestive when the LOD score was between 1.12 and 1.72. For MQM mapping, simulation studies have demonstrated that the thresholds derived for conventional interval mapping are still valid in many situations (Jansen, 1994). After grouping by genotype for the DNA marker flanking the peak of the QTL, phenotype comparison of the F2-animals was performed. Whenever a QTL was found or suggested using the MapQTL® software, unpaired Student's t tests (latency, percentage of time, and average duration) or Mann-Whitney U test (total number) were performed for the markers flanking the peak of the QTL or at the peak of the QTL. The mode of inheritance was chosen as free, additive,

dominant or recessive according to the differences in the mean values of the trait between mice that were homozygous A/J, heterozygous, or homozygous C57BL/6J. Output from JoinMapTM and MapQT® was converted to figures using the graphics program MapChart (Voorrips, 2001).

Interactions

For chromosome 19, all two-marker pairs were tested for epistatic interactions with a two-way ANOVA.

Results

QTL analysis on the (C57BL/6J x CSS19-A/J)F2-population revealed significant LOD scores (Table 1) for 'latency until the first board entry' (Figure 2), 'percentage of time on the board' (Figure 3), 'average duration of a board entry' (Figure 4), but not for 'total number of board entries' (Figure 1). For 'total number of board entries' there was only suggestive evidence for two QTLs, since the LOD scores were 1.18 and 1.52, respectively (Figure 1). The significant QTLs showed either a dominant or a recessive mode of inheritance. Depending on the parameter and the QTL, the C57BL/6J compared to the A/J genome has a decreasing or an increasing effect. The QTL intervals (using the 1-LOD-support interval) are about 1.7 to 13.4 Mb and comprise 15 to 284 known genes (http://www.informatics.jax.org). The 'latency until the first board entry' of each of the three genotypes segregating at locus D19Mit36, which flanks the QTL with peak LOD score of 2.63, is illustrated in Figure 5.

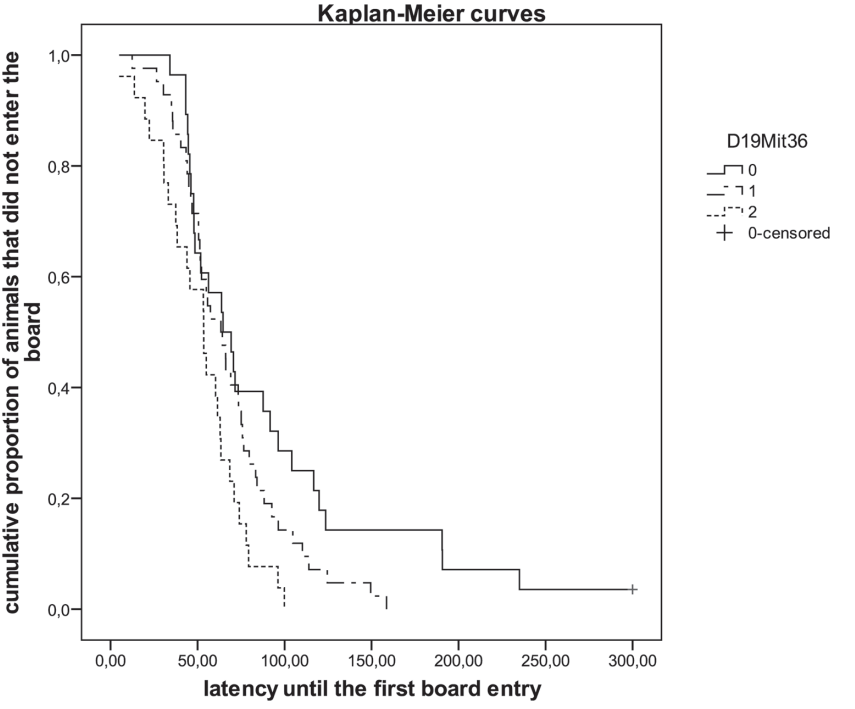


Figure 1 *Kaplan-Meier plots for the parameter latency until the first board entry. Results for 96 male mice from the (C57BL/6J x CSS19)F2-intercross. The mice were classified according to the genotype at the D19Mit36 locus. For some mice from the latency until the first board entry is said to be censored, indicating that the testing period (300 seconds) was cut off before the event (= board entry) occurred. We do not know when (or indeed, whether) these mice will experience the event, only that these mice have not done so by the end of the testing period.*

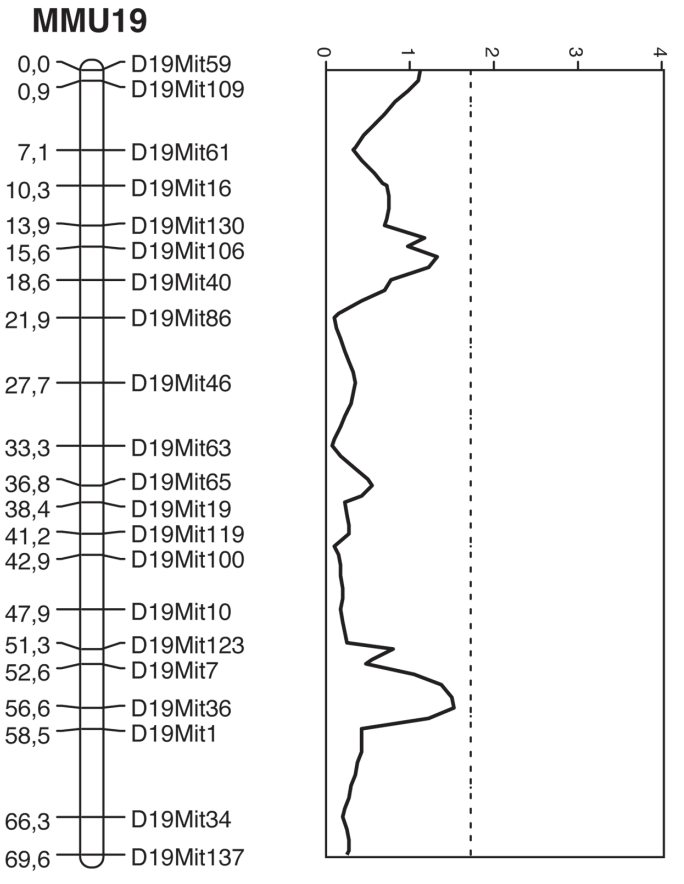


Figure 2 *Genetic map (left; mouse chromosome 19) and LOD score plot (right) for 'total number of board entries' in the (C57BL/6J x CSS19)F2 intercross (n = 96). The dashed vertical line represents the threshold value of the LOD score considered significant for linkage.*

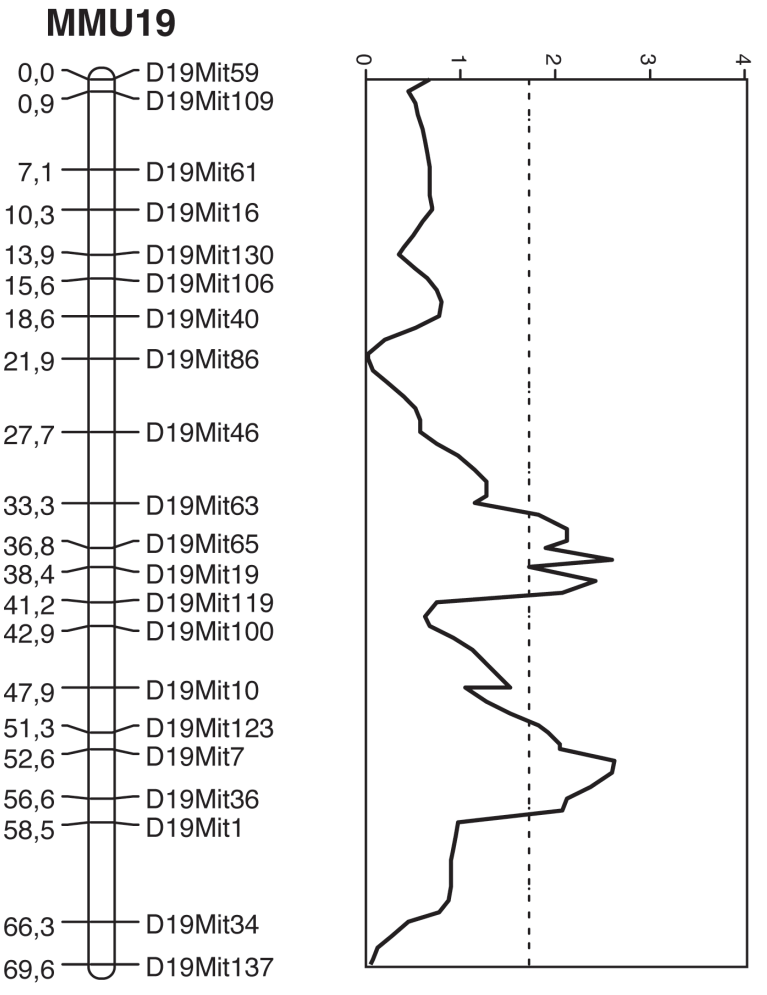


Figure 3 *Genetic map (left; mouse chromosome 19) and LOD score plot (right) for 'latency until the first board entry' in the (C57BL/6J x CSS19)F2 intercross (n = 96). The dashed vertical line represents the threshold value of the LOD score considered significant for linkage.*

Figure 4 *Genetic map (left; mouse chromosome 19) and LOD score plot (right) for 'percentage of time on the board' in the (C57BL/6J x CSS19)F2 intercross (n = 96). The dashed vertical line represents the threshold value of the LOD score considered significant for linkage.*

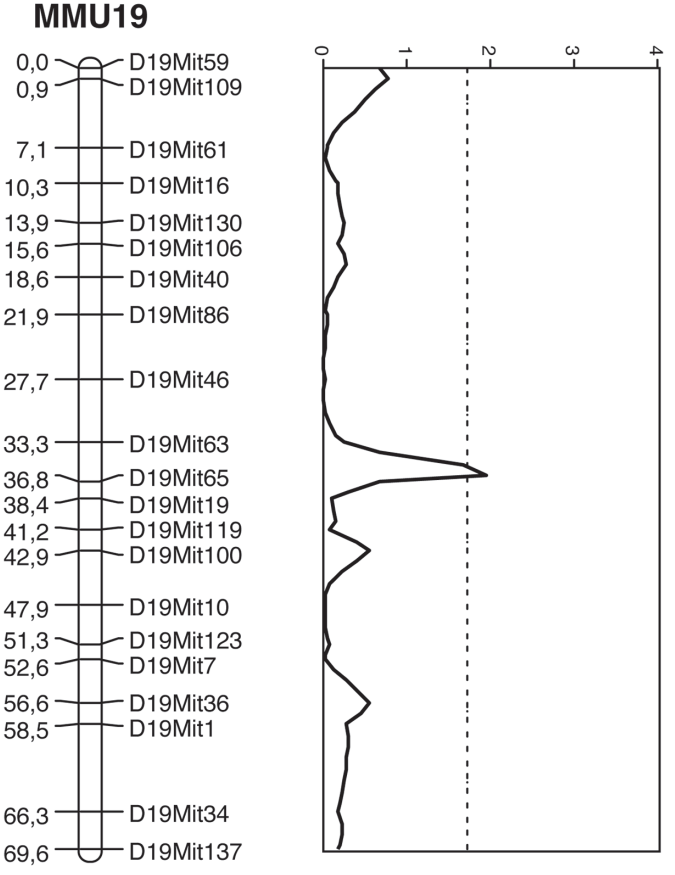


Figure 5 *Genetic map (left; mouse chromosome 19) and LOD score plot (right) for 'average duration of a board entry' in the (C57BL/6J x CSS19)F2 intercross (n = 96). The dashed vertical line represents the threshold value of the LOD score considered significant for linkage.*

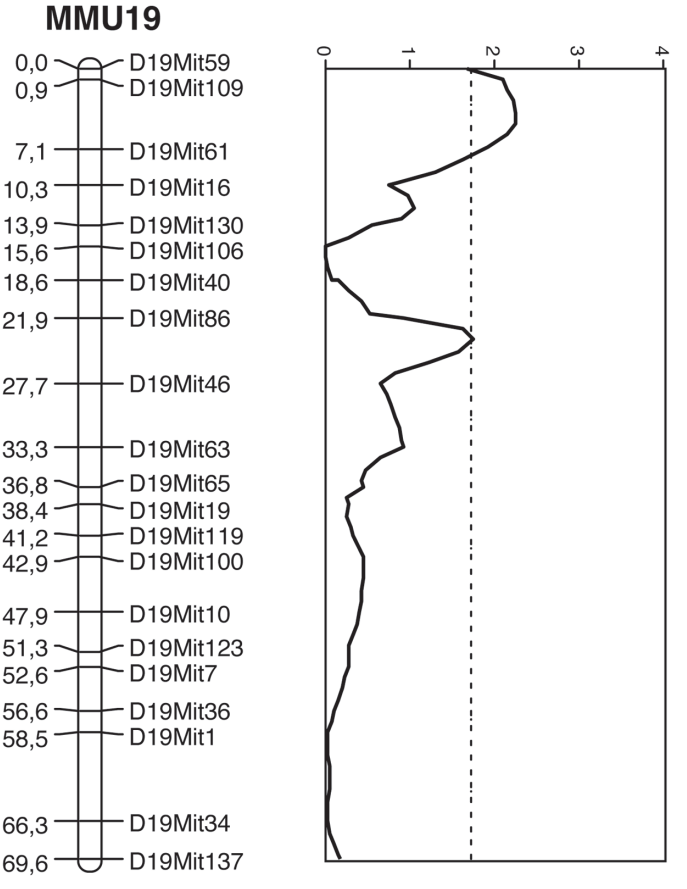
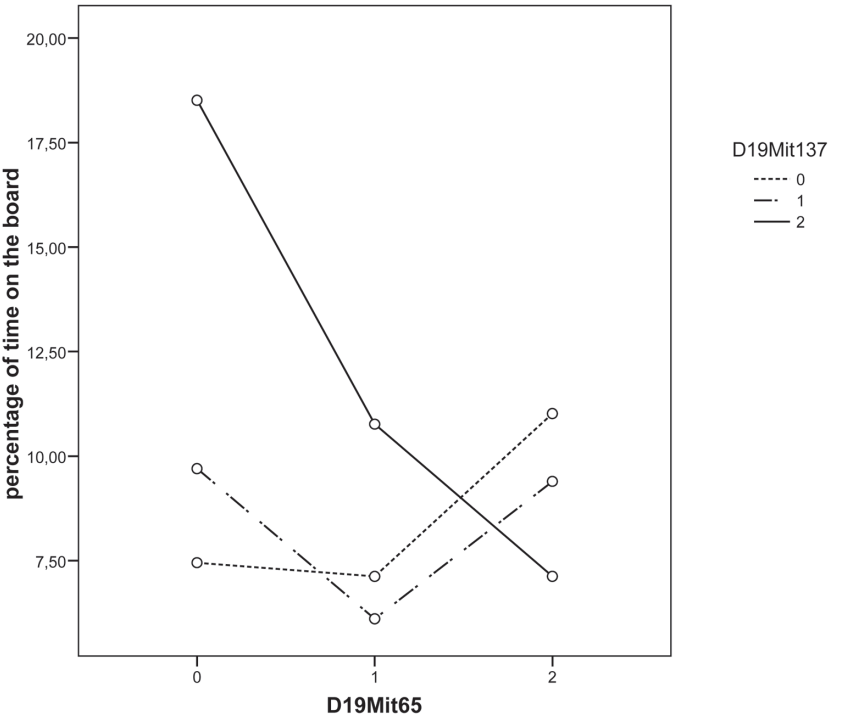


Figure 6 *Graphic presentation of percentage of time on the board in the (C57BL/6J x CSS19)F2 population (n = 96) as the effect of an interaction (P = 0.009) linked to markers D19Mit65 and D19Mit137. Each point represents the mean value for each genotype combination of both markers.*



A two-way ANOVA was used for evaluating within-chromosome epistatic interactions. Table 2 summarizes the significant interactions. Figure 6 illustrates the interaction between the D19Mit65 and D19Mit137 loci for 'percentage of time on the board'. When mice are homozygous or the A/J allele at the D19Mit65 locus and homozygous or the C57BL/6J allele at the D19Mit137 locus, 'percentage of time on the board' was highest.

Table 1 *Summary of the QTLs for anxiety related avoidance behaviour in the modified hole board*[1]

parameter	Model[2]	Peak
Latency until the first board entry[7]	Dominant(+)	
	Recessive(-)	
Percentage of time on the board	Dominant(-)	
Average duration of a board entry[7]	Recessive(-)	
	Recessive(-)	

LOD[3]	QTL position[4]	% Variance[5]	Cofactor(s)[6]
2.60	D19Mit65 – D19Mit19	13.8	D19Mit100 and D19Mit137
(37.8 cM; 36.673.411 – 41.013.122; 47 genes)			
2.63	D19Mit7 – D19Mit36	13.8	D19Mit100 and D19Mit137
(53.6 cM; 49.869.212 – 54.688.753; 15 genes)			
1.95	D19Mit63 – D19Mit65	11.6	D19Mit19
(36.3 cM; 37.172.146 – 38.892.686; 18 genes)			
2.25	D19Mit109 – D19Mit61	13.6	D19Mit106 and D19Mit40
(4.9 cM; 5.321.112 – 18.746.367; 284 genes)			
1.74	D19Mit68 – D19Mit46	15.6	D19Mit106 and D19Mit40
(23.9 cM; 26.361.020 – 32.530.079; 31 genes)			

[1]QTLs were investigated using the MapQTL software on data collected from 96 male (C57BL/6JxCSS19)F_2-intercross mice. The module with cofactors (MQM mapping) was used.

[2]Dominant, recessive or heterosis, as well as plus (+) or minus (-) contribution to the trait was defined with respect to the C57BL/6J grandparent's allele.

[3]Data are shown only when significant results were found. Threshold for significant linkage is 1.72.

[4]Markers flanking the LOD score peak are given. The location (cM) on the chromosome where the LOD score peaked is given in parentheses, as well as the estimated (using linear interpolation calculations) 1-LOD support interval (in bp) and the number of known genes.

[5]Percentage of the genetic variance explained by the QTL.

[6]Cofactors that are used in the MQM mapping procedure.

[7]QTL analyses after logarithmic transformation of the data.

Table 2 Significant (P < 0.05) locus-locus interactions

Parameter	Locus 1 x Locus 2	*P* (interaction) ANOVA[1]
Latency until the first board entry		
	D19Mit61 x D19Mit34	0.008
	D19Mit59 x D19Mit34	0.011
	D19Mit119 x D19Mit34	0.016
	D19Mit16 x D19Mit34	0.017
	D19Mit61 x D19Mit1	0.019
	D19Mit109 x D19Mit34	0.023
	D19Mit109 x D19Mit10	0.024
	D19Mit109 x D19Mit1	0.025
	D19Mit61 x D19Mit10	0.026
	D19Mit109 x D19Mit123	0.040
	D19Mit109 x D19Mit7	0.042
	D19Mit106 x D19Mit34	0.045
	D19Mit61 x D19Mit123	0.047
	D19Mit19 x D19Mit36	0.047

[Table 2 continued]

Parameter	Locus 1 x Locus 2	*P* (interaction) ANOVA[1]
Total number of board entries[2]		
	D19Mit46 x D19Mit137	0.004
	D19Mit116 x D19Mit137	0.007
	D19Mit65 x D19Mit137	0.010
	D19Mit59 x D19Mit63	0.013
	D19Mit65 x D19Mit36	0.013
	D19Mit19 x D19Mit137	0.017
	D19Mit63 x D19Mit137	0.021
	D19Mit109 x D19Mit63	0.024
	D19Mit119 x D19Mit36	0.026
	D19Mit59 x D19Mit86	0.031
	D19Mit40 x D19Mit63	0.034
	D19Mit100 x D19Mit137	0.035
	D19Mit59 x D19Mit119	0.036
Percentage of time on the board		
	D19Mit65 x D19Mit36	0.009
	D19Mit65 x D19Mit137	0.009
	D19Mit59 x D19Mit63	0.011
	D19Mit59 x D19Mit86	0.020
	D19Mit119 x D19Mit1	0.020
	D19Mit19 x D19Mit137	0.021
	D19Mit59 x D19Mit16	0.022
	D19Mit65 x D19Mit1	0.022
	D19Mit65 x D19Mit100	0.025
	D19Mit19 x D19Mit36	0.027
	D19Mit65 x D19Mit123	0.028
	D19Mit119 x D19Mit36	0.028
	D19Mit119 x D19Mit119	0.032
	D19Mit65 x D19Mit7	0.033
	D19Mit109 x D19Mit63	0.045
Average duration of a board entry		
	D19Mit59 x D19Mit119	0.002
	D19Mit109 x D19Mit119	0.002
	D19Mit109 x D19Mit63	0.014
	D19Mit59 x D19Mit65	0.018
	D19Mit59 x D19Mit63	0.023
	D19Mit65 x D19Mit119	0.023
	D19Mit119 x D19Mit100	0.026
	D19Mit109 x D19Mit65	0.027
	D19Mit109 x D19Mit10	0.027
	D19Mit109 x D19Mit100	0.042
	D19Mit59 x D19Mit10	0.043
	D19Mit109 x D19Mit7	0.044
	D19Mit109 x D19Mit86	0.045
	D19Mit59 xD19Mit100	0.048

[1] Two-way ANOVA with main factors locus 1-genotype and locus 2-genotype

[2] Two-way ANOVA after ranking of the data

Discussion

Previous QTL analyses, using A/J and C57BL/6J as parental strains and different mapping populations, have already suggested that mouse chromosome 19 plays a significant role in anxiety-related behavior (Gershenfeld & Paul 1997; Gershenfeld et al. 1997; Gill & Boyle 2005; Singer et al. 2004; Singer et al. 2005; Zhang et al. 2005). Table 3 shows a summary of the QTL mapping results so far for chromosome 19 with respect to the difference in anxiety-related behavior between A/J and C57BL/6J. We used the published results from Gershenfeld et al. (1997), Gershenfeld & Paul (1997), Gill & Boyle (2005) and Zhang et al. (2005) to estimate the associated QTL confidence intervals (1-lod support intervals). The chromosomal region with coordinates 32.551.766 – 42.541.189 bp overlaps these four intervals. In fact the genetic analysis with the advanced intercross lines (Zhang et al. 2005) determines this interval (Table 3). We found for anxiety related avoidance behaviour in the modified hole board test among other the QTL regions 36.673.411 – 41.013.122 bp ('latency until the first board entry') and 37.172.146 – 38.892.686 bp ('percentage of time on the board') (Table 1). By combining the data from multiple crosses between A/J and C57BL/6J (DiPetrillo et al. 2005; Li et al. 2005) it is most likely that the chromosomal region with genome coordinates 37.172.146 – 38.892.686 bp contains an anxiety QTL. This regions contains 18 known genes (Table 1). In this region the gene Cpeb3 (cytoplasmic polyadenylation element binding protein 3) is a promising functional candidate gene. CPEB3 is a member of a protein family that regulates local polyadenylation of mRNAs in the cytoplasm of neurons and oocytes. In mouse hippocampus, the gene is up-regulated transiently after induction of seizure, and it has been implicated in long-term potentiation (Theis et al. 2003). The results of Huang et al. (2006) indicate that CPEB3 is at least one factor that mediates GluR2 mRNA expression. In turn, deletion of the gene encoding the GluR2 subunit of the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (Gria1) in the mouse showed reduced anxiety in the elevated plus maze (Mead et al. 2006).

Table 3 *Summary of QTLs on chromosome 19 influencing the difference in anxiety related avoidance behavior between C57BL/6J and A/J mice.*

Method[1]	Behavioral test (apparatus)	Marker nearest to QTL peak
F_2	Open field	D19Mit46
F_2	Light-dark box	D19Mit86
RIS	Open field	D19Mit10
RCS	Open field	D19Mit10
AIL	Open field	D19Mit19

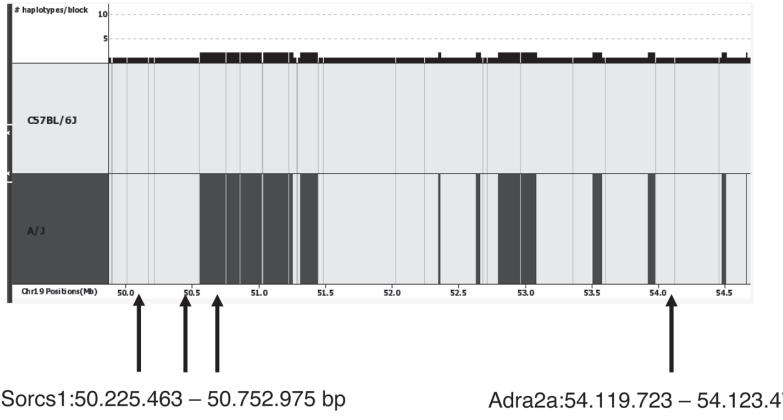
[1] Abbreviations: F_2 = an F_2 intercross population, RIS = a set of recombinant inbred strains, RCS = a set of recombinant congenic strains, AIL = an advanced intercross population, ISCS = a panel of interval-specific congenic strains, CSS = a panel of chromosome substitution strains.

The chromosomal region with genome coordinates 49.869.212 – 54.688.753 bp (Table 1; significant LOD score, 2.63, for 'latency until the first board entry') contains two interesting candidate genes: Sorcs1 (50.225.463 – 50.752.975 bp) and Adra2a (54.119.723 – 54.123.472 bp) (Figure 7). SORCS1 is a VPS10P-domain receptor, being expressed in the central nervous system. The encoded protein is vacuolar protein sorting 10 and SORCS1, or sortilin-related receptor CNS expressed 1 is one of the five members of the VPS10P family found in vertebrates. SORCS1 has been implicated in being involved in Alzheimer's disease and other types of age-related dementia, but also in non-neuronal disorders have been connected to this gene, including type 2 diabetes and myocardinal infarction (Willnow et al. 2008).

Chromosomal region 49.869.212 – 54.688.753 bp also contains the gene Adra2a, which codes for the alpha 2A adrenoceptor. This gene has also been correlated to anxiety related behaviors as well (Schramm et al., 2001). Although the haplotype block structure is unknown for the Adra2a (http://mouse.perlegen.com/preview/mousehap.html) - up until now only one SNP (http://phenome.jax.org/pub-cgi/ phenome/mpdcgi?rtn=snps%2 Fretrieve&gregion= gene&genesym=Adra2a) has been identified in this gene - it is positioned in an area that does not shows any difference between A/J and C57BL/6J (Figure 7). Thus, it is not very likely that the alpha 2A adrenoceptor is involved in anxiety-related avoidance behaviour in the modified hole board in mice. Interestingly, Rosen & Williams (2001) found a QTL within our 1-LOD-support interval for striatal neuron numbers (51.038.004 – 51.038.152 bp). The striatum is a brain region known to be involved in anxiety (Yilmazer-Hanke 2008).

Estimated 1-lod support interval (bp; coordinates)	Reference
23,380,471 - 42,541,189	Gershenfeld *et al.*, 1997
17,393,162 - 53,983,163	Gershenfeld and Paul, 1997
Could not be determined	Gill and Boyle, 2005
15,972,152 - 61,283,022	Gill and Boyle, 2005
32,551,766 - 42,541,189	Zhang *et al.*, 2005

Figure 7 *Haplotype block structure of the genes Sorcs1 and Adra2a for the inbred strains C57BL/6J (host strain) and A/J (donor strain).*



In summary, in the present study we examined anxiety related avoidance behaviour in mice from a (C57BL/6J x CSS19)F2-intercross by using the modified hole board test, and performed QTL analyses. A mouse chromosome 19 contains several regions that influence this phenotype. At least four chromosome 19 segments with significant evidence for additive QTLs could be identified. These QTLs showed either a dominant or a recessive mode of inheritance and the A/J chromosome 19 genome has either a plus or a minus contribution to the phenotype. Further, there was evidence for within-chromosomal (cis) epistatic interactions. Taken together, the results illustrate the complexity of the genetic architecture for anxiety related avoidance behaviour. Future work will be directed towards use of (double) congenic strains, knockout strategies and micro-array analyses to precisely map the QTLs and to confirm the role of the suggested candidate genes.

Chapter 6

Mouse chromosomes 1 and 19 both harbour quantitative trait loci for circulating total cholesterol level – is there a link with experimental behaviour?

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Abstract

Baseline circulating total cholesterol levels are under complex genetic and environmental control. In human and laboratory rodents several genes influencing this phenotype have been identified. Here we report on quantitative trait locus analyses on mouse chromosomes 1 and 19 for baseline circulating total cholesterol concentration, using chromosome substitution strains derived from C57BL/6J (host strain) and A/J (donor strain). For chromosome 19 one QTL (LOD score 5.2; 0.2-Mb support interval) and for chromosome 1 three QTLs (LOD scores 3.4, 3.3, and 4.6; 9.2-, 36.4-, and 20.5-Mb support intervals, respectively) were identified. The individual QTLs explained 17.3 – 34.2% of the phenotypic variance. There also was evidence for within-chromosome epistatic interactions. Furthermore, the consomic survey revealed that the plasma total cholesterol concentration often exceeded the range between C57BL/6J host and A/J donor strains by far. The results clearly illustrate the complexity of the genetic architecture for the regulation of circulating total cholesterol levels. The results clearly illustrate the complexity of the genetic architecture for the regulation of circulating total cholesterol levels, while they do not support a functional relationship between plasma total cholesterol concentration and behavioural measures.

Introduction

In industrialized societies atherosclerosis is one of the major causes of human death. Atherosclerosis is a complex disorder in which both genetic and environmental factors play a role. An important risk factor for the development of this disease is a high blood plasma cholesterol level (Hegele 2009). Circulating cholesterol levels do not exclusively reflect dietary habits; epidemiological studies have revealed consistently higher than average plasma cholesterol levels only in particular individuals after a high dietary cholesterol intake (Beynen & Katan 1988). Individual differences in plasma cholesterol level also exist after a diet with low-fat and/or low-cholesterol content. Similar variability in plasma cholesterol levels can be observed in laboratory animals such as mice (Wang & Paigen 2005), rabbits (de Wolf et al. 2003), and rats (Bonné et al. 2002) in response to control diets. Differences observed between inbred strains of these species indicate that the basal plasma cholesterol concentration is under genetic control.

Thus far, several claims have been made concerning the relationship between plasma cholesterol levels and behavioural measures (e.g. anxiety or locomotor activity parameters) (Elder et al. 2008; Papakostas et al. 2004). Recently, we have performed genetic mapping studies for these behaviours, using a panel of chromosome substitution strains (Laarakker et al. 2008a; de Mooij-van Malsen et al. 2009; Kas et al. 2009). In these studies, we have identified different QTLs for baseline home cage and for open field motor activity levels on mouse chromosome 1 and a QTL for anxiety-related behaviour on mouse chromosome 19. By measuring plasma cholesterol levels in the F2 individuals generated for these studies, we can now assess the relationship between genetic variance on these chromosomes, behaviour and plasma cholesterol levels. The results of the quantitative trait locus (QTL) analyses for total plasma cholesterol concentrations and the correlation of this phenotype with behavioural parameters will be presented and discussed.

Materials & Methods

Ethical note

The protocols of the experiments were reviewed and approved by the Animal Experiments Committee of the Academic Biomedical Centre, Utrecht-The Netherlands. The Animal Experiments Committee based its decision on 'De Wet op de Dierproeven' (The Dutch 'Experiments on Animals Act', 1996) and on the 'Dierproevenbesluit' (The Dutch 'Experiments on Animals Decision', 1996); both are available online (http://www.vet.uu.nl/nca_nl/legislation or http://wetten.overheid.nl/) and are the result of implementation of EC Directive 86/609/EEC (Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes). Further, all animal experiments followed the national

'Code on laboratory animal care and welfare' and the 'Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research' (National Research Council, 2003).

Animals and housing

i) *Parental strains, males and females (Experiment 1)*
Nine males and nine females of the inbred strains A/J (donor strain) and C57BL/6J (host strain) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Housing and behavioural testing of these animals will be described elsewhere.

ii) *Consomic strain survey, males (Experiment 2)*
This study was performed using male mice from the following inbred strains: A/J (the donor strain; n = 30), C57BL/6J (the host strain; n = 27), and the complete set of chromosome substitution strains between these parental strains (n = 6 per consomic line); The Jackson Laboratory. The chromosome substitution strains, whose nomenclature is C57BL/6J-Chr #^{A}/NaJ, are simplified to CSS-#. We tested more host strain animals when compared with consomic mice to improve power to detect a chromosome that contains a QTL. According to Belknap (2003) a 4.5:1 ratio, or twenty-seven C57BL/6J host strain animals and six animals per consomic strain, is the most efficient for selecting chromosome substitution strains that contain a QTL. Housing and behavioural testing of these animals have been described by Laarakker et al. (2008a).

iii) *C57BL/6J x CSS-1 intercross, males and females (Experiment 3)*
To produce F_1 hybrids, C57BL/6J females and males were mated with CSS-1-A/J males and females (A/J = donor strain), respectively. The F_1 hybrids were intercrossed, producing 93 F_2 progeny (45 males and 48 females). Housing and behavioural testing of these animals have been described by de Mooij-van Malsen et al. (2009) and Kas et al. (2009).

iv) *C57BL/6J x CSS-19 intercross, males (Experiment 4)*
To produce F_1 hybrids, C57BL/6J females and males were mated with CSS-19-A/J males and females (A/J = donor strain), respectively. The F_1 hybrids were intercrossed to produce 96 male F_2 segregants. Housing and behavioural testing of these male animals will be described elsewhere, but have already been presented in abstract form (Laarakker et al. 2008b).

v) *C57BL/6J, CSS-19-A/J and CSS-19-PWD/PhJ, males and females (Experiment 5)*
Males (M) and females (F) of the inbred strains C57BL/6J (host strain; n = 27M, 27F), CSS-19-A/J (consomic line; n = 27M, 27F; A/J = donor strain) and CSS-19-PWD/PhJ (consomic line; n = 21M, 18F; PWD/PhJ = donor strain) were purchased from the Jackson Laboratory. Housing and behavioural testing of these animals will be described elsewhere.

Blood sampling and circulating cholesterol determination

Fasted mice were euthanized by decapitation and trunk blood was collected in lithium-heparin-coated tubes. After centrifugation plasma was stored at –80oC until analysis. Before analysis samples were thawed to room temperature and vortexed vigorously to resuspend any precipitated lipids. Total cholesterol in blood plasma was measured enzymatically according to Siedel et al. (1983) using a colorimetric kit supplied by Roche Diagnostics GmbH (Mannheim, Germany). The cholesterol analyses were performed on a Cobas Mira automatic micro-centrifugal analyzer (ABX Diagnostics, Montpellier, France). The inter- and intra-assay coefficients of variation for blood plasma total cholesterol always fell within the limits prescribed by the manufacturer.

DNA samples

Spleens were dissected after decapitation, frozen on dry ice and kept in -80°C until used for DNA isolation. Total genome DNA was isolated using a PureGene DNA isolation kit (Gentra Systems, Venlo, The Netherlands) according to the accompanying protocol for frozen tissues. Isolated DNA was re-suspended in TE buffer (10 mM Tris, 0.2 mM EDTA, pH 8.0) at a concentration of 40 ng/μl. The DNA concentrations were determined by measuring the A_{260} with a PU8700 UV/Visible spectrophotometer (Unicam Analytical Systems B.V., Eindhoven, The Netherlands). DNA samples were stored at 4°C.

SNP analysis of mouse chromosome 1

A total of 15 SNPs were selected across chromosome 1 using the Celera-database based on the presence of allelic differences between the mouse strains A/J and C57BL/6J. For the selected SNPs a Taqman Assay by Design was used (Applied Biosciences, Foster City, CA, USA). SNP analysis was performed as described in Kas et al. (2009).

PCR amplification of mouse chromosome 19 microsatellite loci

For generating a genetic map of chromosome 19, twenty-one microsatellites were chosen from the mouse genome database (Mouse Genome Informatics, http://www.informatics.jax.org/). These markers were dispersed throughout mouse chromosome 19. Primers flanking these microsatellites (MIT mouse MapPair primers) were purchased from Invitrogen Life Sciences (Breda, The Netherlands). Thermal cycling was performed in a GeneAmp thermocycler (PCR System 9700, Applied Biosciences Inc., Foster City, CA, USA). For the following fifteen microsatellites: D19Mit59, D19Mit109, D19Mit61, D19Mit16, D19Mit106, D19Mit86, D19Mit46, D19Mit65, D19Mit119, D19Mit10, D19Mit123, D19Mit36, D19Mit1, D19Mit34 and D19Mit137, the genomic DNA samples were amplified according to the supplied protocol accompanying the microsatellite primers using Super Taq polymerase (SphaeroQ, Gorinchem,

The Netherlands). PCR products were analyzed by electrophoresis on 3% (w/v) agarose gels (Hispanagar MS-8 panarose, SphaeroQ) and visualized by ethidium bromide staining (0.5 μg/ml PCR products).

For microsatellites D19Mit130, D19Mit40, D19Mit63, D19Mit19, D19Mit100 and D10Mit7, a phage M13-based tag (GTTTTCCCAGTCACGAC) was added at the 5' end of the forward primers. PCR amplification was carried out according to the protocol described by Temwichitr et al. (2007). The PCR reactions were diluted 10- to 30-fold with H_2O and 2 μl of the dilution was mixed with 10 μl formamide and 0.2 μl GS500 LIZ or TAMRA size standard (Applied Biosystems). The products were analyzed after capillary electrophoresis and automatically detected using the Genetic Analyzer 3100 (Applied Biosystems). The DNA products were classified by size with Genemapper version 4.0 software (Applied Biosystems) and alleles were assigned (Temwichitr et al. 2007).

Map construction

Segregation ratio of the genotypes of individual markers was checked with the Chi-squared goodness-of-fit-test. None of the markers showed significant segregation distortion. The genetic map distance for the markers was computed with the software package JoinMap™, version 3.0 (van Ooijen & Voorrips 2001). The critical LOD scores used to establish linkage groups and calculate map distances are called 'linklod' and 'maplod', respectively. Marker pairs with a recombination LOD score above a critical 'linklod' are considered to be linked. Only information for marker pairs with a LOD score above 'maplod' is used in the calculation of map distances. To be sure that all markers are placed in the genetic map, a low value for 'maplod' should be used. For the establishment of linkage groups, a critical minimal LOD score ('linklod') of 3.0 was used. For calculation of map distances and estimating most likely gene orders, a critical LOD score ('maplod') of 0.05 was used. Recombination frequencies were converted to map distances in centiMorgans (cM) using the Kosambi function (Kosambi 1944).

Statistical analyses

All statistical analyses were carried out according to Petrie and Watson (1995) and/or Quinn and Keough (2002), using a SPSS® for Windows (version 15.0) computer program (SPSS Inc., IL, USA). Two-sided, exact (i.e. for the non-parametric tests; Mundry & Fischer 1998) probabilities were estimated throughout. The circulating total cholesterol data were summarized as means with standard deviation (SD). The Kolmogorov-Smirnov one sample test was used to check Gaussianity of the cholesterol data. Between total plasma cholesterol concentration and the behavioural parameters Spearman's coefficients of rank correlation (RS) were calculated; significance was assessed by a two-tailed test based on the t statistic.

i) *Parental strains, males and females (Experiment 1)*
   The plasma total cholesterol data were normally distributed and subjected to a two-way analysis of variance (ANOVA) with strain and gender as main factors.

Homoscedasticity was tested using the Levene's test, which is a powerful and robust test based on the F statistic (Lim & Loh 1996). If the ANOVA showed significant effects the group means were further compared with the unpaired Student's t test. The unpaired Student's t tests were performed using pooled (for equal variances) or separate (for unequal variances) variance estimates. The equality of variances was tested with the Levene's test. For the unpaired Student's t test with separate variance estimates, SPSS® uses the Welch-Satterthwaite correction.

ii) *Consomic strain survey, males (Experiment 2)*
Group analyses revealed a parametric distribution of the plasma total cholesterol data. Significant differences in circulating cholesterol level between C57BL/6J and A/J or each consomic strain was calculated using the unpaired Student's t test.

iii) *C57BL/6J x CSS-1 intercross, males and females (Experiment 3)*
Gender appeared to have a significant influence on circulating cholesterol concentration (P = 0.006, unpaired Student's t test). To carry out a genetic analysis using both male and female together for plasma total cholesterol concentration, this phenotype was first normalized to a standard normal distribution (i.e. a distribution with a mean of zero and a variance of one in each gender. The measured individual values were substracted by the mean established for that gender and then divided by the standard deviation of that gender. Within the combined male and female population, this transformed variable had a Gaussian distribution.

iv) *C57BL/6J x CSS-19 intercross, males (Experiment 4)*
The plasma total cholesterol concentration had in this male F2 population a normal distribution.

v) *C57BL/6J, CSS-19-A/J and CSS-19-PWD/PhJ, males and females (Experiment 5)*
Based on gender and haplotype block predictions (Perlegen Sciences Inc., Mountain View, CA, USA; http://mouse.perlegen.com/preview/mousehap.html) the animals were classified into groups. Group analyses revealed a parametric distribution of the plasma total cholesterol data. The circulating total cholesterol data were subjected to a two-way ANOVA with haplotype block structure and gender as main factors. If the ANOVA showed significant effects the group means were further compared with the unpaired Student's t test.

QTL analyses

The location of the QTLs affecting the measured (transformed) quantitative trait (circulating total cholesterol level) and the variance explained by each locus were determined using the MapQTL® software package, version 4.0 (van Ooijen et al. 2002). QTL analysis was performed by MQM (multiple-QTL-model or marker-QTL-marker) mapping, which is more powerful than the traditional interval mapping approach (Jansen 1994). This method is essentially a combination of

interval mapping and multiple regression. The model involves regression both on QTL within an interval and on marker loci outside that interval. Markers take over the role of nearby QTLs and are fitted as cofactors while testing for a single QTL elsewhere in the genome. This way, the cofactors function as a genetic background control and absorb most of the genetic effects of their nearby QTLs from the residual variance. As a result, the power of the QTL analyses is enhanced. Cofactors for the MQM analysis were identified by applying the 'automatic selection of cofactors' option of MapQTL. Results were expressed as LOD scores. Based on Lander & Botstein (1989), having an average distance between markers of 6.3 (mouse chromosome 1) and 3.5 cM (mouse chromosome 19), respectively, and taking into account that a genetic scan was performed across a single, complete chromosome rather than the entire genome, an association was assumed significant when the LOD score was ≥ 1.65 (mouse chromosome 1) and ≥ 1.72 (mouse chromosome 19). For MQM mapping, simulation studies have demonstrated that the thresholds derived for conventional interval mapping are still valid in many situations (Jansen 1994). After grouping by genotype for the DNA marker flanking the peak of the QTL, phenotype comparison of the F_2-animals was performed. The Kolmogorov-Smirnov one-sample test was used to check normality of these data. All data within genotype groups were found to be normally distributed. The co-segregation of the phenotype with alleles at the selected marker locus was evaluated with an ANOVA with post hoc unpaired Student's t test. The mode of inheritance was chosen as free, additive, dominant or recessive according to the differences in the mean values of the circulating cholesterol level between mice that were homozygous C57BL/6J, heterozygous, or homozygous A/J. Output from JoinMapTM and MapQTL® was converted to figures using the graphics program MapChart (Voorrips 2001).

Interactions

For chromosome 1 as well as for chromosome 19, all two-marker pairs were tested for epistatic interactions with a two-way ANOVA.

Thresholds for statistical significance

To take the greater probability of a Type I error due to multiple (post hoc) comparisons into account, a more stringent criterion should be used for statistical significance for the unpaired Student's t tests. For the multiple strain comparisons of Experiment 2 (i.e. host strain versus consomic lines or donor strain) the level of significance for the unpaired Student's t tests was pre-set at $P < 0.004$ (Dunnett's method, as suggested by Belknap, 2003). For experiments 1, 3, 4, and 5 we approached this problem by calculating so-called Dunn-Šidák corrections ($\alpha = 1 - [1 - 0.05]^{1/\gamma}$; γ = number of comparisons). In all other cases (i.e. the Kolmogorov-Smirnov one sample test, Levene's test, ANOVA, Chi-squared goodness-of-fit-test, Spearman's coefficient of rank correlation) the probability of a Type I error < 0.05 was taken as the criterion of significance. Table 1 gives an overview of the

(corrected) thresholds used in each experiment for the (one or two-way) ANOVA's and unpaired Student's t tests. In all other cases (i.e. the Kolmogorov-Smirnov one sample test, Levene's test, Spearman's coefficients of rank correlation, Chi-squared goodness-of-fit-test), the probability of a Type I error < 0.05 was taken as the criterion of significance.

Table 1 *Overview of the (corrected) thresholds for the ANOVA's and Student's t tests*

Study / statistical test	Threshold
Experiment 1 (*significant difference*)	
Two-way ANOVA	$P < 0.05$
Student's *t* tests	
A/J-♂♂ versus C57BL/6J-♂♂	$P < 0.013$
A/J-♀♀ versus C57BL/6J-♀♀	$P < 0.013$
A/J-♂♂ versus A/J-♀♀	$P < 0.013$
C57BL/6J-♂♂ versus C57BL/6J-♀♀	$P < 0.013$
Experiment 2	
Student's *t* tests	
C57BL/6J (n = 27) versus consomic lines (n = 6)	
Evidence that a specific chromosome harbors a QTL:	
Suggestive evidence	$0.004 \leq P < 0.05$
Significant evidence	$P < 0.004$
Experiment 2 (*significant difference*)	
Student's *t* test	
C57BL/6J (n = 27) versus A/J (n = 30)	$P < 0.004$
Experiment 3: cosegregation analyses (*significant difference*)	
One-way or two-way ANOVA	$P < 0.05$
Student's *t* tests	
♂♂ +♀♀, ♂♂, ♀♀	
Homozygous A/J versus heterozygous	$P < 0.009$
Homozygous A/J versus homozygous C57BL/6J	$P < 0.009$
Heterozygous versus homozygous C57BL/6J	$P < 0.009$
♂♂ verus ♀♀	
Homozygous A/J-♂♂ versus homozygous A/J-♀♀	$P < 0.009$
Heterozygous-♂♂ versus heterozygous-♀♀	$P < 0.009$
Homozyg C57BL/6J-♂♂ vs homozyg C57BL/6J-♀♀	$P < 0.009$
Experiment 4: cosegregation analyses (*significant difference*)	
One-way ANOVA	$P < 0.05$
Student's *t* tests	
♂♂	
Homozygous A/J versus heterozygous	$P < 0.017$
Homozygous A/J versus homozygous C57BL/6J	$P < 0.017$
Heterozygous versus homozygous C57BL/6J	$P < 0.017$
Experiments 3 and 4: epistatic interaction analyses (*significant difference*)	
Two-way ANOVA	$P < 0.05$

[Table 1 continued]

Study / statistical test	Threshold
Experiment 5: haplotype analysis (*significant difference*)	
Two-way ANOVA	$P < 0.05$
Student's *t* tests	
C57BL/6J-♂♂ versus C57BL/6J-♀♀	$P < 0.006$
CSS-19-A/J-♂♂ versus CSS-19-A/J-♀♀	$P < 0.006$
CSS-19-PWD/PhJ-♂♂ versus CSS-19-PWD/PhJ-♀♀	$P < 0.006$
C57BL/6J-♂♂ versus CSS-19-A/J-♂♂	$P < 0.006$
C57BL/6J-♂♂ versus CSS-19-PWD/PhJ-♂♂	$P < 0.006$
CSS-19-A/J--♂♂ versus CSS-19-PWD/PhJ-♂♂	$P < 0.006$
C57BL/6J-♀♀ versus CSS-19-A/J-♀♀	$P < 0.006$
C57BL/6J-♀♀ versus CSS-19-PWD/PhJ-♀♀	$P < 0.006$
CSS-19-A/J-♀♀ versus CSS-19-PWD/PhJ-♀♀	$P < 0.006$

Results

Experiment 1: parental strains, males and females

The donor (A/J) and the host (C57BL/6J) strain for the consomic mouse panel had similar circulating cholesterol levels on a commercial, pelleted, control diet (Table 2). The plasma total cholesterol concentration was about 36% and 38% higher in males compared with females from the A/J and C57BL/6J inbred strain, respectively.

Table 2 *Circulating total cholesterol level of A/J and C57BL/6J mice*[1]

	Strain			
	A/J		C57BL/6J	
Measure	**Males**	**Females**	**Males**	**Females**
Plasma total cholesterol level, *μ*mol/L	2179 ± 432	1578 ± 75	2190 ± 208	1607 ± 353

[1] Values are means ± SD for 9 animals per group. Note that a P value of 0.000 does not mean that it is zero, only that it is less than 0.0005.

[2] P values in two-way ANOVA with main factors strain and gender. S indicates effect of strain; G, effect of gender; SxG, interaction. Significant effects (P < 0.05) are indicated in bold characters.

			Post hoc comparisons[3]			
ANOVA[2]			♂♂ vs♀♀		A/J vsC57BL/6J	
S	**G**	**SxG**	**A/J**	**C57BL/6J**	♂♂	♀♀
0.843	**0.000**	0.928	**0.003**[W]	**0.001**[S]	0.947[S]	0.814[W]

[3] P values in unpaired Student's t test. Significance differences (P < 0.013) are indicated in bold characters. S = Student's t test, W = Student's t test with Welch-Satterthwaite correction.

Experiment 2: consomic strain survey, males

Twenty-seven males from the host strain and thirty males from the donor strain were included in this study. In contrast to Experiment 1, C57BL/6J compared with A/J mice had now a 20% higher plasma total cholesterol concentration (Figure 1 and Table 3). When compared to the host strain the consomic panel shows significant evidence for circulating total cholesterol QTLs on mouse chromosomes 1, 9 and 14. There is suggestive evidence for cholesterol QTLs on chromosomes 7, 16, 17, 19, X, and Y. Interestingly, all consomic lines which show evidence for a cholesterol QTL on the substituted chromosome (Table 3), have increased total plasma cholesterol levels compared to the host strain (and also to the donor strain) (Figure 1).

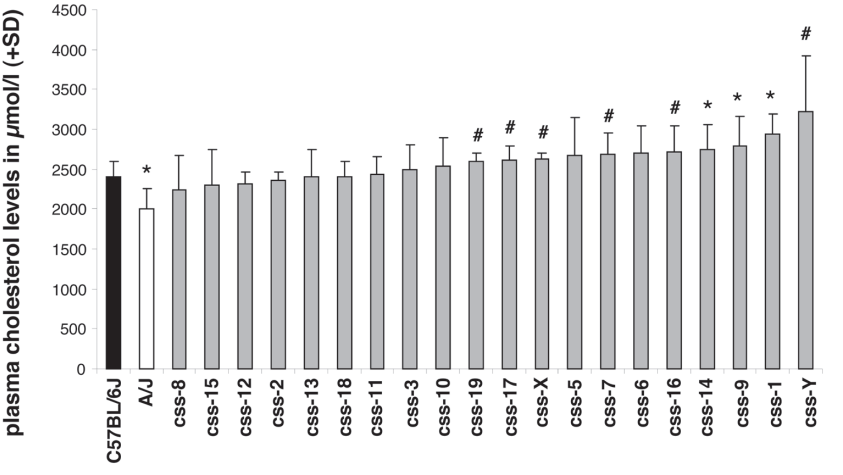

Figure 1 *Baseline circulating total cholesterol level (µmol/L). Results for 19 CSSs (n = 6/consomic strain), the C57BL/6J host strain (n = 27) and the A/J donor strain (n = 30). Results are presented as means + SD. They grey bars (consomic strains) are positioned in order of elevating mean values. Significant (P < 0.004) and suggestive (0.004 ≤ P < 0.05) is indicated by * and #, respectively.*

Table 3 *Suggestive and significant evidence for QTLs influencing the difference in total plasma cholesterol concentration between C57BL/6J (n = 27) and consomic (n = 6) or A/J (n= 30) male mice*[1]

Consomic or donor strain	*P* value
CSS-1	***0.000^{S}**
CSS-2	0.529^{S}
CSS-3	0.366^{S}
CSS-4[2]	-
CSS-5	0.294^{W}
CSS-6	0.116^{W}
CSS-7	*#0.008^{S}*
CSS-8	0.150^{S}
CSS-9	***0.001^{S}**
CSS-10	0.252^{S}
CSS-11	0.847^{S}
CSS-12	0.248^{S}
CSS-13	0.956^{S}
CSS-14	***0.002^{S}**
CSS-15	0.569^{W}
CSS-16	*#0.006^{S}*
CSS-17	*#0.028^{S}*
CSS-18	0.958^{S}
CSS-19	*#0.026^{S}*
CSS-X	*#0.021^{S}*
CSS-Y	*#0.035^{W}*
A/J	**†0.000^{S}**

1 Significant evidence (*, P < 0.004) for a QTL on a chromosome is indicated in bold characters, whereas suggestive evidence (#, 0.004 ≤ P < 0.05) is in italics. † = Donor strain significantly different from host strain (P < 0.004). S = Student's t test, W = Student's t test with Welch-Satterthwaite correction.

2 Due to difficulties with breeding CSS-4 was not available for this study.

Experiment 3: C57BL/6J x CSS-1 intercross, males and females

The results of the QTL analysis for chromosome 1 using the MapQTL software are summarized in Table 4. There was evidence for three QTLs on this chromosome (Figure 2), each with a dominant mode of inheritance. The QTLs explained up to 31.7% of the genetic variance. The presence of multiple peaks on chromosome 1 indicates that several genetic factors on this chromosome may contribute to the control of circulating total cholesterol levels. The three QTL intervals (using the 1-LOD-support interval) are about 9.2, 36.4, and 20.5 Mb and comprise 64, 109, and 146 known genes (http://www.informatics.jax.org/), respectively. Table 5 shows the phenotype of each of the three genotypes (BB, BA, and AA) segregating at locus rs30795049 (i.e. the marker that flanks the QTL with the highest LOD score, = 4.6). The allele frequencies were not statistically different (P > 0.05) from the expected ratio, 1:2:1 (BB:BA:AA). It seems that the C57BL/6J chromosomal 1 genome increased the plasma total cholesterol concentration. As in Experiment 1, males compared to females had a significantly higher plasma total cholesterol concentration.

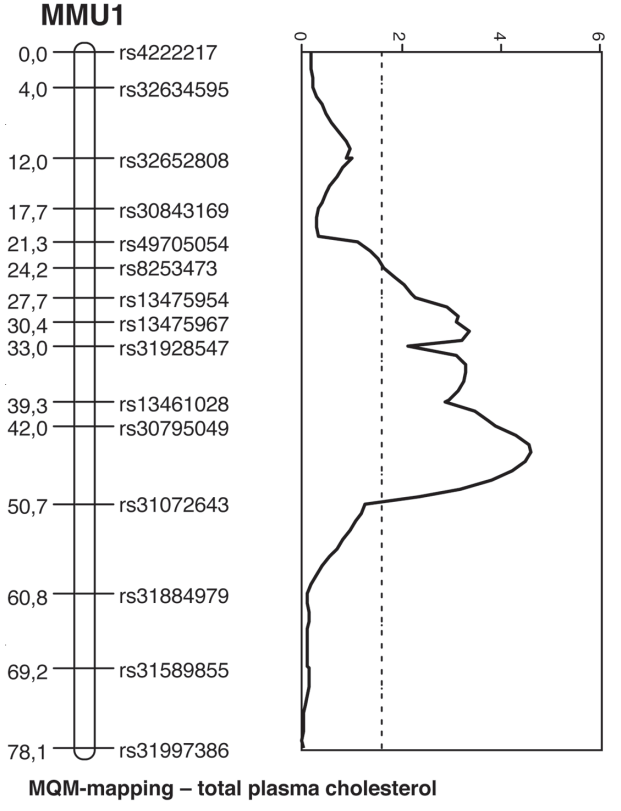

Figure 2 *Genetic map (left; mouse chromosome 1) and LOD score plot (right) for baseline blood total plasma cholesterol concentration in the (C57BL/6J x CSS-1)F2 intercross (n = 93). The dashed vertical line represents the threshold value of the LOD score considered significant for linkage.*

Experiment 4: C57BL/6J x CSS-19 intercross, males

QTL analysis on the (C57BL/6J x CSS-19)F2-population revealed a significant LOD score (5.2) for circulating total cholesterol concentration (Table 4 and Figure 3). It was determined that 34.2% of the variance in the F2-population was accounted for by this QTL. The QTL showed a dominant mode of inheritance. The QTL interval is rather small (204 Kb) and does not contain any known genes. The phenotype of each of the three genotypes segregating at the loci flanking the QTLs (D19Mit1 and D19Mit34) is summarized in Table 6. The allele frequencies were not statistically different (P > 0.05) from the expected ratio. In contrast to chromosome 1, the C57BL/6J compared to the A/J chromosomal 19 genome decreased the plasma total cholesterol concentration.

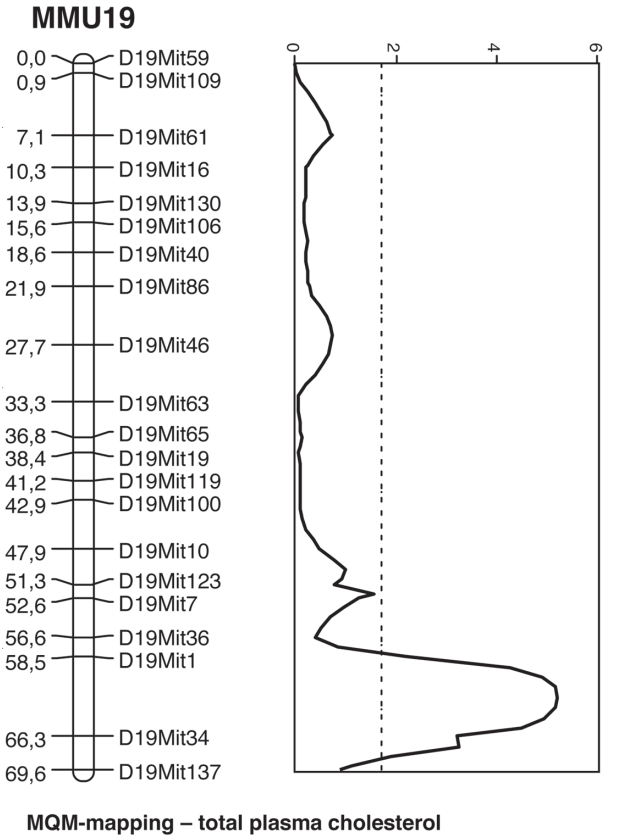

Figure 3 *Genetic map (left; mouse chromosome 19) and LOD score plot (right) for baseline blood total plasma cholesterol concentration in the (C57BL/6J x CSS-19)F2 intercross (n = 96). The dashed vertical line represents the threshold value of the LOD score considered significant for linkage.*

Table 4 *Summary of the QTLs for circulating total cholesterol level detected in Experiments 3 and 4*[1]

Mouse chromosome	Population	Model[2]	Peak LOD[3]
1	♂♂ ± ♀♀	Dominant(+)	3.4
1	♂♂ ± ♀♀	Dominant(+)	3.3
1	♂♂ ± ♀♀	Dominant(+)	4.6
19	♂♂	Dominant(-)	5.2

[1] QTLs were investigated using the MapQTL software on data collected from 93 (C57BL/6JxCSS-1)F2-intercross mice (Experiment 3; males and females) and 96 (C57BL/6JxCSS-19)F2-intercross mice (Experiment 4; males). The module with cofactors (MQM mapping) was used.

[2] Dominant as well as plus (+) or minus (-) contribution to the trait was defined with respect to the C57BL/6J grandparent's allele.

QTL position[4]	% Variance[5]	Cofactor(s)[6]
rs13475967-rs31928547 (31.4 cM; 83.508.390 – 92.755.316)	17.3	rs30843169 and rs31884979
rs31928547-rs13461028 (36.0 cM; 93.572.108 – 130.010.342)	19.9	rs30843169 and rs31884979
rs30795049-rs31072643 (45.0 cM; 119.918.605 – 140.429.802)	31.7	rs30843169 and rs31884979
D19Mit1-D19Mit34 (62.5 cM; 54.897.086 – 55.101.075)	34.2	D19Mit36

[3] Data were shown only when significant results were found. Thresholds for significant linkage are found in the Materials and Methods.

[4] Markers flanking the LOD score peak are given. The location on the chromosome where the LOD score peaked is given in parentheses, as well as the estimated (using lineair interpolation calculations) 1-LOD support interval (in bp).

[5] Percentage of the genetic variance explained by the QTL.

[6] Cofactors that are used in the MQM mapping procedure.

Table 5 *Co-segregation analysis for marker rs30795049 in F2 progeny of C57BL/6J and CSS-1 mice1*

		***Genotype*[2]**
	BB	***BA***
Population	***(n=11M,13F)***	***(n=26M, 26F)***
♂♂ + ♀♀	2177 ± 314	2246 ± 519
♂♂ + ♀♀, *normalized*	0.068 ± 0.640 (0.724)[6]	0.156 ± 0.980 (0.782)
♂♂	2231 ± 408	2453 ± 492
♀♀	2131 ± 213	2040 ± 468
	BB vs. BA	*BB vs. AA*
♂♂ + ♀♀	0.549[S]	0.075[W]
♂♂ + ♀♀, *normalized*	0.689[S]	0.067[W]
♂♂	0.198[W]	0.267[S]
♀♀	0.506[S]	0.148[W]

	P value Chi-squared test[8]
♂♂ + ♀♀	0.317
♂♂	0.489
♀♀	0.652

1 Circulating total cholesterol level values (_mol/L) are means ± SD; n is the number of male (M) and female (F) animals per group. Note that a P value of 0.000 does not mean that it is zero, only that it is less than 0.0005.

2 B indicates C57BL/6J allele; A, A/J allele.

3 LOD scores reported are at the marker indicated. The LOD score between markers is higher (see Figure 2).

4 One-way ANOVA with main factor genotype. Significant effects ($P < 0.05$) are indicated in bold characters.

AA (n=8M,9F)	LOD score[3]	ANOVA (P value) One-way[4] genotype	Two-way[5] genotype	gender	genotype x gender
1871 ± 621		**0.029**			
-0.574 ± 1.265 (-0.852)	3.89	**0.028**			
1945 ± 680		0.051			
1805 ± 596		0.235	**0.024**	0.334	0.051

P value post hoc comparisons[7]

BA vs. AA	BB♂ vs. BB♀	BA♂ vs. BA♀	AA♂ vs. AA♀
0.016[S]			
0.040[W]			
0.026[S]			
0.234[S]	0.476[W]	**0.003**[S]	0.656[S]

5 Two-way ANOVA with main factors genotype and gender. Significant effects (P < 0.05) are indicated in bold characters.

6 Adjusted means (calculated by MapQTL).

7 Contrast significance (unpaired Student's t test). Significant differences (P < 0.009) are indicated in bold characters. S = Student's t test, W = Student's t test with Welch-Satterthwaite correction.

8 Segregation ratio of the genotypes of marker rs30795049 was checked by means of the Chi-squared goodness-of-fit test.

Table 6 *Co-segregation analysis for two markers in F2 progeny of C57BL/6J and CSS-19 mice*[1]

	Genotype[2]			
Marker	**BB**	**BA**	**AA**	**LOD score**[3]
D19Mit1	2641 ± 200 (2498; n =28)[7]	2436 ± 530 (2310; n = 42)	2673 ± 259 (2798; n = 24)	2.2
D19Mit34	2634 ± 276 (2497; n = 31)	2420 ± 504 (2311; n = 43)	2733 ± 193 (2874; n = 20)	3.2

1 Circulating total cholesterol level values (_mol/L) are means ± SD; n is the number of (male) animals per group. Some DNA samples failed to give a conclusive genotype, hence the number of mice typed varied slightly with each locus. Note that a P value of 0.000 does not mean that it is zero, only that it is less than 0.0005.

2 B indicates C57BL/6J allele; A, A/J allele.

3 LOD scores reported are at the marker indicated. The LOD score between markers is higher (see Figure 3).

4 Segregation ratio of the genotypes of marker rs30795049 was checked by means of the Chi-squared goodness-of-fit test.

Interactions: Experiments 3 and 4

A two-way ANOVA was used for evaluating within-chromosome epistatic interactions. Table 7 summarizes the significant interactions. Figure 4 illustrates the interaction between the D19Mit100 and D19Mit36 loci for circulating total cholesterol concentration. When mice are homozygous for the A/J allele at the D19Mit36 locus and homozygous for the C57BL/6J allele at the D19Mit100 locus, the total plasma cholesterol concentrations were lowest. Interestingly, the D19Mit36 locus was used as cofactor in the QTL-analysis (Table 4). For chromosome 1 there was a significant interaction between the cofactors (rs30843169 or rs31884979, see Table 4) and SNP marker rs31997386 (Table 7).

P value				
Chi-squared test[4]	One-way ANOVA[5]	Post hoc comparisons[6]		
		BB vs. BA	BB vs. AA	BA vs. AA
0.258	**0.030**	0.027[W]	0.614[S]	0.044[S]
0.179	**0.006**	0.036[S]	0.171[S]	**0.001**[W]

[5] One-way ANOVA with main factor genotype. Significant effects (P < 0.05) are indicated in bold characters.

[6] Contrast significance (unpaired Student's t test). Significant differences (P < 0.017) are indicated in bold characters. S = Student's t test, W = Student's t test with Welch-Satterthwaite correction.

[7] Adjusted means (calculated by MapQTL).

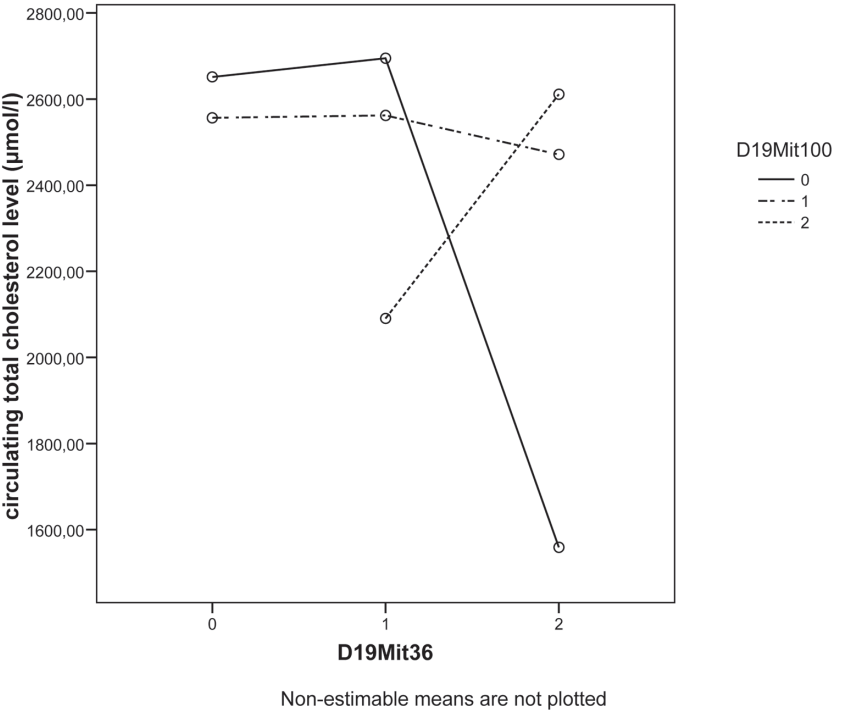

Figure 4 *Graphic presentation of baseline circulating total cholesterol level in the (C57BL/6J x CSS-19)F2 population (n = 96) as the effect of an interaction (P = 0.003) linked to markers D19Mit100 and D19Mit36. Each point represents the mean value for each genotype combination of both markers.*

Table 7 *Significant (P < 0.05) locus-locus interactions*

Chromosome	Locus 1 x Locus 2	*P* (interaction) ANOVA[1]
1	rs30843169 x rs31997386	0.044
	rs31072643 x rs31997386	0.049
	rs31884979 x rs31997386	0.000
19	D19Mit59 x D19Mit106	0.036
	D19Mit109 x D19Mit106	0.048
	D19Mit109 x D19Mit86	0.028
	D19Mit63 x D19Mit36	0.040
	D19Mit100 x D19Mit36	0.003
	D19Mit10 x D19Mit36	0.024
	D19Mit123 x D19Mit36	0.034

[1] Two-way ANOVA with main factors locus 1-genotype and locus 2- genotype.

Experiment 5: C57BL/6J, CSS-19-A/J and CSS-19-PWD/PhJ, males and females

CSS-1-PWD/PhJ mice had higher total plasma cholesterol concentrations than host strain and CSS-19-A/J mice. C57BL/6J and CSS-19-A/J mice had a similar plasma total cholesterol concentration. Gender has a significant effect on circulating total cholesterol concentration in the C57BL/6J and CSS-19-A/J strain, but not in the CSS-19-PWD/PhJ. The haplotype block structure for the QTL interval on chromosome 19 (54.897.086 – 55.101.075 bp, see Table 4) with respect to the C57BL/6J, A/J and PWD/PhJ strains is shown in Figure 5. We searched for a haplotype that is different between these three strains. Thus, also between C57BL/6J and A/J, because although no difference in circulating total cholesterol level exists between these two strains, we still detected a QTL on chromosome 19 (Tables 4 and 6). Using this criterion the left (54.897.086 – 54.929.335 bp) or the right (55.082.525 – 55.101.075 bp) chromosomal region of Figure 5 is most likely involved in regulating plasma cholesterol levels in strains C57BL/6J, A/J and PWD/PhJ.

Associations

For individual mice (i.e. consomic, host and donor strain mice) we studied per experiment the association between circulating total cholesterol level and behavioural parameters of the modified hole board (Experiments 1, 2, 4, and 5), home cage monitoring (Experiment 3), or the open field (Experiment 3). There were several weak, but significant, correlations between the behavioural parameters and total plasma cholesterol concentration (Table 9).

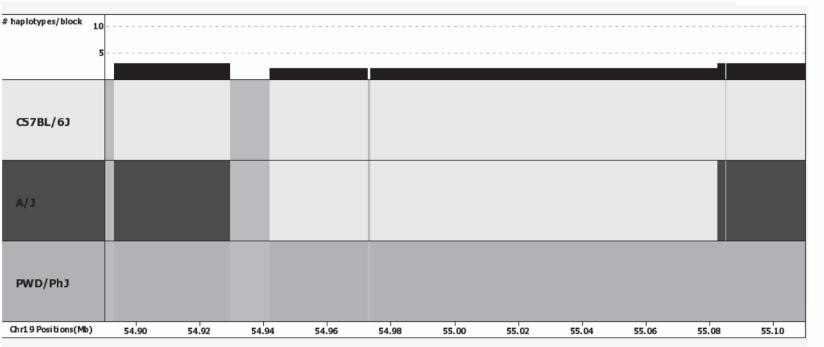

Figure 5 *Haplotype block structure of mouse chromosome 19 region 54.897.086 – 55.101.0756 bp for the inbred strains C57BL/6J (host strain), A/J (donor strain) and PWD/PhJ (donor strain).*

Discussion

Research suggests that patients with anxiety disorders may have significantly elevated plasma cholesterol levels compared to healthy controls (Peter et al., 1999; Papakostas et al. 2004). In contrast, we found for mice that the anxious A/J strain (Laarakker et al. 2008a) had – at least in Experiment 2 - a significantly lower total plasma cholesterol concentration than the non-anxious C57BL/6J (Laarakker et al. 2008a) inbred strain (Table 3 and Figure 1). This strain difference in plasma total cholesterol concentration corroborates previous work (Gallou-Kabani et al. 2007; Paigen 1995; Singer et al. 2004). For individual mice we computed between total plasma cholesterol concentration and total number of board entries a weak, but significant R_S of 0.173 (Experiment 2) and –0.186 (Experiment 5). Thus, if there is a relationship between plasma total cholesterol and anxiety-related behaviour in mice, it is certainly not a simple one. Female A/J and C57BL/6J mice when compared with their male counterparts have a lower circulating total cholesterol level (Tables 1 and 8). A similar difference in total plasma cholesterol concentration between females and males on a control diet has been reported in the literature (Gallou-Kabani et al. 2007).

Table 3 and Figure 1 give an overview of the chromosomes for which there is evidence for a QTL affecting baseline plasma total cholesterol concentration. Our results indicate the presence of QTLs on at least 9 chromosomes (1, 7, 9, 14, 16, 17, 19, X, and Y). Singer et al. (2004) measured also levels of circulating total cholesterol in 19 CSSs and the parental strains (A/J, donor strain; C57BL/6J, host strain). They identified QTLs for total plasma cholesterol concentration on similar mouse chromosomes as we did (chromosomes 16, 17, X, and Y) but also on other chromosomes (4, 8, 11, and 12). Since it is well known that the diet fed to laboratory animals is one of many variables that can confound research

results (Beynen, 1985), this discrepancy might be explained by the fact that the male mice used by Singer et al. (2004) were fed a regular chow from Research Diets Inc. (New Brunswick, NJ, USA), whereas our mice were fed a commercial diet. One concern is the ever present possibility of genotype by diet interactions, thus, not only biochemist (Beynen 1989), but also (laboratory) animal geneticist and experimental ethologist should use standardized diets (Hartley et al. 2003; Wainwright 2001).

Inspection of the International database resource for the laboratory mouse (MGI; http://www.informatics.jax.org/) by using cholesterol as 'Marker Symbol/ Name', we found that fourteen out of twenty-one chromosomes, with the exception of chromosomes 10, 11, 15, 16, 18, X, and Y, carry QTLs that control circulating cholesterols levels (i.e. total, HDL, non-HDL). By performing an additional literature search - using '((chromosome #) OR MMU#) AND QTL* AND cholesterol* AND (mouse OR mice)' as search-term (# = mouse chromosome number, X or Y)) - on PubMed or Web of Science®, there were also reports that describe circulating cholesterol QTLs on the remaining mouse chromosomes (10, 11, 15, 16, 18, X, and Y). Combining these results, and also based on the review by Wang & Paigen (2005), we may conclude that all mouse chromosomes (including both sex chromosomes) harbor genes that influence circulating (total, non-HDL, HDL) cholesterol levels in the laboratory mouse.

The phenotypic difference between the two parental strains was -401 μmol/L (Figure 1) with♂♂A/J <♂♂C57BL/6J. In contrast the phenotypic effect of the 9 significant CSSs compared to C57BL/6J was positive: the phenotypic difference ranged from +191 (CSS-19) to +815 (CSS-Y) μmol/L. These findings point to strong epistasis, and as a consequence the sum of the effects of the 9 consomic strains (+3235 μmol/L) dramatically exceeded the difference between the two parental strains (-401 μmol/L). Recently, Takada et al. (2008) and Shao et al. (2008) reported similar findings with CSSs derived from C57BL/6J (host strain) and MSM/Ms (donor strain) or A/J (donor strain), respectively.

Several genetic analyses have been published that revealed QTLs on mouse chromosome 1 influencing plasma cholesterol levels (Anuciado et al. 2003; Lyons et al. 2004; Machleder et al. 1997; Schwarz et al. 2001; Su et al. 2009; Suto & Sekikawa 2003; Suto et al. 2004; Suto et al. 2005; Suto et al. 2007; Srivastava et al. 2006; Wang & Paigen 2005; Wergedal et al. 2007). All of these QTLs, with two exceptions (Anunciado et al 2003; Su et al. 2009), do not overlap with the 1-LOD support intervals of our three chromosome 1 QTLs. The QTL (for circulating total cholesterol level) found by Anunciado (2003) colocalizes with our QTL region 83.508.390 – 92.755.316 bp, whereas the QTL (for circulating HDL cholesterol level) detected by Su et al. (2009) colocalizes with our QTL region 93.572.108 – 130.010.342 bp. Su et al. (2009) narrowed their QTL region to 2 genes, Farp2 and Stk25, with considerable evidence for both. Kas et al. (2009) reported differential gene expression in F_2 animals for Stk25. Further, the haplotype block structure (Figure 6) was more different between C57BL/6J and A/J. Taken together Stk25 might be a good candidate gene controlling circulating total cholesterol levels in the C57BL/6J x CSS-1 intercross.

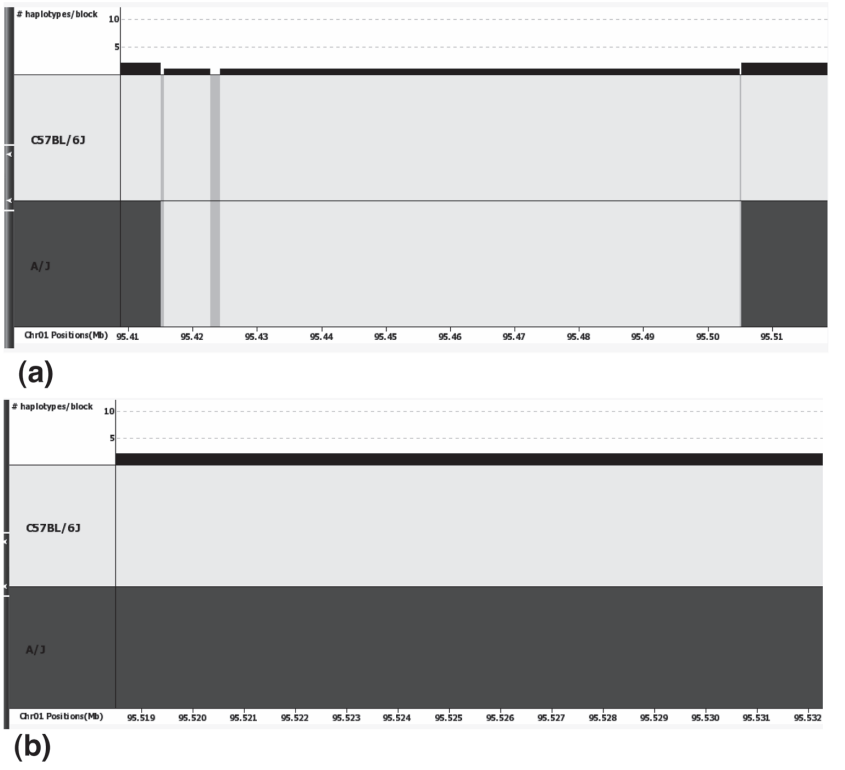

Figure 6 *Haplotype block structure of the genes Fap2 (a) and Stk25 (b) for the inbred strains C57BL/6J (host strain) and A/J (donor strain).*

We also found evidence for interaction between regions of chromosome 1, e.g. between the region that contains the gene Zbtb37 (162.938.931 – 162.964.980 bp; rs31884979) and the segment harbouring Dusp10 (185.858.325 – 185.899.429 bp; rs31997386). In the vicinity of gene Zbtb37 two QTLs (158.554.749 - 158.554.869 bp and 167.119.686 - 167.119.780 bp) influencing (HDL) cholesterol levels are located (Srivastava et al. 2006; Wergedal et al. 2007). The QTLs for total plasma cholesterol concentrations as reported by Schwarz et al. (2001) and Suto (2005) are closely linked to Dusp10.

Locomotor activity is essential for both human and animal species to explore their environment for food and social interaction. Therefore, the genetic variation that contributes to locomotor activity has for long been subject of research. The Department of Neuroscience and Pharmacology, University Medical Centre Utrecht, Utrecht, has also studied the genetic background of locomotor activity levels in the mouse using the open field test (de Mooij-van Malsen et al. 2009) and home cage monitoring (Kas et al. 2009), and focussed on chromosome 1 of this species (Experiment 3). With the modified hole board test locomotor activity can also be measured (Laarakker et al. 2008a; Experiments 1, 2, 4, and 5). A clinical correlation between total plasma cholesterol concentrations and locomotor

activity has been described. However, evidence for such a relationship using animal models is poor (Elder et al. 2008). In an attempt to study this relationship too, we collected blood plasma from mice that were measured for locomotor activity levels, and determined the circulating total cholesterol level. The results were conflicting (Table 9) and speak against a clear relationship between plasma total cholesterol and locomotor activity. Since by using the modified hole board as behavioural test, we found in Experiments 1 and 2 a positive association between total plasma cholesterol concentration and 'total number of line crossings", whereas in Experiment 5 this association was negative. In the open field (novelty based motor activity levels) and the home cage (baseline motor activity levels) the relation was negative (Experiment 3).

The Division of Laboratory Animal Science, Utrecht University is specifically interested in the identification of genetic factors underlying the development of (pathological) anxiety. While other researchers, e.g. the Wellcome Trust Centre for Human Genetics in Oxford, UK (Fullerton 2006; Willis-Owen & Flint 2006), focus with respect to anxiety-related behaviour on mouse chromosome 1, we have special interest for mouse chromosome 19. An F2-intercross between C57BL/6J and CSS-19 was produced and QTL analysis of the male progeny resulted in significant QTLs (Laarakker et al. 2008b). In the course of this study (Experiment 4) we had the opportunity to measure total plasma cholesterol concentrations in male F2 mice and performed a QTL analysis (Tables 4 and 6). The chromosomal segment between markers D19Mit1 and D19Mit34 controls circulating total cholesterol concentration in a dominant fashion. Wergedal et

Table 8 *Circulating total cholesterol level (_mol/L) of C57BL/6J, CSS-19-A/J and CSS-19-PWD/PhJ mice classified into gender and haplotype blocks from the mouse chromosome 19 region with genome coordinates 54897086- 55101075 bp (1-LOD support interval)*[1]

	Haplotype block			ANOVA[3]		
Gender	**'1'**[2]	**'2'**[2]	**'3'**	**H**	**G**	**HxG**
C57BL/6J = '1', CSS-19-A/J = '2', CSS-19-PWD/PhJ = '3'						
♂♂	2411 ± 191 (*n* = 25)	2441 ± 230 (*n* =26)	3163 ± 523 (*n* = 21)	**0.000**	**0.000**	**0.000**
♀♀	1850 ± 289 (*n* = 22)	1922 ± 292 (*n* = 25)	3276 ± 387 (*n* = 18)	($3.7x10^{-34}$)	($6.9x10^{-8}$)	($3.6x10^{-6}$)[5]
C57BL/6J = CSS-19-A/J = '1', CSS-19-PWD/PhJ = '3'						
♂♂	2426 ± 210 (*n* = 51)		3163 ± 523 (*n* = 21)	**0.000**	**0.001**	**0.000**
♀♀	1888 ± 290 (*n* = 47)		3276 ± 387 (*n* = 18)	($1.3x10^{-35}$)	($7.7x10^{-4}$)	($5.5x10^{-7}$)

1 Values are means ± SD. Number of animals per group is given in parentheses. Note that a P value of 0.000 does not mean that it is zero, only that it is less than 0.0005.
Haplotype blocks for the 1-LOD support interval are illustrated in Figure 4.

2 From some animals there was no blood plasma available for determination of circulating total cholesterol level.

al. (2007) reported significant linkage of the marker D19Mit1 with total plasma cholesterol concentrations in a study with (combined) intercrosses derived that from NZB/B1NJ, RF/J, MRL/MpJ and SJL/J mice. This QTL region co-localizes with the QTL region 54.897.086 – 55.101.075 bp as found in the present study (Table 4). Via interval-specific haplotype analysis (DiPetrillo et al. 2005) we were able to narrow down the QTL interval on chromosome 19 into two candidate segments (32 Kb and 19 Kb; Figure 5; Table 8). Unfortunately no genes have been assigned to these segments. A new class of regulatory genes, the microRNAs, also appear to be involved in the regulation of cholesterol metabolism (Esau et al. 2006; Krützfeldt & Stoffel 2006). Up until now several microRNAs map to mouse chromosome 19 (http://microrna.sanger.ac.uk/; Zhu et al. 2007), but alas not to our QTL region. However, it is anticipated that more microRNAs will be assigned to mouse chromosome 19, maybe one of these new genes is located in the 1-LOD support interval of the chromosome 19 QTL.

In the present study there was evidence for interaction between segments of chromosome 19, e.g. between the region that contains D19Mit100 (42.474.814 – 42.474.967 bp) and the segment harbouring D19Mit36 (53.997.400 – 53.997.563). Interestingly, in the vicinity of marker D19Mit100 a QTL (42.469.099 – 42.469.245 bp) influencing HDL cholesterol levels after an atherogenic diet is located (Korstanje et al, 2004), whereas at a distance of about 971 Kb from D19Mit36, Wergedal et al. (2007) found the aforementioned QTL for total plasma cholesterol concentrations.

Post hoc comparisons[4]								
♂♂ **versus** ♀♀			♂♂			♀♀		
'1'	**'2'**	**'3'**	**'1'vs'2'**	**'1'vs'3'**	**'2'vs'3'**	**'1'vs'2'**	**'1'vs'3'**	**'2'vs'3'**
0.000^{S}	**0.000**^{S}	0.456^{S}	0.615^{S}	**0.000**^{W}	**0.000**^{W}	0.405^{S}	**0.000**^{W}	**0.000**^{S}
0.000^{S}		0.456^{S}		**0.000**^{W}			**0.000**^{S}	

3 P values in two-way ANOVA with main factors haplotype block and sex. H indicates effect of haplotype block; G, effect of sex; HxG, interaction. Significant effects (P < 0.05) are indicated in bold characters.

4 P values in unpaired Student's t test. Significance differences (P < 0.006) are indicated in bold characters. S = Student's t test, W = Student's t test with Welch-Satterthwaite correction.

5 Precise P values in the ANOVA are in parentheses.

Table 9 *Associations (Spearman's RS) between circulating total cholesterol concentration and behavioural parameters*

Experiment	*n*	Behavioural dimension / parameter	R_S	P value[4]
1	36	*Locomotion*[1]		
		Total number of line crossings	0.378	0.024
		Latency until the first line crossing	- 0.354	0.034
2	177	*Anxiety*[1]		
		Total number of board entries	0.173	0.024
		Undirected exploration[1]		
		Total number of rearings in the box	0.192	0.013
		Total number of rearings on the board	- 0.195	0.011
		Latency until the first rearing on the board	0.200	0.009
		Total number of hole explorations	0.193	0.012
		Directed exploration[1]		
		Total number of new object explorations	0.170	0.027
		Memory[1]		
		Total number of familiar object explorations	0.299	0.000
		Percentage of time being busy with familiar object explorations	0.213	0.005
		Locomotion[1]		
		Total number of line crossings	0.372	0.000
		Arousal[1]		
		Percentage of time being busy with Grooming	-0.235	0.002
		Other behaviour[1]		
		Percentage of time in the box	0.240	0.002
3	93	*Locomotion*[2]		
		Average distance moved, day 1	-0.245	0.026
		Average distance moved, day 2	-0.291	0.008
		Average distance moved, day 3	-0.246	0.027
		Locomotion[3]		
		Horizontal distance moved	-0.280	0.007

Experiment	*n*	Behavioural dimension / parameter	R_S	P value[4]
4	96	*Directed exploration*[1]		
		Total number of unfamiliar object explorations	-0.210	0.042
		Memory[1]		
		Latency until the first familiar object exploration	-0.254	0.013
5	138	*Anxiety*[1]		
		Total number of board entries	- 0.186	0.035
		Undirected exploration[1]		
		Total number of rearings in the box	- 0.299	0.001
		Directed exploration[1]		
		Total number of holes visited	- 0.198	0.025
		Latency until the first hole visited	- 0.201	0.022
		Percentage of time being busy with unfamiliar object explorations	0.249	0.004
		Locomotion[1]		
		Total number of line crossings	- 0.234	0.008
		Latency until the first line crossing	0.318	0.000
		Arousal[1]		
		Latency until the first grooming	0.179	0.042
		Percentage of time being busy with Grooming	- 0.232	0.008
		Other behaviour[1]		
		Number of times the mouse is in the box	0.260	0.003

1 Behavioural parameters measured in the modified hole board (Laarakker et al., 2008a).
2 Behavioural parameters measured in a home cage environment (Kas et al., 2009).
3 Behavioural parameter measured in the open field (De Mooij-van Malsen, 2009).
4 Only significant ($P < 0.05$) associations are shown.

In summary, in the present study we examined circulating cholesterol levels in a set of chromosome substitution strains as well as in the host and donor strains, we showed the QTL analyses for this phenotype with respect to mouse chromosomes 1 and 19, and studied the correlation of this phenotype with behavioural parameters. Taken together the results form the consomic strain survey and the QTL results for mouse chromosomes 1 and 19 clearly illustrate the complexity of the genetic architecture for the regulation of circulating total cholesterol levels, as already suggested by Stylianou et al. (2006), Shao et al. (2008) and Takada et al. (2008). Further, it can be concluded that if there is a relationship between plasma total cholesterol and experimental behaviour in mice, it is certainly not a simple one and may depend on the experiment.

Discussion & Future research

In this thesis we integrated a behavioural genetic approach with novel statistical strategies and a multidimensional behavioural test design. A consomic line was selected, which revealed a specific and stable high novelty-avoidance phenotype. By means of a minimised number of offspring individuals, several possible candidate genes for anxiety-related behaviour could be identified in chromosome substitution strain 19. In the following, the relevance of the findings will be discussed and methodological aspects of our approach will be critically reviewed.

Behavioural phenotyping

The parental strains of the consomic lines used in this study revealed profound strain difference with respect to anxiety related behaviour. The high anxiety phenotype in A/J mice was found to be a primary strain characteristic and thus, chromosome substitution strains derived from B6 and A/J as parental strains obviously are an interesting tool to search for genes that are involved in the regulation of anxiety-related behaviour in mice. It further was found that males show a more pronounced phenotypic strain difference than female mice. Therefore, the genetic analysis was performed in male mice. Finally, the parameter 'latency to the first board entry', indicating the animals' avoidance behaviour towards a novel and exposed area, appeared to be the most robust anxiety-related parameter and therefore formed the basis for the complex statistical calculation necessary to design a quantitative genetic approach.

In the mHB, several parameters are understood as being indicative for a behavioural phenotype that is classified as 'anxiety-related' (Ohl 2004; Ohl et al. 2001a). Anxiety-parameters are in the first instance related to avoidance of 'novelty'. Next to avoidance towards an unprotected, novel area, avoidance of an unknown (novel) object has been reported to be indicative for anxiety in mice (Griebel et al. 1993) and is therefore measured in the mHB as well. Further, risk assessment is a anxiety-related exploratory behaviour that is expressed by a stretched attends (Augustsson & Meyerson 2004; Berton et al. 1998; Blanchard et al. 1990b; Blanchard et al. 1993). These mHB parameters have all been pharmacologically validated in mice, using anxiolytic compounds that showed to ameliorate the anxious phenotype (Ohl et al. 2001b; Ohl et al. 2003a).

In the mHB, we repeatedly found that the mouse strains A/J and C57BL/6J differ in almost all motivational systems (Laarakker et al. 2006). Further, both

strains also differ in terms of sensitivity for benzodiazepines (Mathis et al. 1995) and have previously been used for anxiety-related research purposes (Bouwknecht & Paylor 2002). Our findings correspond to these results and further indicate that the highly anxious phenotype in A/J mice is not secondary to their low activity level. Therefore, the consomic A/J and C57BL/6J panel was used for further genetic studies.

From this consomic panel the phenotype of the chromosome substitution strain 19 was identified as the most interesting strain for further investigations. This strain not only revealed a specific high-anxiety phenotype but in addition, showed no alterations with respect to locomotor activity of exploration related parameters, which confirms that the anxiety phenotype is not secondary to other, more unspecific behavioural characteristics. This finding again corresponded to the literature where several other research groups identified chromosome 19 as being involved in anxiety-related behaviour in mice as well (Gershenfeld et al. 1997; Gershenfeld & Paul 1997; Gill & Boyle 2005).

Reducing the number of animals needed by use of statistical approaches

To minimise the number of animals necessary for the genetic analysis, a specific statistical approach was used. We adjusted α to 0.004 according to Belknap's calculations (Belknap 2003) and in addition applied a multiple phase approach. This multiple phase approach is a form of sequential analysis, which was already suggested by Russell and Burch (1959, reprinted 1992) as one method to reduce the number of animals used in experiments. A significant reduction of the number of animals was successfully applied in the consomic survey. Further, prelimanry results indicate that using a sequential sampling procedure, when compared with a fixed sample size, can provide a substantial decrease in mean sample size required for detecting QTLs.

The importance of reducing animal numbers may at the first sight be hypothesised as conflicting with the use of chromosome substitution strains, since those even seem to increase the number of animals needed, as showed in the following calculation example: If there are 10 chromosomes that supposable contain a QTL for a trait of interest (Laarakker et al. 2008b), and we still follow Belknap's suggestion (27:6 ratio) and our two stage testing approach the following calculation can be made:

27	B6 parental mice
10 x 27 = 270	animals from 10 chromosomal lines containing a QTL
11 x 6 = 66	animals from the chromosomal lines without a QTL
---	
323 total	

For the F2 intercross 10 (QTLs) x 196 (84 per gender) animals are needed, resulting in additional 1960 mice. This is a total of 323 + 1960 = 2323 animals. In a traditional F2 intercross that can be used for a total genome association study, only 200 animals are needed (approximately 100 per gender) plus the initial parents to generate the F1 and F2 –generation. If one would start off with 5 breeding pairs (= 10 mice), generating on average 6 pups per nest (5 x 6 = 35 F1 mice) that would be used to breed the F2 –generation (two nests per pair necessary to generate 200 F2's), another 10 + 35 = 45 mice would be necessary. Adding these to the initial 200 animals, an F2 cross would result in 245 mice, thus in significantly less then 2323 animals needed in the consomic approach.

Further, in the F2 approach, epistatic interactions between chromosomes can be detected, and the whole genome could be genotyped at once. In the consomic approach only intra-chromosome epistatic interactions can be detected. The advantage of using consomics is that they have greater power to detect smaller QTLs compared to traditional intercrosses. Therefore, it has to be concluded that the use of consomics only reduces the number of mice needed if one focuses on only one chromosome, as done in our studies.

QTLs and candidate genes

The consomic survey showed that mouse chromosomes 5, 8, 10, 15, 18, 19, and Y each contain at least one QTL that is involved in anxiety-related behaviour in mice. It was further concluded that in addition to using the two-stage approach, the quality of the conclusions from the consomic survey could be increased by performing both univariate and multivariate (bivariate) analyses. The analysis led to a special focus on mouse chromosome 19 because of its specific association with anxiety-related behaviour. Thus, mapping of the QTL(s) for anxiety on chromosome 19 by using an F2 intercross between CSS-19 and the C57BL/6J host strain was the subject of a subsequent study.

Table 1 shows a summary of the QTL mapping results so far for chromosome 19 with respect to the difference in anxiety-related behavior between A/J and C57BL/6J. We used the published results from Gershenfeld & Paul 1997; Gershenfeld et al. 1997; Gill & Boyle 2005 and Zhang et al. 2005 to estimate the associated QTL confidence intervals (1-lod support intervals). The chromosomal region with coordinates 32,551,766 - 42,541,189 bp overlaps these four intervals (Table 1). Thus, by combining the data from multiple crosses between A/J and C57BL/6J (DiPetrillo et al. 2005; Li et al. 2005) it is most likely that this chromosomal region contains the anxiety QTL. Linkage for an anxiety proneness phenotype (early onset susceptibility to anxiety disorders) was suggested on the q-arm of human chromosome 10 (Smoller et al. 2001a). Mouse chromosome 19 has homologous regions on human chromosomes 9, 10 and 11 (Poirier & Guénet 1998). Unfortunately, the region of human chromosome 10q to which the anxiety QTL mapped does not correspond to the murine chromosome 19

region with coordinates 32,551,766 - 42,541,189 bp. Again, via comparative genomics (DiPetrillo et al. 2005) it is not possible to narrow the QTL interval in the mouse.

Table 1 *Summary of QTLs on chromosome 19 influencing the difference in anxiety-related behavior between C57BL/6J and A/J mice*

Method	Behavioral test (apparatus)	Marker nearest to QTL peak	Estimated 1-lod support interval (bp; coordinates)	Reference
F_2	Open field	D19Mit46	23,380,471 - 42,541,189	Gershenfeld et al., 1997
F_2	Light-dark box	D19Mit86	17,393,162 - 53,983,163	Gershenfeld & Paul, 1997
RIS	Open field	D19Mit10	Could not be determined	Gill & Boyle, 2005
RCS	Open field	D19Mit10	15,972,152 - 61,283,022	Gill & Boyle, 2005
AIL	Open field	D19Mit19	32,551,766 - 42,541,189	Zhang et al., 2005

As to the chromosome 19 genes involved in the difference in anxiety-related behaviour between A/J and C57BL/6J we can only speculate. The gene CPEB3 (cytoplasmic polyadenylation element binding protein 3) ia a promising candidate gene. CPEB3 is a member of a protein family that regulates local polyadenylation of mRNAs in the cytoplasm of neurons and oocytes. In mouse hippocampus, the gene is up-regulated transiently after induction of seizure and it has been implicated in long-term potentiation (Theis et al. 2003). The results of Huang et al. (2006) indicate that CPEB3 is at least one factor that mediates GluR2 mRNA expression. In turn, deletion of the gene encoding the GluR2 subunit of the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (Gria1) in the mouse showed reduced anxiety in the elevated plus maze (Mead et al. 2006). Interestingly, CPEB3 falls within the range of the one of the 1-LOD support intervals described in chapter 5 and is therefore still considered as a possible candidate.

Analysis of the parameter 'latency time until the first board entry' resulted in two highly significant QTLs and further fine-mapping of the chromosomal regions of interest by haplotype-analysis resulted in several candidate genes. Both QTLs influenced 'latency time' in opposite directions: the QTL decreasing 'latency time' contained the gene Cpeb3 that has been related to the glutamate pathway, which has been shown to be involved in anxiety related behaviour in mice (Huang et al. 2006). The other QTL that seems to increase anxiety contains two candidates, a SORCS gene and the gene Pten, both known to be involved in neurological

pathways related to anxiety (Kwon et al. 2006; Willnow et al. 2008). It is of note that these genes as well are involved in cognitive processing (Ransome & Turnley 2008; Turnley 2005). Since it has repeatedly been hypothesised that high anxiety may strongly be influenced by distinct cognitive processes, it will be of interest to further evaluate whether the identified genes may be of importance for the integration of cognitive-emotional processes (Ohl et al. 2003b).

We further found a significant correlation between distinct behavioural characteristics including locomotion, exploratory behaviour and anxiety, and cholesterol levels related to mouse chromosomes 1 and 19. Thus, we as well investigated whether chromosomes 1 and/or 19 of the mouse contain a QTL for total plasma cholesterol. Mouse chromosomes 1 was found to contain three and chromosome 19 to contain one QTL for cholesterol. One of the three QTLs on chromosome 1 contains two interesting candidate genes, Farp2 and Stk25, both earlier shown to be involved in cholesterol variations in mice, as well as their homologues in humans (Su et al. 2009). Interestingly, Stk25 has also been related to locomotion, a behavioural trait often been mapped to chromosome 1 of the mouse. Both, cholesterol levels and locomotion have been linked to neurological pathways involving the hippocampus, which can probably explain the significant correlations we have found between locomotor activity and cholesterol levels in CSS1 (Elder et al. 2008a; Mulder et al. 2007). The other two QTLs of chromosome 1 do not contain any known genes or QTLs and future investigations will have to show whether these chromosomal locations are involved in the discussed mechanism. The QTL found on chromosome 19 is mapped to a location that has almost exactly been found earlier, and being mapped to HDL cholesterol levels (Wergedal et al. 2007). However, no known genes have been found in this interval, based on gender and haplotype block predictions (Perlegen Sciences Inc., Mountain View, CA, USA, http://mouse.perlegen.com/preview/mousehap.html).

The alpha A2-adrenoceptor gene

It has extensively been reported that in rodents and in humans the alpha A2-adrenoceptor (adra2a) is involved in anxiety and arousal related behaviour and acute stress response (Domes et al. 2004; Ruffolo et al. 1991; Ruffolo et al. 1993; Stamatakis et al. 2008). Since the alpha A2-adrenoceptor gene is expressed on mouse chromosome 19, the adra2a gene, after identification of chromosome substitution strain 19, seemed to be an interesting candidate gene. We therefore performed a pharmacological study to evaluate whether this receptor is involved in the regulation of avoidance behaviour in the mHB as well. The results clearly showed that the adra2a receptor is not primarily involved in anxiety-related behaviour but in arousal and stress-coping in mice. Still an epistatic effect of the adra2a gene remains possible and should be investigated further.

Notably, haplotype analysis for fine mapping the identified QTLs after analysis of the F2 -generation revealed no haplotype difference between A/J and B6, underlining that the adra2a is no candidate gene for avoidance behaviour.

Although the adra2a gene is positioned in a region with unknown haplotypes for A/J and B6, the surrounding regions are quite homozygous for B6 alleles, and it is therefore unlikely that the small region containing the adra2a gene in contrast does differ.

Gender differences and interactions

During the consomic survey preceding the actual QTL analysis, a gender difference was found in the CSS19 strain. CSS19 male mice showed a high anxiety profile compared to B6, in contrast to the females, which did not show this behavioural difference. Interestingly, we could show that the y-chromosome contains a QTL for anxiety related behaviour in the mHB, something never been shown before in the literature. The y-chromosome only contains the Sry-gene, sex-determining region y, leading to a male organism. One possible explanation for this gender difference may be that this sex-specific gene interacts with possible other candidate genes on chromosome 19 and thus may cause the found gender difference.

Another possible explanation for the gender difference is the fact that we found two additive QTLs on mouse chromosome 19 that act in different directions. The QTL increasing anxiety seems to be higher, and may thus influence the phenotype stronger than the QTL decreasing anxiety. It is possible that in females both QTL are present as well but having the same height, which would not result in a visible phenotypical effect.

Further, due to the gender difference found in the parental strains, only male mice of the complete consomic panel were screened, while female mice were tested from the selected chromosome substitution line 19 only. If chromosome 19 influences anxiety in a gender-specific manner, perhaps due to interactions with the y-chromosome, no phenotype can be expected in females. However, it can not be excluded that a female-specific chromosome would have been identified if the complete consomic survey would have been accomplished in female animals as well. Notably, a gender-specificity especially with respect to the susceptibility for anxiety disorders is well known for the human situation: women are at least twice as likely to develop an anxiety disorder and/or depression, although in general, psychiatric disorders are equally common in men en women (Brown 2001).

Methodological considerations

Social housing conditions

In animal experiments and especially in behavioural (genetic) research environmental and housing factors are of crucial importance as potentially confounding factor and should be standardized as much as possible during the whole course of the study. In The Netherlands and many other countries animal experimentation is strictly regulated by law, which includes housing conditions of animals. For example social animals are not allowed to be housed individually, except for specific scientific

reasons. For the experiments described in our first chapter of this thesis, male mice were therefore housed in groups. We however found that social housing resulted in intermale aggression between cage mates, potentially interfering with testing behaviour. Since a trend of testing order effects in the mHB data was revealed (data not shown in this thesis), we had to conclude that for statistical analysis, not the individual animal could be used as experimental unit, but instead the entire cage-group would have to be used, potentially resulting in extreme large numbers of animals per experiment. Further, no stress effect of individual housing was found in other experiments (Arndt et al. 2009). Therefore, the conclusion was drawn that individually housed male mice had to be used for further studies.

Disadvantages of multidimensional testing

Using a multidimentional test allows for the assessment of a variety of different motivational systems in parallel and therefore for dissection of motivational systems. This approach has been shown to represent an efficient and reliable behavioural phenotyping procedure (Ohl 2003; Ohl 2004; Ohl et al. 2001). However, there are also disadvantages of using a multidimensional test. Firstly, the test cannot be automated and is therefore quite labour-intense. Secondly, multidimensional tests involve a variety of different parameters that can be measured, which can result in a difficult to interpret overall-picture due to potentially contradicting parameters. Thirdly, the mHB, like most novelty tests in rodents is based on a (artificial) testing time of 5 minutes, which can result in high variation for distinct parameters within experimental groups, either weakening the statistical power of increasing the group-size necessary to obtain significant results. However, the use of a test battery, i.e. the investigation of different motivational systems in a series of unidimensional tests, would lead to either a significantly longer experimental phase per animal or even higher numbers of animals needed (Ohl et al. 2001a).

Statistical developments

Irrespective of the careful statistical consideration done for this study, different statistical approaches can be found in this thesis: in chapter 2 (Laarakker et al. 2006) the value of _ (alpha) was not corrected for the two stage approach. Further literature research showed that the correct way for performing the statistical analysis twice is to divide _ conform the Bonferroni adaptation by two (for each time the statistical analysis is done) (Petrie & Watson 1995). Re-calculation of the data from chapter 3 revealed that the final conclusions of the experiments do not change after correcting the statistics.

Further, in chapter 2 the Bonferroni adaptation was used to correct _ for the increasing risk of a type I error, while in all other chapters the Dunn-Sidak method was used due to the large number of parameters measured in the mHB. Not only the number of valid comparisons between experimental groups and other relevant factors should be corrected, but the numbers of parameters measured in the behavioural test have to be corrected as well. Usually the conservative Bonferroni-

correction is used, mostly resulting in unacceptable low p-values. As mentioned above, the less conservative Dunn-Sidak correction is therefore advised to be used for behavioural data.

Data sharing

A recent development in biomedical research is the trend of data-sharing and making raw data publicly available in a database. These databases help reducing numbers of animals as well, since for example other researcher's data can be used for meta-analysis studies or fine-mapping QTLs by haplotype analysis. There are different databases available for genetic data, with the best known probably being GeneNetworks with the program MapQTL hosted by Williams and collegues (Rosen et al. 2003), containing data of the BxD recombinant inbred panel. The here presented data will be published (in prep.) on PhenomeNetworks, a database publishing data on a variety of consomic panels for a broad variety of organisms, including plants.

Complementary consomic lines

Recently, other consomic panels (mouse and rat) than the A/J and B6 panel become commercially available. For our research, a potentially interesting strain was another CSS19 line, where again B6 served as the host strain, but this time chromosome 19 was substituted by the corresponding chromosome of the PWD/PhJ (PWD) mouse, the latter being a wild derived inbred strain that is thought to be more anxious compared to B6 (Holmes et al. 2000; Fernandes et al. 2004a; Fernandes et al. 2004b). Crossing these PWD with CSS19 with an A/J substitution would show whether the candidate genes of the recessive A/J trait could be found as well on the PWD chromosome. However, behavioural phenotyping of the PWD strain in the mHB did not reveal a high anxiety phenotype compared to B6. Therefore additional complementary experiments were not performed.

Future research

In future studies, the involvement of the here identified candidate genes in affecting anxiety related behaviour in mice has to be validated. Complementary genetic approaches using other consomic mouse strains can confirm the relevance of these candidate genes for the mouse in general. Already existing and future consomic rat strains would allow for the translation of our results to another rodent species, thus proving the potential translational value of our findings. Further, knock-out mice could further help to confirm the possible candidate genes. Thereafter, real-time PCR and/or micro-array techniques could show whether the found genes differ in expression levels between A/J and C57BL/6J. In addition, sequencing the genetic region of interest in both, host and donor strains of our consomic panel, can determine eventual amino acid changes that can also contribute to the possible

differences between A/J and C57BL/6J. Ultimately, after confirming the gene(s) involved in anxiety-related variations in laboratory mice, the translations to the human situation would provide crucial information about the pathway involved. DNA of anxiety patients and healthy controls divided in two groups scoring either high or low for anxiety based on the 'State - Trait Anxiety Inventory' (Spielberger et al. 1983) should be screened to hopefully show that the patient group contains the 'anxiety-variant' of the gene, and that the low-anxiety controls contain the 'non-anxiety variant'. An interesting finding would be if the highest high-anxiety controls would also contain the 'anxiety-variant' of the gene.

Further, potential epistatic effects have to be elucidated and the specific neurobiological pathways regulated by the identified candidate genes have to be further investigated with respect to their functional involvement in anxiety related processes. However, the use of consomics only allows assessing for interactions between cis – interacting genes, thus being located on the same chromosome. The use of recombinant inbred strains (RIS) for future studies of epistatic effects would therefore be of additive value.

If in the future 'behaviour-genes' are mapped, chromosomal regions can be compared to the same regions of other mammals, including humans. Comparing chromosome positions of genes between different species showed that distinct parts of the genome are conserved during evolution. It can therefore be expected that yet unknown genes involved in behavioural traits can be identified in animal models and translated to the human situation. Such knowledge hopefully can, in the long run, lead to the discovery of new targets and strategies for the prevention and treatment of psychiatric disorders.

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Summary

Chapter 1 describes the profound strain difference with respect to anxiety related behaviour revealed for the parental strains of the consomic lines used in this study. The high anxiety phenotype in A/J mice was found to be a primary strain characteristic, and thus, chromosome substitution strains derived from C57BL/6J and A/J as parental strains obviously are an interesting tool to search for genes that are involved in the regulation of anxiety-related behaviour in mice. It further was found that males show a more pronounced phenotypic strain difference than female mice. Therefore, the genetic analysis was performed in male mice. Finally, the parameter 'latency to the first board entry' appeared to be the most robust anxiety-related parameter and therefore formed the basis for the complex statistical calculation necessary to design a quantitative genetic approach.

To minimize the number of animals necessary for the genetic analysis, a specific statistical approach was elaborated in chapter 2. We adjusted _ to 0.004 according to Belknap's calculations (Belknap, 2003) and in addition applied a multiple phase approach. A significant reduction of the number of animals could be achieved, and was successfully applied in the consomic survey.

In chapter 3 and 5 we show by means of a consomic survey that mouse chromosomes 5, 8, 10, 15, 18, 19, and Y each contain at least one QTL that is involved in anxiety-related behaviour in mice. It is concluded that in addition to using the two-stage approach, the quality of the conclusions from the consomic survey could be increased by performing both univariate and multivariate (bivariate) analyses. The analysis led to a special focus on mouse chromosome 19 because of its specific association with anxiety-related behaviour. Thus, mapping of the QTL(s) for anxiety on chromosome 19 by using an F2 intercross between CSS-19 and the C57BL/6J host strain was the subject of a subsequent study.

Since the alpha A2-adrenoceptor (Adra2a) gene is expressed on mouse chromosome 19, we performed a pharmacological study to investigate whether this receptor is involved in avoidance behaviour in the mHB, and the Adra2a gene may be a possible candidate gene (chapter 4). The results show that the adra2a receptor is not primarily involved in anxiety-related behaviour but in arousal and stress-coping in mice. Still an epistatic effect of the adra2a gene remains possible.

Analysis of the parameter for anxiety related avoidance behaviour resulted in four significant QTLs and several within-chromosomal (cis) epistatic interactions.

Functional candidate genes located in the vicinity of two of these QTLs were identified: Cpeb3 and Sorcs1. These two QTL regions influenced anxiety related avoidance behaviour in opposite directions. One region contained the gene Cpeb3 that has been related to the glutamate pathway, which has been shown to be involved in anxiety related behaviour in mice. The other QTL region contains the Sorcs1 gene, which is known to be involved in neurological pathways related to anxiety.

Chapter 6 describes weak, but significant correlation between distinct behavioural characteristics and cholesterol levels related to mouse chromosomes 1 and 19. Thus, we as well investigated whether chromosomes 1 and/or 19 of the mouse contain a QTL for plasma total cholesterol level. Mouse chromosomes 1 was found to contain three and chromosome 19 to contain one QTL for cholesterol. One of the three QTLs on chromosome 1 contains two interesting candidate genes, Farp2 and Stk25, both earlier shown to be involved in cholesterol variations in mice, as well as their homologues in humans. Interestingly, Stk25 has also been related to locomotion, a behavioural trait often been mapped to chromosome 1 of the mouse. The other two QTLs of chromosome 1 do not contain any known genes or QTLs. The QTL found on chromosome 19 was mapped to a location that has almost exactly been found earlier, and which is being linked to HDL cholesterol levels. However, no known genes have been found in this interval.

Dutch summary / Nederlandse samenvatting

In hoofdstuk 1 worden de gevonden stamverschillen die betrekking hebben op gedrag dat is gerelateerd aan angst beschreven. Deze gedragskarakterisering is in eerste instantie onderzocht bij de ouderstammen (donorstam A/J, gastheerstam C57BL/6J) van de consome lijnen die in deze studie gebruikt zijn. Het hoogangstige fenotype in A/J muizen bleek als een primair stamkenmerk beschouwd te kunnen worden. In het licht hiervan is het panel van chromosoomsubstitutie-stammen (CSS) waarbij de C57BL/6J en A/J ouderstammen als ouderstammen fungeren uitgesproken interessant gereedschap voor de zoektocht naar genen die betrokken zijn bij het ontstaan en de regulatie van angstgedrag. Er kon bovendien aangetoond worden dat mannelijke muizen meer uitgesproken stamverschillen in het fenotype laten zien dan vrouwtjes. Daarom is er ook voor gekozen om de studie met mannelijke muizen uit te voeren. Ten slotte bleek de parameter 'latentietijd tot dat eerste toegang tot het bord' het meest robuust te zijn en zal daarom ook de basis vormen voor complexe statistische berekeningen die nodig zin om een goede kwantitatieve genetische aanpak op te zetten.

Om het aantal dieren dat nodig is voor een betrouwbare genetische analyse zo veel mogelijk terug te dringen, is in hoofdstuk 2 een specifieke experimenteerwijze en statistische analyse toegepast. Hiervoor is _ volgens de methode van Belknap (Belknap, 2003) naar 0.004 aangepast en een zogenaamde sequent proefschema gehanteerd. Een aanzienlijke vermindering van het aantal benodigde dieren kon daardoor bereikt worden. Deze aanpak is vervolgens succesvol toegepast in het consome panel onderzoek (hoofdstuk 5).

In hoofdstuk 3 laat het consome panel onderzoek zien dat chromosomen 5, 8, 10, 15, 18, 19, en Y elk op zijn minst één QTL bevat die betrokken is bij gedrag dat in relatie gebracht wordt met angst bij muizen. Er kon ook geconcludeerd worden dat naast de statistische twee-fase aanpak, de kwaliteit van de conclusies van het consome panel onderzoek verhoogd kon worden door zowel univariate als multivariate (bivariate) statistiek toe te passen. De resultaten van deze consome studie duiden erop dat chromosoom 19 van de muis specifiek geassocieerd is aan angstgedrag, wat aanleiding is om het verdere onderzoek te concentreren op dit chromosoom. Het hoofddoel van de daarop volgende studie was daarom ook het lokaliseren van QTL(s) voor angst op chromosoom 19 in een F2 inter-kruising tussen CSS19 en de C57BL/6J gastheerstam.

Gezien het alfa 2A-adrenoceptor (Adra2a) gen op chromosoom 19 van de muis gelocaliseerd is en bij mensen in verband wordt gebracht met opwinding en stress, is er een farmacologische studie uitgevoerd om de mogelijke betrokkenheid van dit gen bij angstgedrag te onderzoeken (hoofdstuk 4). De resultaten laten echter zien dat deze receptor niet primair bij het ontstaan van gedrag dat in verband gebracht wordt met angst betrokken is, maar eerder bij stresshanteringsgedrag in muizen. Een epistatisch effect van het Adra2a-gen kan desalniettemin niet worden uitgesloten.

Analyse van de parameters welke angst gerelateerd vermijdingsgedrag beschrijven, resulteerde in vier significante QTLs en verschillende intra-chromosomale (cis) epistatische interacties. Functionele kandidaat-genen die gepositioneerd zijn in het betrouwbaarheidsinterval van twee van deze QTLs zijn gevonden: Cpeb3 en Sorcs1. Deze twee QTL regio's beïnvloeden aan angst gerelateerd vermijdingsgedrag in tegenovergestelde richting. Eén regio bevat het gen Cpeb3 dat onderdeel van de glutamaat-pathway is en eerder in verband is gebracht met angstgedrag. De andere QTL regio bevat Sorcs1, een gen dat is betrokken bij verschillende neurologische pathways in relatie tot angst.

Hoofdstuk 6 beschrijft een zwakke maar significante correlatie tussen bepaalde gedragsfenotypen en de bloedplasma cholesterol concentratie in de muis. In dit hoofdstuk is tevens naar QTLs voor bloedplasma cholesterol waarden op de chromosomen 1 en 19 van de muis gezocht. Op chromosoom 1 bleken drie cholesterol QTLs te liggen, en op chromosoom 19 één. Het betrouwbaarheidsinterval van één van de drie chromosoom 1 QTLs bevat twee interessante, functionele kandidaat-genen: Farp2 en Stk25, beiden al eerder in verband gebracht met HDL cholesterol variaties in muizen, net zo als hun humane homologen dat zijn. Interessant is dat Stk25 ook in verband is gebracht met locomotie, een gedragsdimensie die vaak aan chromosoom 1 van de muis wordt gecorreleerd. De betrouwbaarheidsintervallen van de andere twee QTLs van chromosoom 1 bevatten noch genen waarvan bekend is dat zij betrokken zijn bij de regulatie van de bloedplasma cholesterol spiegels, noch aan bloedplasma-cholesterol gerelateerde QTLs. Het QTL-segment welke op chromosoom 19 is aangetroffen komt perfect overeen met het chromosoom 19 segment dat door andere onderzoekers in verband is gebracht met de regulatie van de HDL cholesterol spiegel. Ook dit interval bevat tot heden echter geen bekende functionele kandidaat-genen.

German summary / Deutsche Zusammenfassung

Das erste Kapitel beschreibt die grundlegenden Unterschiede im Angstverhalten der Elternstämme der in dieser Studie benutzten Chromosomensubstitutionsstämme (CSS). Die Resultate zeigen, dass der Angstfenotyp der A/J Inzuchtmäuse eine primärere Karakerstik des Mausstammes ist. Hieraus kann geschlussfolgert werden, dass CCS-Stämme, die von C57BL/6J und A/J Elternstämmen abstammen, ein interessantes Model sind um Gene zu lokalisieren, die bei der Regulation von Angstverhalten von Mäusen eine Rolle spielen. Ausserdem wurde festgestellt, dass der Stammunterschied bei maennlichen Tieren deutlicher ausgepraegt ist als bei weiblichen Tieren. Daher wurde die genetische Analyse bei männlichen Mäusen durchgeführt. Schliesslich wurde der Parameter 'Latenz bis zum ersten board Eintritt' als der robusteste befunden und wurde daher als Basis für die komplexen statistischen Berechnungen gebraucht, die nöting sind, um ein quantitatives genetisches Studiendesign zu entwerfen.

Um die minimale Anzahl Tiere zu berechnen, die nötig für eine korrekte genetische Analyse sind, wurde eine spezifische statistische Methode entwickelt, welche in Kapitel 2 ausgeführt wird. Der Wert von alpha wurde, den Berechnungen von Belknap (Belknap, 2003) folgend, auf 0.004 gesetzt und zu einer 2-Phasen Methode gefügt. Hierdurch konnte eine signifikante Reduktion der Anzahl der benoetigten Tiere erzielt werden, die erfolgreich auf die Fenotypierung der CCS-Stämme angewendet werden konnte.

In Kapitel 3 und 5 koennen anhand der Analyse der CCS-Stämme zeigen, dass die Mauschromosomen 5, 8, 10, 15, 18, 19 und Y jewels mindestens einen QTL umfassen, der in die Regulation des Angstverhaltens der Maus involviert ist. Weiterhin wird ausgefuehrt, dass die Qualität der Resultate aus der CCS -Studie erheblich verbessert werden kann, indem zusaetzlich zu der 2-Phasen Methode sowohl eine univariate als auch eine multivariate (bivariate) Statistik ausgeführt wird. Die statistische Analyse erwies schlussendlich eine spezifische Assozation von Chromosom 19 mit dem Angstverhalten der Maus. Im weiteren Verlauf wurde daher auf die QTL Analyse potentieller Angstgene auf Chromosom 19 der F2–intercross Generation zwischen CSS19 und dem C57BL/6J Gaststam fokussiert.

Da der A2 adrenerge Rezeptor (Adra2a) Gen auf dem Mauschromosom 19 exprimiert wird, wurde eine pharmakologische Studie ausgeführt, die nachweisen

sollte, ob dieser Rezeptor in das Vermeidungsverhalten der Maus involviert und das Adra2a Gen somit ein mögliches Kandidatengen ist (Kapitel 4). Die Resultete zeigen jedoch, dass Adra2a nicht primär in die Regulation des Angstverhaltens verwickelt ist, vielmehr jedoch Erregung und Stress-coping bei Mäusen beeinflusst. Epistatische Effekte von adra2a sind aufgrund dieser Ergebnisse jedoch nicht auszuschliessen.

Die Analyse der Angstverhaltensparameter resultierte schlussendlich in vier signifikanten QTLs und verschiedenen Interaktionen zwischene Genen auf demselben Chromosom (cis– epistatische Interaktionen). Funktionale Kandidatengene, die sich in zwei dieser QTLs befinden, wurden als Cpeb3 und Sorcs1 identifiziert. Diese beiden QTLs beeinflussen angstrelatiertes Vermeidungsverhalten in entgegen gesetzten Richtungen. Von Cpeb3 ist bereits bekannt, dass es eine Rolle spielt bei der Regualtion von Glutamat, was wiederum mit Angstverhalten bei Mäusen in Verbindung gebracht werden konnte. Sorcs1 ist involviert in neurologische Prozesse, welche ihrerseits mit Angst in Bezug stehen koennen.

Kapitel 6 beschreibt eine schwache aber signifikante Korrelation zwischen bestimmten Verhaltenskarakteristiken und Cholesterolwerten, die in Relation stehen zu den Chromosomen 1 und 19 der Maus. Daher haben wir untersucht, ob die Chromosomen 1 und/ oder 19 moegliche QTLs für Plasmacholesterolwerte aufweisen. Auf Mauschromosom 1 lassen sich 3 QTLs, auf Mauschromosom 19 dagegen 1 QTL nachweisen. Einer der drei QTLs von Chromosom 1 beinhaltet zwei interessante Gene, Farp2 und Stk25, die beide bereits zuvor in Studien zu Cholesterolvariationen bei der Maus gefunden wurden, ebenso wie auch ihre Homologe beim Menschen. Interesasant ist, dass Stk25 auch in Bezug zu Lokomotion gesetzt werden konnte, ein Verhaltensaspekt, der in Verbindung zu Chromosom 1 der Maus steht. Fuer die anderen beiden QTLs von Chromosom 1 sind keine Gene oder QTLs bekannt. Der QTL, der auf Chromosom 19 gefunden wurde, betrifft eine Region, die bereits zuvor annaehernd exakt für Variationen in HDL Cholesterolwerte beschrieben wurde. Dennoch konnten in diesem Interval bis jetzt keine bekannten Gene nachgewiesen werden.

List of abbriviations

A	A/J (inbred mouse strain)
A	adenosine
AIL	advanced inbred line
AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AN	anxiety
ANOVA	analysis of variance
AR	arousal
ati	atipamezol (Antisedan®)
B6	C57BL/6J (inbred mouse strain)
C	cytosine
cM	centi Morgan (= genetic distance)
cort	corticosterone
Cpeb3	cytoplasmic polyadenylation element binding protein
CSS19	chromosome substitution strain (19 reveres to substituted chromosome with official strain name C57BL/6J-Chr 19A/NaJ)
dex	dexmedethomidine (Dexdormitor®)
DI	directed exploration
DNA	deoxyribonucleic acid
DW	dry weight
EPM	elevated plus maze
F_1	Filial 1 hybrid generation
F_2	Filial 2 hybrid generation
FST	forced swim test
G	guanine
GAD	generalized anxiety disorder
GluR2	metabotropic glutamate receptor 2
Gria1	ionotropic glutamate receptor 1
HS	heterogeneous stock
ip	intra peritoneal
IQR	inter quartile range
ISCS	interval-specific congenic strain
LO	locomotion
LOD	logarithm (base of 10) of odds

ME	memory
mHB	modified hole board test
MMU	Mus musculus (eventually followed by chromosome number)
mRNA	messenger ribonucleic acid
OCD	obsessive compulsive disorder
OF	open field
OT	other behaviour
P	parental generation
PCR	polymerase chain reaction
Pten	phosphate and tensin homolog
PTSD	post traumatic stress disorder
PWD	PWD/PhJ (inbred mouse strain)
QTL	quantitative trait locus/loci
RCS	recombinant congenic strain
RI	risk assessment
RIS	recombinant inbred strain
SAD	social anxiety disorder
SD	standard diviation
SEM	standard error of the mean
SNP	single nucleotide polymorphism
Sry	sex determining region on chromosome Y
SSLP	simple sequence length polymophism
T	tyrosine
UN	undirected exploration

Curriculum vitae

Marijke C. Laarakker was born on July, 25th 1982 in Nördlingen, Germany. Her family moved back to the Netherlands in 1997 where she passed her final exams at the Openbare Regionale Scholengemeenschap Lek & Linge in Culemborg in 2000. The following four years she studied higher laboratorial education at the Hogeschool van Utrecht. In order to receive her bachelor degree in zoology, she went to the United States for 9 months to investigate the role of the autotransporter protein MisL on intestinal colonization of Salmonella enterica serovar Typhimurium. Furthermore, the phenotypic and molecular features of this protein were characterized. This internship was supervised by Dr. Caleb Dorsey and Prof. dr. Andreas Bäumler at the Department of Microbiology and Immunology of the Texas A&M University System Health Science Centre in College Station, Texas. Here, Marijke became enthusiastic about scientific research and in 2004 she started working as a PhD-student at the Department of Animals, Science & Society, Division Laboratory Animal Science, at the Veterinary Faculty of the Utrecht University. Her project contributes to unraveling the genetic background of anxiety-related avoidance behaviour in laboratory mice and in the future to better diagnostics and therapeutics of anxiety disorders in humans. Marijke's PhD-project was supervised by Dr. Hein van Lith and Prof. dr. Frauke Ohl. The results that were obtained during this project have been presented at various (international) scientific meetings and finally described and discussed in this thesis.

Marijke started working as a post-doc, doing research on the genetics schizophrenia and autism with Dr. Roel Ophoff and Dr. Martien Kas at the Rudolf Magnus Institute for Neurosciences, on April, 1st of this year. Meanwhile she is still training to take the black-belt karate exam at the end of this year.

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Weening EH, Dorsey CW, Laarakker MC, Humphries AD, Kingsley RA, Tsolis RM and Bäumler AJ (2004) *Seven Fimbrial Operons of Salmonella Typhimurium Play a Role in Intestinal Persistence in CBA/J Mice.* 104th General Meeting of the American Society for Microbiology. New Orleans, LA

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