Genetic analysis of emotionality using consomic mouse strains established from C57BL/6J and MSM/Ms

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Contents

Abstract

Emotionality, such as fear and anxiety, is an evolutionally conserved trait in many animals to prepare for and react against danger. However, excess level of emotionality interrupts their normal life, and it will be diagnosed as psychological disorder in human (e.g. anxiety disorder). It is known that emotionality has genetic bases as well as environmental effect, and recently a number of genes contributing to anxiety have been progressively found. In the animal model, several behavioral tests and indices are developed and used to measure emotionality of animals. However, it has been noticed that all of those emotionality-related indices do not have consistent correlation within individuals, even between measurements in the same test. That is, those indices are measuring several different aspects. In psychological studies, emotionality has been considered as a "complex of factors" rather than a single alternative construct. However, not many studies that aim to identify genes associated with emotionality have concerned this multifactorial architecture of emotionality. There are some attempts by combining genetic analysis and multivariate analysis of behavior to identify genetic loci related to the "complex of factors", but those are just a beginning. In this study, I examined the structure of those multiple factors of emotionality, validated those factors genetically, and tried to identify genetic loci related to those factors. I focused on the open-field test, which is the first model for measuring emotionality, and still common today.

At the start point of this study, I examined genetic contribution to the open-field behavior by using conventional measurements, ambulation and defecation, and some ethological measurements in a variety of wild-derived mouse strains. By describing open-field behavior in detail and examining temporal changes, I was able to identify the prominent behavioral features of each strain of mice. Conventional simple measurements lose substantial information, such as the variety of behaviors that can be displayed, and the use of too few indices might

easily lead to confusion in interpreting the genetic mechanisms underlying open-field behavior or “emotionality”. Principal component analysis showed that the open-field behavior consisted of three dimensions of psychological trait: “locomotor activity”, “thigmotaxis”, and “anxious tension state”.

In order to perform genetic mapping of open-field behavior, I used consomic strains of mouse established from C57BL/6J and MSM/Ms (B6-Chr^{MSM}CSSs) in which one of each chromosome of C57BL/6J was substituted by a corresponding chromosome of MSM. By analyzing a series of CSSs, I was able to map the chromosomes associated with a certain phenotype. In addition to open-field test, two kinds of other emotionality-related tests, elevated plus-maze and social interaction test, were examined. By analyzing a panel of CSSs, I identified multiple chromosomes that have a QTL or QTLs related to conventional and ethological measurements of open-field behaviors, elevated-plus maze, and social interaction test. Many CSSs had substantially large effect QTLs due to the non-additive effect, and thus they were expected to be superior tool for the next step of QTL analysis: identifying the quantitative trait gene. By analyzing both males and females of CSSs, I found that there were many sex-dependent QTLs. Principal component analysis of a series of CSSs validated the three factors underlying open-field behavior as in wild-derived mouse strains. Because behaviors loaded on “anxious tension state” factor have rarely been analyzed in most behavior genetic analysis, I focused on this factor for the further analysis.

One CSS, B6-17MSM, that has substituted chromosome 17 from MSM, showed increase of the “anxious tension state” factor. They also exhibited reduced novelty- induced activity and highly increased social interaction behavior, but no differences in their home-cage activity. Thus, it was expected that there is a genetic locus/loci related to some aspect of “emotionality” on the chromosome 17. For characterizing B6-17MSM in more detail, I conducted several behavioral tests and brain morphological analysis. Fear conditioning tests revealed B6-17MSM had an increased fear memory in the cue-fear conditioning but not in the context-fear conditioning.

Thus, it was expected there is a genetic locus/loci related to cue-specific fear learning on the chromosome 17. On the other hand, this strain had increased incidence of hydrocephalus. Histological analysis revealed that externally-normal individuals of B6-17MSM had enlarged brain ventricle size than C57BL/6J. Despite the hydrocephalus phenotype, B6-17MSM showed normal sensorimotor gating and motor coordination as C57BL/6J.

The analysis of reciprocal F1 intercross of B6-17MSM and C57BL/6J revealed that there are prominent maternal effects on their behavior. To identify genetic loci related to those behaviors and the hydrocephalus-like phenotype, I established a series of congenic mouse strains of B6-17MSM. By analyzing those congenic strains, I successfully revealed novel genetic loci associated with the brain ventricle size on the chromosome 17. Behavioral analysis also identified several genetic loci related to each behavior. Although social interaction behavior was prominently high in B6-17MSM, any congenic strains showed increased duration of social contact. It was supposed that there are interacting epistatic genes for inducing social interaction on this chromosome.

So far, I conducted the factor analyses of open-field measurements in the wild-derived strains and consomic mouse strains, and confirmed that there are “locomotor activity”, “thigmotaxis”, and “anxious tension state” factors related to their behaviors. Behavioral analysis of congenic strains also revealed the existence and independence of those factors. The analysis of open-field behavior revealed two interesting congenic strains, C10 and C15; C10 has relation to “locomotor activity” factor, and C15 is associated with both “locomotor activity” and “anxious tension state” factors. Further behavioral characterization of these congenic strains showed differences of home-cage activity and fear conditioning between C10 and C15. This result suggested that the “locomotor activity” factor and “anxious tension state” factor are independent traits and have relation to different genetic and biological pathways.

In addition to the above study, I conducted genetic analysis of other important emotion, aggression. Aggression has considerable importance for animal’s living and is evolutionally

ancient behavior. Because the wild-derived strain MSM/Ms still retains considerable aggression, it was expected that B6-Chr^{MSM}CSSs would have advantages to identify genetic loci associated with the aggressive behavior. In this study, I focused on one CSS, B6-15MSM, which has substituted chromosome 15 from MSM, and examined their aggression in the resident-intruder paradigm. Resident-intruder test revealed that B6-15MSM shows elevated aggressive behavior toward the same genotype intruder compared to C57BL/6J. By analyzing both homogenous pairs and reciprocal, heterogenous pairs in the resident-intruder test, I found prominent effect of the opponent (intruder) in their aggressive behavior: aggressive behavior was increased when the intruder was B6-15MSM but not C57BL/6J. The analysis of reciprocal F1 progeny indicated there are dominance effect on the tail-rattling and submission behavior, and also maternal effect on attack behavior. Preliminary analysis of congenic strains showed the possibility to identify the genetic loci associated with the aggressive behavior of B6-15MSM, and suggested there are multiple genetic loci related to the aggressive behavior on chromosome 15.

CHAPTER 1

Introduction

1.1 What is "emotionality"?

When you are faced with a danger (e.g. runaway truck rush toward you) or when you are in a situation that contains implicit danger (e.g. midnight test of courage), you will be stirred strong emotion, fear and anxiety. At the time strong emotion arises in you, your body responds in many aspects; increasing muscle tension, heightening adrenaline level, elevating heart rate, sweating, shaking, and/or freezing.

From the Latin *emovere* (*e* = out, *movere* = to move), the word "emotion" originated out of the idea of "moving out", in the sense of agitation or perturbation of the psychological state (Ramos and Mormede, 1998). Fear, angry, sadness, and happiness are generally recognized as emotions. However, the definition of emotion is rather vague and not standardized among researchers in different study fields (Plutchik, 1980). At least, most researchers agree that emotions have subjective, behavioral, and physiological aspects. Also, it should be noted that emotion has a role for adaptation or self preservation.

Since 1872, when Charles Darwin published his book *The Expression of the Emotions in Man and Animals*, emotion has been thought as a conserved trait in many animals, and it has evolved via natural selection. Emotional responses, in this case negative emotions, lead animals to prepare for and react to the opponent or situation that have potentially reduce their fitness, and thus they increase animal's survivability. Darwin described physical displays of emotion including body language of many animals and facial expressions in humans, and discussed the consistency in emotional expression between species. Based on broad recognition for the idea of Darwin, animal models have been widely used for understanding neural, hormonal,

physiological, and genetic basis of emotion. Because negative emotions (fear, anxiety, and anger) are stronger than positive emotions and easier to observe in animals, most of research on the emotions has been confined to fear/anxiety and anger in animals.

Besides conserved properties of emotion among species, there is a large individual difference in the levels of emotional responses toward the same emotional stimuli. This individual difference of emotional reactivity is regarded as a psychological trait, named "emotionality" (Hall, 1934a), and it is consistent across situations within the individual. Like many other quantitative traits, the distribution of the levels of emotionality in a population is expected to follow the normal distribution. Although emotional responses have advantages for the survivability of the individual, an extreme level of emotionality interferes with its normal life and will be diagnosed as psychological disorder (e.g. anxiety disorder). The word emotionality is mostly regarded as individual differences of timidity: fear and anxiety in the animal studies (Hall, 1934b). Behavioral geneticists have been interested in the genetic effect on the individual differences of behavior, and the object that one of the earliest behavioral genetics investigations focused on was emotionality (Hall, 1951).

1.2 Open-field study and emotionality

Open-field test is one of tests that use the simplest apparatus for the behavioral study. In this test, an animal is simply placed into a novel, brightly lit large arena from which escape is barred, which is expected to evoke negative emotion from the animals. Because of its simplicity, this apparatus has been widely used in psychology, pharmacology, and genetics: nowadays behavioral screening for genetically engineered mouse certainly includes the open-field test (Crawley, 1999). This test has been used for a wide variety of animals, include cockroaches, honeybees, lobsters, calves, pigs, lambs, chickens, pullets, gerbils, primates, and also human infants (Prut and Belzung, 2003; Walsh and Cummins, 1976).

Open-field test was invented in 1934 by Calvin Hall as the first test to measure individual differences of emotionality in the rat. He first measured the days spent to start eating food put in the middle of arena of the strange open-field situation in food-deprived rats, and found that there were individual differences. It was hypothesized that the drive for food was inhibited by emotional responses, thus the measurement was thought to be affected by emotionality (Hall, 1934a). Then, he proposed defecation as a valid index of emotionality for the following reasons: a) it occurs in a situation recognized to be emotionally arousing in character, b) these responses are linked with other reactions set off by impulses traveling over the autonomic nervous system, c) it tends to be eliminated with repeated experience in an originally strange situation, and d) as the number of defecating animals decreases, the number of animals eating in the originally strange situation increases (Hall, 1934b). Hall also mentioned the negative correlation between ambulatory activity in the open-field and emotionality (Hall, 1936). Rats with high emotionality tended to show low activity and to avoid central enclosure of the field.

Since his seminal work, defecation and ambulation have been used as major indices of open-field test. At the same time, many other indices have also shown up for measuring the emotionality. However, in contrast to its popularity among many researchers, the open-field test has a history of controversy that has not yet been settled (Archer, 1973; Walsh and Cummins, 1976). The issue is very critical: What is the open-field and what, in general terms, does it really measure? (Walsh and Cummins, 1976; Denenberg, 1969)

Readers may feel odd with this issue because I have previously mentioned that open-field was invented for measuring the emotionality. This controversy came along with the basic question about the validity of defecation and ambulation as consistent indices of emotionality for several species. Hall and other researchers demonstrated the validity of those measurements by showing the changes over trials or the effect of different stress level (e.g. illumination) on the defecation and ambulation in the rat (Hall, 1934b, 1936; Broadhurst, 1957; 1960). However, the

interpretation of open-field indices is not fully validated in species other than rat, such as chicken (Gallup et al., 1976), guinea pig (Suarez and Gallup, 1982), and mouse (Blizard, 1971; Bruell, 1969; Collins, 1966). For example, it has been suggested that the defecation response, which has been shown to decrease over trials in the rat (Hall, 1934b), changes in the opposite direction in specific mouse strains (Collins, 1966). Another controversy occurred when emotionality was considered as single construct. Several researchers considered the emotionality as general construct and that affects various kinds of emotional behaviors stably (Broadhurst, 1957, 1975; Denenberg, 1964, Savage and Eysenck, 1964). If behaviors in the open-field are related to unitary hypothetical construct "emotionality", it is expected that all measurements change consistently within an individual. However in mice, and even in rats, the relationships among open-field indices vary according to strains or testing conditions (Archer, 1973; Blizard et al, 2006; Ivinskis, 1970; McReynolds et al., 1967; Thompson, 1953). Studies of heritability estimates and genetic correlations of ambulation and defecation showed that degree of genetic association between those two measurements varies (Broadhurst, 1969; Hegmann and DeFries, 1968; Henderson, 1967).

Ambulation in a novel environment has been used not only as an inverse measure of emotionality but also to denote exploration (Archer, 1973). Actually, the open-field has been used for different aims depending on researchers; some used this apparatus to measure "exploratory drive" (Thompson, 1953; McClearn, 1959, Pare, 1964), and others attempted simply to measure spontaneous "activity" (DeFries and Hegmann, 1970; McClean, 1960; Makino, 1983). These cases suggested that there may be multiple factors determining the qualitative and quantitative nature of behaviors evinced by a subject in the open-field rather than the existence of emotionality as a unitary construct (Archer, 1973; Russell, 1973; Walsh and Cummins, 1976).

Principal component analysis has been applied to reveal those factors, and showed multiple factors related to the open-field behavior. Joseph R. Royce (1977) reviewed many principal

component analysis studies, and showed it is more reasonable to consider there are multiple, mainly three factors (autonomic balance, territoriality, and motor discharge), embedded in the open-field measures. He mentioned that because these factor analyses are performed just for five kinds of measurements, additional or more precious factors may be extracted by analyzing more detailed indices (Royce, 1977). After studies by C. S. Hall, many other measurements of open-field behavior have appeared (Walsh and Cummins, 1976). Over 30 measurements have been reported so far: including major body movement (type of movement, locations of field, part body movement), autonomic nervous system, adrenal activity, and electrophysiology.

In conclusion, it is better to understand emotionality as a "complex of factors" (Archer, 1964). Actually, the inventor of open-field first mentioned that emotionality "is merely a convenient concept for describing a complex of factors" (Hall, 1934b). It is required to confirm the structures of those factors by using multiple behavioral measurements for extracting precious factors, and to elucidate biological or genetic pathways related to each factor for examining construct validity of those factors of emotionality. This may give us the answer for the questions: What is the open-field and what, in general terms, does it really measure?

1.3 Behavioral genetics of emotionality

Behavioral genetics have aimed to reveal the following four issues: 1) to discover whether a given behavior pattern is transmitted from generation to generation, 2) to determine the number and nature of the genetic factors involved in the trait, 3) to locate the gene or genes on the chromosomes, and 4) to determine the manner in which the genes act to produce the trait (Hall, 1951). Here I will overview some classical and recent methods of behavioral genetics studies of emotionality.

Classical studies of behavioral geneticists have employed the method of selective breeding and comparison of different strains, breeds, or species.

Strain comparison was the most elementary analysis. Because laboratory animals rear in virtually regulated environment, we can directly observe behaviors that reflect genetic differences between strains. Inbred strains have been established by brother-sister mating over 20 generations, and then animals within the strain can be regarded for most purposes as genetically identical (Rules for Nomenclature of Mouse and Rat Strains, MGI, http://www.informatics.jax.org/). A number of inbred strains of mouse/rat have been established, and over 100 inbred strains of mice are available (Plomin et al., 2001). Analysis of intercross and backcross between two inbred strains has been used to estimate the heritability of open-field behaviors (Henderson, 1967; Crusio et al., 1989). Inbred strains have been used not only for showing the genetic influences on the behaviors (Archer, 1973; Thompson, 1953), but also for testing the effects of gene-environment interactions on the behaviors related to emotionality (Izidio et al., 2005; Joffe, 1969; Poley and Royce, 1970; Priebe et al., 2005).

Selective breeding is performed by selectively crossing animals that have high or low levels of the trait over many generations. If a desired trait is successfully selected by selective breeding, it means that heredity plays a part in the determination of the trait. Hall (1951), inventor of the open-field, selectively bred rats for the number of defecation in the open-field. This was one of the first selection studies for a complex psychological trait. Since then, many selection studies for a variety of behavioral measurements of open-field behavior have been performed in rats and mice: defecation (Broadhurst, 1975), activity (DeFries and Hegman, 1970; Makino, 1983), activity in the central part of open-field (Ramos et al., 2003), thigmotaxis (Leppanen et al., 2005), and rearing behavior (van Abeelen, 1970). Also, there have been selection studies for other aspects of emotionality: ultrasonic vocalizations as isolation stress in infants (Brunelli, 2005), elevated-plus maze test (Liebsch et al., 1998), runway test (Fujita, 1984), avoidance learning (Bignami et al., 1965; Brush et al., 1979; Ryzova et al., 1983), anxiolytic diazepam-sensitivity

(Gallaher et al., 1991), and 5-HT_{1A} receptor sensitivity (Knapp et al., 2000). This method takes on significance when it examines the relationships between the selected trait and other behavioral, physiological, and neurological traits (Blizard and Adams, 2002). That is, those strains can be used for understanding the construct validity of a trait, emotionality.

By recent advances in genetics, it has become able to identify the effects of a single gene on complex behavior by reverse genetics approach using genetically altered animals, such as transgenic or knock out/in mouse (Belzung and Griebel, 2001, Crawley, 1999). More than 50 genes are reported as genes for "anxiety" trait, judging from the database (NCBI Entrez Gene, http://www.ncbi.nlm.nih.gov/). However, the overall contribution of large effect Mendelian mutations to behavioral variation is minute. Most normal behavioral variation is quantitative in nature (Plomin et al., 2001). Individual differences in almost all behaviors can be attributable in part to quantitative genetic variation, and genetic loci that contain the responsible allelic variations are termed quantitative trait loci, or QTL (Flint, 2003).

QTL analysis has been developed to reveal the chromosomal positions of genetic loci for a certain behavior. In most cases, QTL analyses are performed for F2 intercross or N2 backcross populations by crossing two different laboratory strains of mouse/rat. It is advisable to include strains that have large genetic and behavioral variations between them for identifying complex genetic mechanisms of behavior. Some advantageous crosses have been established for rising the precision or ability of replication of the QTL analysis: recombinant inbred strain, heterogeneous stock, and consomic mouse strains (Churchill et al., 2004; Mott et al., 2000; Nadeau et al., 2000). The advantages of consomic strains will be described in detail in a later chapter (Chapter 3). To date, a vast number of QTLs related to emotionality have been reported in the rat and mouse (Fernandez-Teruel et al., 2002; Flint et al., 1995; Gershenfeld et al., 1997; Gershenfeld & Paul, 1997; Talbot et al., 1999; Turri et al., 2001a, 2001b; Ramos et al., 1999). Jonathan Flint (2002) described in his review that sixteen of the mouse's chromosomes are

implicated in influencing behavior in at least one test and some chromosomal regions appear to influence almost a dozen different, but correlated, measures of anxiety. This method is useful not only for showing genetic loci related to a phenotype on the chromosome, but also for examining whether some behavioral traits have the same genetic regulations or not. For example, QTLs for open-field activity and fear conditioning, both emotionality related traits, were mapped on discrete position of chromosome 1 (Talbot, 2003). Henderson (2004) performed multivariate analysis on several behavioral tests for emotionality to extract common factors for all the tests, and tried to map QTLs for those factors. In this way, QTL analysis has advantages to understand the architecture of emotionality.

What is the role of behavior genetics? In a very seminal paper, Vale (1973) discussed behavior genetics could help to make advances in genetics or in psychology. He considered the former possibility is unlikely, and explained "although genes are vital for behavior, behaviors are not well suited to the study of genetics". Today, the technology has developed, and we have successfully found many genes related to behavior by reverse genetics approach, and also a vast number of QTLs by forward genetics approach. However, still it may be true that "behavior genetics will be of most value if its emphasis shifts to the analysis of the behavior rather than the analysis of the genetics" (Hay, 1978).

1.4 Purpose of this study

In this study, I try to elucidate the genetic basis of emotionality. As discussed so far, emotionality is not a simple trait, but rather consists of multiple factors. In conducting the genetic analysis of emotionality, it is essential for researchers to consider those multiple factors. However, not many genetic studies focused on this multiplicity of emotionality, though many genes or genetic loci related to "anxiety " have been reported in the studies of gene-altered

mouse or QTL studies so far. Therefore, this study aims to capture the structure of the multiple factors of emotionality and to explore the genetic mechanisms underlying those factors. I decided to give my focus in this study on the historical object, the open-field test, because this test is still commonly used today.

In chapter 2, I examine the genetic effect of open-field behavior by using conventional measurements, ambulation and defecation, and some ethological measurements in a huge variety of wild-derived mouse strains. By using multiple measurements and a number of mouse strains, it is expected that the refined structure of factors underlying open-field behavior can be extracted. This study was actually performed for my master thesis, but it has become an important start point for my PhD study. Therefore, I present those data with some recalculation. Then, chromosomal mapping in those open-field behaviors are conducted by using consomic mouse strains established from C57BL/6J and MSM (Chapter 3). In this chapter, two kinds of other emotionality-related tests, elevated plus-maze and social interaction test, were also performed. One strain, B6-17MSM that has the substituted chromosome 17 from MSM, exhibited interesting phenotypes for this study: reduced novelty-induced activity but normal home-cage activity, increased risk assessment behavior, and extended social interaction. Thus, further behavioral and brain morphological examinations were performed in B6-17MSM (Chapter 4), and congenic strains that have a narrowed-down MSM region on chromosome 17 are established and analyzed to elucidate genetic loci related to “emotionality” (Chapter 5). Through all these chapters, I am trying to confirm the structure of multiple factors for emotionality from the genetic basis, and to identify the genetic loci associated with those factors.

Chapter 6 is a kind of independent chapter from the entire story of this study. I found increased aggressive behavior in one comsomic mouse strain, B6-15MSM. Genetic analysis was performed by using F1, and congenic mouse strains were established. This chapter indicated the possibility of forward genetics approaches to aggressive behavior by using consomic strains, which has been thought as difficult in the previous study. Because aggression is also an important aspect of

the emotion, I include this chapter in this thesis.

CHAPTER 2

Ethological and Multivariate Analysis of Open-field Behavior in Wild-derived Mouse Strains

2.1 Introduction

Inbred mouse strain is the most representative tool in animal behavioral genetics. Behavioral genetics studies have been conducted in those inbred strains by comparing their different behavioral characters and genetic polymorphisms. However, because most standard inbred strains were derived from the so-called fancy mouse, they have undergone extensive artificial selection and domestication before inbreeding. Several behavioral responses have been changed or sometimes attenuated in these standard strains (Blanchard et al.,1998; Fernandes et al., 2004; Holms et al., 2000, Koide et al., 2000). Furthermore, most standard inbred strains have limited genetic diversity since they all derived from the same small original population belonging largely to *Mus musculus domesticus* (Bonhomme and Guenet, 1996; Ferris et al., 1982; Wade et al., 2002; Yonekawa et al., 1982).

To deal with the problem of commonly used inbred strains, we and other groups have been continuing work on establishing and examining wild-derived inbred strains of the mouse (Bonhome and Guenet, 1996; Fernandes et al., 2004; Furuse et al., 2002a; Gregorová and Forejt, 2000; Koide et al., 2000). These mice were established as inbred strains derived from wild mice around the world. These inbred strains are known to have a wide genetic diversity by examining the variation of microsatellite markers (Koide et al., 1998, 2000) or triplet repeats (Ogasawara et al., 2005), since they were captured from many countries and belong to several different subspecies (Bonhomme and Guenet, 1996; Moriwaki, 1994). Wild-derived strains have been shown to exhibit enormous behavioral differences from standard laboratory inbred strains and

also within wild-derived strains (Fernandes et al., 2004; Furuse et al., 2002a, 2002b, 2003; Holmes et al., 2000; Koide et al., 2000), and are expected to be a major resource for the genetic study of behavior.

When the animals are placed into the open-field, they show a series of behaviors such as sniffing, rearing, running and grooming. An animal's behavior is complex, consisting of many particular patterns of bodily movement, and these patterns of behavior change with time. It is necessary to describe those behavioral patterns to understand animal's behavior precisely. However, these behaviors are ignored in many tests. Some researchers have stressed the importance of describing the animals' behavior in detail (Bindra, 1961; Gray, 1965; Streng, 1971; van Abeelen, 1963). They demonstrated that the frequency and pattern of the temporal changes of each behavior (sniffing, rearing, grooming, freezing, etc.,) were strain-dependent (Crusio et al., 1989; Makino et al., 1991; van Oortmerssen, 1971; Streng, 1971; Vadasz et al., 1992), and differed by sex (Gray, 1965) or context (Bindra and Spinner, 1958, van Oortmerssen, 1971) in both the rat and the mouse. To deal with this complexity, an ethological approach has been adopted and applied to elucidate drug effects (Antoniou et al., 1998; Blanchard et al., 1993; Choleris et al., 2001). This is thought to be a more sensitive way of detecting differences in pharmacological effects, since drugs that have a similar effect on overall levels of ambulation showed different effects on discrete behaviors (Antoniou and Kafetzopoulos, 1991). Thus, it is expected that those ethological measurements could be useful to understand behavioral characters in detail and more correctly.

In this chapter, I aimed 1) to profile behavioral characteristics of wild-derived mouse strains by both conventional indices, such as the ambulation and defecation, and 12 ethograms supported by detailed temporal observation of open-field behavior, and 2) to find fundamental structure that underlies the open-field behavior by principal component analysis.

2.2 Methods

Animals

Twelve inbred strains were used in this experiment (Table 2.1). Ten inbred mouse strains, PGN2, BFM/2, HMI, NJL, BLG2, CHD, SWN, KJR, MSM and JF1, were established as inbred strains after 20 generations of brother-sister mating at the National Institute of Genetics (NIG, Mishima, Japan). The place of collection and establishment of these strains has been reported previously (Furuse, 2002a; Koide et al., 1998, 2000). Because the coat color allele "s", which JF1 possesses, is known to relate to auditory disability, I used a spontaneous revertant at this allele with a black coat color, JF1-s+, in this experiment. C57BL/6J and CAST/Ei were purchased from The Jackson Laboratory (Bar Harbor, Me., USA). All the strains were maintained at NIG under a 12-h light/dark cycle (light from 8:00 to 20:00) in a temperature-controlled room (23 ± 2 ˚C). The mice were housed in same sex groups (2-5 per cages) in standard sized plastic cages on wood shavings, and one week before the test, they were housed individually. Food and water were available ad libitum. Ten males and ten females from each strain were used in this test at the age of 10 weeks.

Apparatus

The open-field consisted of a square arena (60 x 60 cm) made of white polyvinylchloride plastic board with walls 40 cm high. The arena was lit by incandescent lighting placed 90 cm above the arena. The light level was 365 lux at the center of the arena. The open-field was surrounded by a black curtain except for one side from where the experimenter directly observed the animal's behavior. For analyzing ambulation, the arena was continuously recorded by a video camera placed over its center and relayed to a video tracking system (Image OF, O'hara & Co. Ltd., Tokyo, Japan).

Procedure

At least 1 hour before the beginning of the test, animals were brought into the test room to minimize the effect of transfer. Each mouse was gently picked up by its tail with tweezers and placed in the same corner of the open-field. During the ten-minute trial, their behavior was observed directly and recorded using the multi-event time sampling method (Makino et al., 1991). The behaviors collected included the following 12 behavioral items.

Sniffing: sniffing the arena and air, identified by characteristic movements of the nose and whiskers.

Locomotion: walking and running around the arena.

Stretched attend posture (Stretching): stretching its whole body forward while keeping the hindlimbs in place.

Leaning-against-wall (Leaning): standing on the hindlimbs with the forelimbs against the wall.

Rearing: standing on the hindlimbs without touching the wall.

Grooming: licking and/or scratching its fur, licking its genitalia and tail.

Face-washing: scrubbing its face with the forelimbs, not followed by grooming.

Digging: trying to dig a hole in the arena or the corner of the wall.

Gnawing: gnawing mainly on the corner of the wall.

Jumping: jumping vertically.

Pausing: a brief moment of inactivity.

Freezing: stationary state lasting more than 3 seconds regardless of posture.

The presence or absence of each behavior was recorded as 1/0 in each 5-sec period.

At the end of the sessions, the number of defecations and presence of urination were recorded, after which the arena was cleaned with a damp cloth. All the tests were carried out during the light period (13:00-17:00).

Statistical analysis

The image of the arena was divided into 16 squares (4 × 4), and the number of squares transited was counted as ambulation by the computer software (Image OF). Ambulation in the central area has also been used, since rodents naturally avoid staying in open spaces. This is known as thigmotaxis (Treit and Fundytus, 1989). I also calculated the percentage of central ambulation (the number of transitions in the central 4 blocks / total 16 blocks × 100). All measures—ambulation, central ambulation, percentage of central ambulation, the number of fecal pellets (defecation), and the frequencies of behavioral items were subjected to one-way ANOVA. Most variables did not show any sex differences, and thus, I decided not to deal with the effect of sex in this analysis, and combined data across males and females. Because individual differences with respect to urination were very large, statistical analysis was not performed on this index. Post hoc comparisons were carried out using the HSD test ($p<.05$). In some behavioral items, locomotion, stretching, leaning, and rearing, a minute-by-minute frequency was calculated to analyze the temporal factor, and 1-way ANOVA for repeated measures of continuous variable (time) was also performed on their frequency.

A principal component analysis was performed using SPSS version 14.0J software packages. Means were calculated for each sex of each strain in each variable, and those mean scores were used to calculate the genetic correlations. Then I performed principal component analysis with Varimax rotation for the genetic correlation. I adopted genetic correlation for the principal component analysis rather phenotypic correlation, calculated by individual values, throughout this study. It has reported that phenotypic correlation was influenced by test session and environmental history (assumption errors), and using genetic correlation is expected to avoid those entangled effects (Crusio, 2001, Henderson et al., 2004). Although, using mean values involved the problem of reduction of the number of dependent variables analyzed, and principal component analysis on genetic correlation has traditionally been viewed as caution. It is said that it is desirable to analyze three times more animals (dependent variables) than the number

of behavioral items (independent variables) for the principal component analysis. However, recent work showed that the ratio of variables to observations is not a critical element in the stability of factor analytic solutions (Preacher and MacCallum, 2002). Thus, genetic correlation is expected to extract better factor structure without the effect of errors.

2.3 Result

2.3.1 Conventional variables

Data in three conventional variables (open-field ambulation, percentage of central ambulation, and the number of defecations over 10 min) are summarized in Table 2.2. ANOVA revealed significant main effects of strain on all three measures [all $F(11,228) \geq 12.982$, $p<.0001$]. In the ambulation, two wild strains, KJR and SWN, and a laboratory strain C57BL/6J were characterized as high activity strains, whereas the wild strain MSM and the fancy strain JF1 were low activity strains. The central ambulation was higher for the wild strains BLG2 and BFM/2, and the lowest in JF1. The wild KJR also showed relatively low central ambulation. The wild KJR and the fancy JF1 showed a high frequency of defecation, while it was low in the wild BFM/2 and the laboratory C57BL/6J. The correlations between each conventional variable were calculated, and they were just modestly correlated. (Concerning phenotypic correlation: $r = -0.22$ for the ambulation and defecation, $r = 0.43$ for the ambulation and % central ambulation, and $r = -0.27$ for the % central ambulation and defecation, $p<.001$ for all correlations). MSM, BFM/2 and BLG2 showed the urination in many animals, while C57BL/6J and PGN2 rarely urinated.

2.3.2 Ethological behavior measures

ANOVA indicated a significant effect of strain on all the twelve behavioral items [$F(11,228) \geq 6.766$, $p<.0001$]. Those results were summarized in Table 2.2. Sniffing was the most frequently observed behavior. Especially C57BL/6J showed this behavior very often, while CHD exhibited it less. C57BL/6J showed higher locomotion, while JF1 and MSM were lower. This was almost parallel to the results of total ambulation. CAST/Ei, NJL and JF1 showed higher stretching, while PGN2, BLG2, SWN, and KJR seldom showed this behavior. KJR exhibited the highest

frequency of leaning. On the other hand, CHD and JF1 showed significantly lower leaning than other strains. Rearing was higher for PGN2, while JF1 seldom showed rearing. MSM showed the highest frequency of grooming, while six strains—C57BL/6J, PGN2, BFM/2, HMI, CAST/Ei, and BLG2—showed this behavior infrequently. Face-washing was higher for BLG2, while it was low in the NJL, KJR, MSM, and JF1. Digging and gnawing appeared less frequently than other behavioral items. BFM/2 and BLG2 showed the highest frequency of digging and gnawing, respectively. On the other hand, HMI, CHD, and C57BL/6J seldom showed digging, and JF1 rarely exhibited either digging or gnawing. PGN2 showed the highest frequency of jumping, while C57BL/6J, MSM, and JF1 seldom showed this behavior. Pausing was higher for MSM, JF1, and CHD, while it was low in the C57BL/6J. JF1 showed the highest frequency of freezing, while five strains— CAST/Ei, NJL, BLG2, KJR, and C57BL/6J—seldom or never showed freezing.

2.3.3 Temporal changes of the behavioral measurements

The temporal changes in some behavioral items, locomotion, stretching, leaning, and rearing, are summarized in Figure 2.1. Repeated-measure ANOVA indicated significant strain×time interactions on those four measurements [$F(99,2052) \geq 2.126$, $p < .0001$].

Locomotion: Ten strains, except for the C57BL/6J and CAST/Ei, showed significant changes across time ($p<.05$). Most exhibited an initial increase in frequency, which then stabilized or in some showed a gradual decrease.

Stretching: Ten strains, except for KJR and PGN2, exhibited a significant effect of time ($p<.05$). Most showed the highest frequency during the first minute, which did not recur, but some strains, such as CAST/Ei, CHD and JF1, exhibited stretching later in the session.

Leaning: Eleven strains, except for CAST/Ei, showed significant changes across time ($p<.01$). The temporal changes were similar to those for locomotion, with most strains showing an initial increase, which then stabilized or gradually decreased.

Rearing: Ten strains, with the exception of CHD and JF1, showed a gradually increasing frequency ($p<.05$).

2.3.4 Principal component analysis

To identify the relationship between these behavior items and conventional variables, and to extract some common components underlying the open-field behavior of all strains, I performed principal component analysis. Some variables that showed a low frequency (digging, gnawing) or consistently high frequency (sniffing) were excluded from the analysis. Although some variables, jumping and freezing, showed skewness from normal distribution, the factors extracted from raw mean data and transformed data (1/0) had almost the same factor structures. Thus, I adopted the factors extracted from the raw data.

The factor loadings obtained from principal component analysis with Valimax rotation based on the mean values from the wild-derived mouse strains are shown in Table 2.3. Three factors with eigenvalues higher than one were extracted and that accounted for 77.3 % of total variance. As the different factors were orthogonal to each other, it was generally assumed they reflect distinct biological traits. Variables that loaded highly on Factor 1 were ambulation, locomotion and leaning for positively, and pausing and freezing in the reverse direction. This first factor represented 34.2 % of total variance. Factor 2 represented 21.7 % of total variance, and it had positive loadings from rearing and jumping, whereas negative loadings from stretching and grooming. Factor 3 represented 21.3 % of total variance, and it included central ambulation, face-washing and defecation.

2.4 Discussion

2.4.1 Conventional variables

In open-field tests, it has been expected that animals that show relatively low total ambulation have low central ambulation and high defecation based on experiments with the rat (Hall, 1934b, 1936). Although this test has not been thoroughly validated in the mouse (Blizard, 1971; Collins, 1966), these indices are widely used in mouse studies as well. I first investigated the relationship of these traditional variables in the present battery of strains. The ambulation data indicated that KJR, SWN, and C57BL/6J were characterized as high activity strains, while MSM and JF1 were low activity strains. A number of studies have reported that C57BL/6J shows higher activity in the open-field when compared with other laboratory strains (Crawley et al., 1997; Logue et al., 1997; Marks et al., 1986; Streng, 1971; Thompson, 1953). The present data suggest that C57BL/6J showed high ambulation even compared to wild-derived mouse strains. This strain also showed higher frequency of central ambulation and lower defecation in the field. Meanwhile, JF1 showed the opposite pattern: low activity, low central ambulation, and high defecation. These results are consistent with the traditionally expected relationships between the open-field indices. However, there were many exceptions. For instance, wild-derived KJR mice, which were also high activity strain in the open-field, exhibited a lower level of central ambulation and the highest defecation of all strains. Some other strains also exhibited inter-relationships between those measurements that are inconsistent with Hall's original proposal (Table 2.2). Because some strains showed the expected relationship between those measurements but some strains did not, there were only modest correlations between them. While the traditional association between key indices of open-field behavior has already been questioned (Archer, 1973), this result provides ample evidence of a variable relationship between these measures within strains in the Mishima battery.

2.4.2 Temporal changes of the ethological measurements

Detailed observations of open-field behavior revealed the temporal character of each behavioral component. In most strains, stretching was exhibited during the first few minutes but was seldom evident later in the session. This behavior was considered to be related to risk assessment and approach-avoid conflict (Blanchard et al., 1991a; Carola et al., 2002; Rodgers and Johnson, 1995); therefore C57BL/6J and NJL, which were characterized by a high level of stretching early in the session, were thought to have a high risk-assessment tendency at the beginning of the session. By comparison, CAST/Ei and JF1 kept this behavior throughout the session, so these strains might need a longer time to reduce the strong tension that induced their risk assessment behavior. In other words, it took longer for them to get used to the situation. Leaning and rearing, both standing postures, had different temporal changes. The temporal pattern of leaning was fairly similar to the behavioral component of 'locomotion'. These two behaviors, leaning and locomotion, were frequently observed concurrently in a short segment of time (5 seconds) in the open-field (Makino et al., 1991). Thus, leaning is thought to have a close relationship to locomotor activity. In contrast, rearing showed a gradual increase throughout the session. A similar temporal pattern has been reported in previous studies, and rearing has been viewed as a behavior expressed when animals habituate to the environment (Vadasz et al., 1992). Most strains demonstrated an increased pattern of rearing over time, whereas JF1 never showed this behavior. This again suggests the difficulty of characterizing habituation in JF1. However, I need longer periods of observation before concluding this, as there is a possibility that the JF1 strain exhibits different behavioral patterns in the habituated situation.

Some behavioral components were characteristic of or specific to several strains. Grooming was characteristic in MSM, and they exhibited long bouts of grooming late in the session (data not shown). Meanwhile, jumping was especially pronounced in PGN2. This strain also exhibited a high frequency of rearing but not locomotion; therefore, PGN2 mice were very active only on the

vertical axis. It was previously reported that some behaviors, freezing and jumping, were observed in many wild-derived strains but not in laboratory strains (Fernandes et al., 2004; Holmes et al., 2000). In the present findings, again, the laboratory strain C57BL/6J seldom exhibited these behaviors. There may be some differences in the behavioral patterns exhibited in a novel situation between laboratory and wild-derived strains. Thus, it seems that the effect of domestication is associated with a change in their behavior, however, there was also a large variety within the wild strains. Some sophisticated studies proposed that the behavioral differences between strains reflect the ecological context in which the strains evolved (Dudek et al., 1971; Oortmerssen, 1971). I may conclude therefore that the strain differences of the open-field behavior reflect both the process of domestication as well as their natural ecological context. Taking these data together, it is assumed that each behavioral component reflects a particular psychological state (especially when considered within a temporal context) but the expression of particular behaviors must also be considered within their strain-specific context.

2.4.3 Principal component analysis

To find the fundamental structure that underlies the open-field behavior of various mouse strains, I conducted a principal component analysis of ethological measurements and confirmed that there are multiple, at least three, factors related to the open-field test. The first factor was described as 'locomotor activity' because it correlated positively with ambulation and leaning and negatively with the freezing and pausing. Some factor analyses of open-field behavior interpreted the factor describing these behaviors in terms of general motor activity (Carola et al., 2002; Pardon et al., 2000; Royce, 1977; Trullas and Skolnick, 1993). A similar factorial structure was reported by using many laboratory inbred mouse strains that included the information of temporal changes of behavior (Makino et al., 1991, Takahashi et al., 2004). Many factor studies of open-field behavior extracted similar "locomotor activity" factor as the first factor that explains the greatest variance, and thus this factor is thought to be the main component of the

open-field behavior. Factor 2 had negative loadings from stretching and grooming, while positive loadings from rearing and jumping. In the interpretation of this factor, variables that have negative loading may provide a hint. As mentioned before, stretching was considered to be related to risk assessment and approach-avoid conflict, and this behavior was reduced by anxiolytic drugs in the mouse (Blanchard et al., 2003; Choleris et al., 2001). On the other hand, grooming was considered to be related to two opposite psychological states: situations in high and low stress (File et al., 1988; Lawler and Cohen, 1988; Moody et al., 1988; Kalueff and Tuohimaa, 2004). Low-stress comfort grooming is a spontaneous body care which occurs as a transition from rest to activity (Fentress, 1977), whereas high-stress grooming is a behavioral response to stressors (van Erp et al., 1994, Moyaho and Valencia, 2002). Grooming behavior in open-field is viewed as high-stress grooming, and this behavior is also reduced by anxiolytic drugs in the mouse and rats (Barros et al., 1994; Choleris et al., 2001; De souza Spinosa et al., 2000; Kalueff and Tuohimaa, 2005). In contrast, positively loaded rearing and jumping tended to occur late the session, and rearing has often been considered as exploratory behavior that occurs when reducing the strong emotional response (Vadasz et al., 1992; Crusio, 2001). Thus, variables that negatively loaded on Factor 2 may reflect "anxious tension state" of the animals. Factor 3 had contribution from % of centre ambulation and defecation in the opposite direction. Thus, this factor may reflect "thigmotaxis and autonomic emotional response".

2.4.4 Summary

By describing open-field behavior in detail and examining its temporal changes, I was able to identify the prominent behavioral features of each strain of mice. The results of this experiment make it possible to assert that conventional simple measurements lose substantial information, such as temporal changes and the variety of behaviors that can be displayed, and that the use of too few indices might easily lead to confusion in interpreting the genetic mechanisms underlying open-field behavior or "emotionality". In this chapter, I showed that the open-field behavior

consisted of three dimensions of psychological trait among the wild-derived mouse strains. Behaviors that loaded on each factor are commonly considered as measures of emotionality or anxiety. However, as many principal component analysis studies have argued (Archer, 1973; Walsh and Cummins, 1976; Ramos and Mormede, 1998), I here suggested that those measurements may reflect a different aspect of emotionality, and in turn, related to a different "complex of factor" of emotionality.

From these data, I hypothesize that there are three independent biological pathways involved in the open-field behavior. However, because factors extracted in this experiment were obtained from behavioral correlations in inbred strains, more consideration is required before conclude it. Note that correlation could be affected by one extreme outlier. When a certain strain showed strong phenotypes on two behaviors that are actually affected by independent biological pathway or genetic loci, we may find correlations between those behaviors. One of my major goals of this study is to examine this multiple dimension of emotionality at biological levels. In the next and later chapters, I am going to discuss about the assurance of these behavioral correlations from the genetic level, and examine whether these dimensions are true or pseudo.

CHAPTER 3

Chromosomal mapping of emotionality-related behavioral traits in Consomic Mouse Strains established by C57BL/6J and MSM/Ms

3.1 Introduction

In chapter 2, I showed genetic effects on the conventional and the ethological measurements of open-field behavior in a variety of wild-derived mouse strains. As described before, most behavioral variation is quantitative in nature rather than arise from few large effects of Mendelian mutations. Several studies have been adopted to map the chromosomal location of those quantitative trait loci (QTL) for behavior of rodents after the first QTL study in 1990s (Plomin et al., 1991). To date, a vast number of QTLs related to anxiety-like behaviors has been reported in mice and rats by using F2 intercross, N2 backcross, recombinant inbred strains, and heterozygote stock (Flint, 2002, 2003; Valdar et al., 2006). It was expected that genes in the QTL would be identified soon, however, researchers have gradually noticed that this was rough and thorny path. Meta analysis revealed that most QTLs have just a small effect contributing, on average, 5.8% and 5.3 % of the total variance for behavioral and physiological phenotypes, respectively (Flint et al., 2005). Also, extensive genome-wide high-resolution mapping using heterogeneous stock mice revealed 843 QTLs for a variety of phenotypes including behavior, and only 10 QTLs have effect of greater than 5% and 109 QTLs effect less than 2 % (Valder et al., 2006). Because of this small effect, it is difficult to identify the gene from the information of a genetic locus.

Consomic strains (CSSs), also known as chromosome substituted strains, are one of the favorable resources to overcome this small effect issue. A series of strains are established by replacing one chromosome of host strain with a corresponding chromosome of donor strain. For

the constructon, F1 mice made by cross of two parental strains are backcrossed into a host strain and followed by one-directional backcross for over 10 generations. During the backcross, intended chromosome is screened to inherit as an intact chromosome from the donor strain. Finally, heterosomic mice are intercrossed to make homosomic for the substituted chromosome. Thus they have the same genetic background as the host strain except one chromosome from the donor strain (Nadeu et al., 2000). Despite a large amount of effort and a long time requirement for establishing consomic strains, analysis with CSSs has many advantages over other QTL analysis: no requirement of genotyping for QTL mapping, reproducibility of the result, statistical significance of QTL detection, and rapid progress for making congenic strains (see more detail, Nadeu et al., 2000; Belknap, 2003). Genetic studies with CSSs made by crossing C57BL/6J and A/J (named as B6-Chr$^{A/J}$ CSSs) have successfully shown that many chromosomes affecting serum levels of sterols and amino acids, diet-induced obesity, pubertal timing, and behavioral traits (Singer et al., 2004, 2005; Petryshen et al., 2005; Krewson et al., 2004). They have indicated that CSSs have prominently large phenotypic differences from their parental strain, that means the substituted chromosome has large effects on those phenotypes. Shao and colleagues (submitted) analyzed the effect of QTLs in B6-Chr$^{A/J}$ CSSs for several physiological phenotypes, and showed that those QTLs have considerably large effect, an average of 50.0%. These CSSs thus possess advantages for the further analysis to identify the gene. Transcriptional variation in liver was also analyzed in those CSSs, and there were a large number of genes (around 4200 genes) that are differentially expressed as compared to parental C57BL/6J.

Recently, a new set of CSSs were established from C57BL/6J and MSM at mammalian genetics laboratory in NIG, Mishima, Japan. These CSSs, named as B6-Chr^{MSM}CSS, are established by replacing one chromosome of C57BL/6J strain with the corresponding chromosome of MSM strain. As mentioned in Chapter 2, MSM was derived from a Japanese wild mouse and they considerably differ genetically and behaviorally from C57BL/6J. Thus, these CSSs are expected

to be valuable resource for genetics and behavioral studies. It has been reported that males of B6-XMSM with substituted X chromosome are sterile, and QTLs associated with the hybrid sterility were mapped (Oka et al., 2004, 2006). Furthermore, the resistant allele for age-related hearing loss in C57BL/6J was mapped on the substituted chromosome 17 by using the B6-Chr^{MSM}CSS (Nemoto et al., 2004). For the behavioral phenotype, B6-6CMSM showed reduced spontaneous activity and different emotionality, and identified several QTLs related to those phenotypes on chromosome 6 (Nishi, doctoral thesis).

In this chapter, I examine the open-field behavior in a series of B6-Chr^{MSM}CSSs. The open-field is one of the initial behavioral tests used for QTL analyses (Plomin et al., 1991; Flint et al., 1995). Most of those QTL studies have measured conventional indices such as ambulation and defecation, but detailed ethological behaviors have been rarely analyzed. As discussed in Chapter 2, conventional simple measurements lack substantial information, and we should not ignore the importance of the ethological measurements. Therefore, I conducted detailed observation for the characterization of B6-Chr^{MSM}CSSs and performed chromosome mapping for those ethological measurements. In addition to examining detailed open-field behavior, I conducted other tests that are considered to be related to the emotionality: elevated plus-maze and social interaction test. In this study, I analyzed both males and females to examine sex difference. As males and females have different sex chromosomes, X and Y, the data of males and females were analyzed independently in each consomic strain to avoid influence from sex chromosomes. By analyzing both sexes of animals, I expected to find sex-dependent QTLs which have interaction with those sex differences.

In this chapter, I aimed to map the chromosomes associated with emotionality-related behavioral traits, and to find out sex-dependent effect in CSSs. In addition, as large-effect QTLs were reported for blood, bone and metabolic traits in B6-Chr$^{A/J}$CSSs (Shao et al., submitted), I estimate the effect size by using our B6-Chr^{MSM} CSSs for the behavioral traits to confirm whether the large-effect of CSS was common phenomenon for any crosses of CSSs and also

whether large-effect could be observed for behavioral traits. Finally, correlations between the multiple behavioral measurements are examined in the CSSs to confirm the architecture of multiple factors related to the open-field behavior extracted from wild-derived mouse strains (Chapter 2).

3.2 Method

Animals

MSM/Ms (MSM) was established as inbred strains after 20 generations of brother-sister mating at the NIG (Mishima, Japan), and C57BL/6J (occasionally abbreviated as B6) was purchased from CLEA Japan, Inc (Tokyo, Japan) and bred at NIG. Figure 3.1 shows a panel of CSSs used in this study. Establishment of B6-Chr^{MSM}CSSs has been described in detail by Oka et al. (2004). Briefly, MSM was backcrossed to C57BL/6J for 10 generations. In each generation, genotyping were performed by using MIT markers distributed on the desired chromosome (Figure 3.1). All CSSs have the same genetic background as C57BL/6J except for one pair of chromosomes which are replaced with corresponding MSM chromosomes. Because some chromosomes (2, 6, 7, and 12) had difficulty to substitute entire chromosome for unclear reason, these chromosomes were separated into two segments, centromere side (C) and telomere side (T), of the chromosome and introduced into two independent strains. Some CSSs were not bred well and their behavioral characterization has not been completed yet (Figure 3.1 gray colored). The name of each CSS was formally described as B57BL/6-Chr#^{MSM}, but for the sake of simplify, it was abbreviated as B6-#MSM where # indicate the chromosomal number. All animals are maintained at NIG under the 12-h light/dark cycle (light from 8:00 to 20:00) in a temperature-controlled room (23±2°C). The mice were weaned around 3 weeks of age and housed in same sex groups in standard sized plastic cages on wood chips. Food and water were available *ad libitum*. Mice were maintained according to NIG guidelines, and all procedures were carried out with approval by our institutional animal care and use committee.

Behavioral testing

A battery of tests for behavioral characterization of B6-Chr^{MSM}CSSs were performed as a

project of Mouse Genomics Resource Laboratory, NIG. To detect a wide range of behavioral characters in each strain, we evaluated several kinds of behaviors, spontaneous activity, emotionality-related behaviors, and pain sensitivity with a series of tests: novel cage test, home-cage test, open-field test, light/dark box test, elevated plus maze test, hot-plate test, and tail flick test. Every mouse experienced a series of behavioral tests in the same order with at least one day's rest between the consecutive tests. About 15 males and 15 females from each strain were used. This behavioral battery started at the age of 9 to 12 weeks, and ended at 12 to 15 weeks old. Several people were involved in this project. Dr. Nishi and Ms. Ishii for spontaneous activity test and light-dark box test, and Ms. Kusakari and Dr. Koide for pain sensitivity test. In this chapter, I show the data of open-field test and elevated plus maze test that I conducted. Note that social interaction test was performed by myself as an independent experiment using a new set of animals.

Open-Field Test

The open-field used consisted of a square arena (60 × 60 × 40 cm) made of white polyvinylchloride plastic board, and divided into 16 equal squares. The arena was brightly lit by incandescent lighting (365 lux). During the 10 min trial, we observed their behavior directly and recorded the presence or absence of the following 12 behavioral items in each 5-s period; sniffing, stretching, locomotion, leaning, rearing, grooming, face-washing, digging, gnawing, jumping, pause, and freezing (for more detail, see Chapter 2). For analyzing ambulation (number of square transit), central ambulation, % of central ambulation, and time spent in the center, the arena was continuously recorded by a video camera placed over its center and relayed to a video tracking system (Image OF; O'hara & Co. Ltd., Tokyo, Japan). At the end of the sessions, whether animal defecated or not was recorded. All animals experienced this test twice on two consecutive days. All tests were carried out during the light period (13:00-19:00).

Elevated Plus-Maze Test

Elevated plus-maze test was invented as another form of apparatus to measure anxiety in rodents (Lister, 1987). This apparatus consists of a plus-shaped maze placed in an elevated position from the floor. Two opposite arms have high walls to prevent animals from falling off; the other two do not have walls and animals are able to look around/down. This test is based on the aversion of rodents to open spaces (Treit et al., 1993). Behavioral pharmacological studies frequently use this apparatus for investigating sensitivity for anxiolytic or anxiogenic drugs, anxiolytic drug increase open-arm exploration (Pellow and File, 1986).

The apparatus used in this study, made of white acrylic board, consisted of two open arms with low edge (30 × 5 × 0.25 cm) and two closed arms enclosed by clear acrylic plastic wall (30 × 5 × 15 cm) that extended from a central platform (5 × 5 cm), and was elevated 60 cm above the floor (Figure 3.2A). The apparatus was dimly lit (150 lux). Mice were placed individually in the central platform, and allowed to move freely for 10 min. Ambulatory activity (cm), number of entries into the open-arm or closed-arm, and duration in the open-arm or closed-arm were measured by a video tracking system (Image EPM; O'hara & Co. Ltd., Tokyo, Japan). Because one ethological behavioral measurement, head-dipping, that is propensity of animals that look down from the open/closed arm, have been purported to be a valuable indicator of anxiety on the elevated plus-maze (Espejo, 1997; Rodgers and Johnson, 1995), I also observed their behavior directly to count the head-dipping behavior. Head dipping was defined as peer down behavior in which their nose goes underneath the floor of the maze. The presence or absence of head-dipping was recorded as 1/0 in each 5-sec period, and head-dips performed in the open-arm and in the central platform or the closed-arm were separately counted as open head-dipping and protected head-dipping, respectively. All tests were carried out during the light period (13:00-19:00).

Social Interaction Test

Social interaction test measures the number and duration of social contact between two

animals in a novel environment (e.g. open-field). This test was developed as the first animal test of anxiety (File and Hyde, 1978). Fearful situation (high light level and unfamiliarity) decrease social interaction between animals: social interaction is highest when rats are tested in a familiar arena lit by low light (File and Seth, 2003). This apparatus is expected to have association with both animal's sociality, affiliation or aggression toward other individual, and anxiety that inhibits animal social behavior.

Two individuals that were littermates of the same sex, aged 10 weeks, were used for each test. The mice were weaned around 3 weeks of age and housed in same sex groups. Ten days before the test, all the animals were separated into isolated cages and kept individually until the test. The apparatus used in this study was same as the one used for the open-field test (60 × 60 × 40 cm), which was lit less bright than normal open-field (80 lux, Figure 3.2B). For analyzing total number and duration of social contact, movement of the mice in the arena was continuously recorded by a video camera placed over its center and relayed to a video tracking system (Image SI; O'hara & Co. Ltd., Tokyo, Japan). When the two mice came close to less than or equal to 20 pixels, which was about 12 cm between the center of animals, the behavior of these animals was recognized as a state of social contact. All tests were carried out during the light period (16:00-19:30).

Statistical analysis

Data analysis was performed using the SPSS version 14.0J software packages. Significance of each CSS was determined in a Dunnett's t-test between each CSS and the C57BL/6J control. In this analysis, males and females were analyzed separately.

For calculating the effect size of each CSS, I adopted a modified method (Shao et al., submitted). Effect size is the percentage of total phenotypic variance contributed by a particular QTL (Valder et al., 2006). However, it is not able to apply the conventional method to CSS, because genetic variation in a population is required for calculating effect size while individual from the

same CSS have identical genetic background, thus genetic variance is zero in the case of CSS. Thus, in order to deal with this problem, we used the following equation to calculate effect size according to the Shao's method:

$$ES_i = 100 * |(CSS_i - B6) / (Hi - Lo)|$$

Where ES_i represents the effect size of hypothesized-QTL for the ith CSS, Hi is the highest mean phenotypic value among the progenitor strains and the CSS panel, and Lo is the lowest mean phenotypic value. To prevent confusion with the conventional method, it is named as "phenotypic effect" instead of the term "effect size".

Direction and magnitude of QTL action was calculated by following equation:

$$DM_i = (B6 - CSS_i) / (B6 - MSM)$$

Where DM_i represents the direction and magnitude of hypothesized-QTL action for the ith CSS. When the hypothesized QTLs on ith CSS have actions in the same direction toward MSM from C57BL/6J, DM_i will be positive. And if it has effect in the inverse direction, DM_i will be negative. DM_i is zero if there is no QTL action on a CSS, and 1 if the direction and magnitude of the hypothesized-QTL action on a CSS are equal to those on MSM.

A principal component analysis was also performed using SPSS version 14.0J software packages. Principal component analysis with Varimax rotation was performed to reveal the genetic correlation. Factor scores for individual animals were estimated by summing up each value that is weighted with eigen vector of each factor. Those factor scores were subjected to two-way ANOVA to examine strain-sex interaction, and then to Dunnet's t-test ($p < .05$).

3.3 Result

3.3.1 Mapping the chromosomes associated with emotionality-related traits

We first identified the chromosomes related to behavior by comparing each CSS with C57BL/6J in three behavioral tests by 32 variables. In consideration of sex dependent QTLs, we performed Dunnet's t-test for males and females independently. Note that in some pairs of CSSs (B6-2CMSM/B6-2TMSM, B6-6CMSM/B6-6TMSM and B6-12CMSM/ B6-12TMSM) different parts of a chromosome are substituted (Chr 2, 6 and 12), and they have MSM region overlapped in the middle of chromosome (Figure 3.1). Thus, to avoid overestimation, we consider that it has one CSS even if both of them (C and T) showed significant behavioral differences from C57BL/6J.

Figure 3.3 demonstrates the data of the ambulation in the open-field as a representation of all measurements. Over half of CSSs, 11 CSSs, showed significant differences from C57BL/6J in either or both sex. MSM showed considerably decreased ambulation compared to C57BL/6J. Most CSSs that had significant differences showed decreased ambulation from C57BL/6J and situated between C57BL/6J and MSM. However, both sexes of B6-9MSM and females of B6-3MSM, B6-13MSM and B6-14MSM exhibited increased ambulation compared to C57BL/6J.

For all 32 variables, there was a total of 117 CSSs in male and 130 CSSs in female that showed significant differences from C57BL/6J ($p<.05$, Table 3.1). Fifty-seven of them showed significant differences in both male and female. The data suggested that there were about twice more CSSs that exhibited significant changes in either male or female, named sex-dependent QTLs, than CSSs that have significant differences in both sexes (133 CSSs and 57 CSSs, respectively). Two-way ANOVA revealed significant sex-genotype interaction for 1st OF ambulation, % of central ambulation, EPM total arm entry, and closed-arm entry, SI contact number ($p<.01$), and 1st OF center ambulation, stretching, 2nd OF ambulation, locomotion, leaning, grooming, and

EP total distance ($p<.05$).

In many behavioral variables, multiple CSSs showed significant differences from C57BL/6J. The number of variables that were related in more than two CSSs, named multigenic traits (Flint et al., 2005), were 18 (56%) in male and 21 (66%) in female for these 32 variables. Activity in the novel situation (open-field and elevated plus-maze) tended to be contributed by many CSSs. In contrast, we failed to find any CSSs related to grooming which is prominent character of MSM. Jumping was especially characteristic in one strain, B6-3MSM, while both parental strains less likely show this behavior.

Most CSSs had QTLs for multiple behavioral traits. Especially, B6-1MSM, B6-6CMSM, and B6-17MSM exhibited significant differences from C57BL/6J in more than 10 variables in both sexes. However, three strains, B6-7TMSM, B6-19MSM, and B6-YMSM showed no differences in any measurements from C57BL/6J.

Open-field test was performed for two consecutive trials, and the number of contributed CSSs for second trial was reduced in male (from first to second trial, 48 CSSs to 39 CSSs) but slightly increased in female (44 CSSs to 46 CSSs) from first trial. Those were classified into first trial specific QTLs (25 CSSs and 17 CSSs in male and female, respectively), second trial specific QTLs (16 CSSs and 19 CSSs), and common QTLs for both trials (23 CSSs and 27 CSSs). Most measurements had effect from these three kinds of QTLs (Table 3.2). However, measurements for central ambulation were affected by first trial specific QTLs in large part, but not by common QTLs, in male. In contrast, rearing did not have effect from first specific QTLs, but was affected by many second specific QTLs. Ambulation had effects mainly from common QTLs.

3.3.2 Phenotypic effects in each CSSs

Next, I estimated the phenotypic effect of each CSS by the modified method for the effect size (Shao et al., submitted). The result indicated that the phenotypic effects in CSSs, which had significant differences from C57BL/6J for any behavioral trait, were quite large (Fig 3.4). The

average phenotypic effects for a CSS were 44% for male (range 18% to 99%) and 47 % for female (range 25% to 100%).

Stretching was explained 100% of variance by B6-9MSM (44%) and B6-17MSM (56%) in male, and B6-6CMSM (40%) and B6-XCMSM (59%) in female for the day 1 trial. Because I found there was significant sex-genotype interaction for stretching behavior ($p<.05$), this behavior may have different genetic contribution between male and female. Social contact-duration was also explained about 100% of variance by two strains, B6-6CMSM and B6-17MSM, in both sexes.

Figure 3.5 shows the direction and magnitude of hypothetical-QTL action in each CSSs. This histogram contains *DM* value for all CSSs including non-significant CSSs. Most CSSs (92%) had effect for the same direction as MSM related to C57BL/6J. Among them, 77% were positioned same as C57BL/6J or between C57BL/6J and MSM, while other 15% showed the effect more than two times in magnitude than the difference between C57BL/6J and MSM. On the other hand, 8% of CSSs exhibited inverse direction from MSM. Among them, 3% were positioned within two times in magnitude between C57BL/6J and MSM, while other 5% showed the effect more than two times in magnitude.

3.3.3 Multivariate analysis of behavioral measurements in CSSs

Table 3.3 shows phenotypic and genetic correlations between each 32 measurements. Principal component analysis was performed using genetic correlation by taking advantages for removing entangled effect of test session and environmental history (Chapter 2).

First, measurements of open-field test in first trial were subjected for principal component analysis to compare with the factor structures that were extracted from wild mouse strains (Chapter 2). As jumping shows large skewness and leptokurtosis because of vastly high value in B6-3MSM, this index seems to affect the factor structure irrelevantly. Thus, I excluded this measurement from this analysis. Table 3.4 shows the factor loadings obtained from principal component analysis followed by Valimax rotation. Three factors that accounted for 76 % of total

variance were extracted with eigen values higher than 1. Variables that loaded highly on Factor 1 were ambulation, locomotion, and central ambulation for positively, and defecation, grooming, and pausing loaded in the reverse direction. This first factor represented 35.8 % of total variance. Factor 2 represented 26.5 % of total variance, and it had positive loadings from percentage of center ambulation and central stay time, whereas negative loadings from leaning and face-washing. Factor 3 represented 13.7 % of total variance, and it included stretching and rearing in two directions. To confirm the reliability of this factor structure, I performed principal component analysis several times by subtracting some variables that have high correlation with other measurements: central stay time and locomotion (correlate with % of center ambulation (r= .93) and ambulation (r= .91), respectively). Although some indices were changed by each computation, I could find almost similar factor structure to the first analysis (data not shown).

To examine whether there are common factors underlying different emotionality-related behavioral tests, I next performed principal component analysis on all measurements of this study including first trial of open-field test (OF), elevated plus-maze (EPM) and social interaction test (SI). Five factors accounting for 81.1% of total variance were extracted with eigen value higher than 1 (Table 3.5). Factor 1 represented 21.9% of total variance, and it had positive loadings from EPM ambulation, total arm entry, protected head-dipping, and OF leaning, whereas negative loadings from grooming. Factor 2 represented 20.9% of total variance, and it included OF ambulation and rearing positively, and defecation and pausing negatively. SI variables also loaded on this factor; duration of social contact for positively and number for negatively. Factor 3 accounted for 15.0% of variance, and it had positive loadings from OF center measurements, and negative loading from grooming. Factor 4 represented 14.4% of total variance, and highly positive loading from EPM open-arm entry and open head dipping. The final factor had loading from OF stretching and rearing.

3.3.4 Representation of each CSS in three factors

Character of each consomic strain is able to be represented by using these factors. Three factors extracted from open-field measurements (Table 3.4) were used for the representation. Factor score in each individual was estimated by summing up each value that is weighted with eigen vectors of each factor. Figure 3.6 shows the average factor scores in each CSS. Two-way ANOVA revealed significant effect of strain for all three factors [$F(22,686) > 13.6$, $p < .001$]. Strain×sex interaction was significant in Factor 2 and 3 [$F(21,686) > 1.7$, $p < .05$]. Because there was no strain-sex interaction in Factor 1, subsequence analysis was performed by combining the data of both sexes for this factor. Dunnet's t-test revealed that nine CSSs (with substituted chromosome 1, 4, 6, 8, 11, 12, 15, 16, and 17) showed significant decrease of Factor 1 score, whereas one CSS, B6-3MSM, exhibited increased score. In the Factors 2 and 3, male and female were separately analyzed. Significant decreases of Factor 2 score were observed in six strains, B6-1MSM, B6-9MSM, and B6-11MSM in both sexes, males of B6-6CMSM and B6-13AMSM, and females of B6-2CMSM. Only males of B6-12TMSM indicated increased Factor 2 score. In Factor 3, both sexes of B6-6CMSM, male of B6-17MSM, and female of B6-XCMSM showed significant decrease of the score. Significant increases of Factor 3 score were observed in both sexes of B6-9MSM, female of B6-2TMSM, and male of B6-16MSM. Two dimensional representations of each CSSs were shown Figure 3.7. Parental strains and three CSSs, B6-1MSM, B6-6CMSM, and B6-17MSM, that showed significant differences from C57BL/6J in two or more factors were indicated as colored points.

3.4 Discussion

3.4.1 Mapping the chromosomes associated with emotionality-related traits

Recent QTL studies using F2 intercross, heterozygote stock or recombinant inbred strains have demonstrated that there are a vast number of QTLs related to anxiety and that they are distributed widely to nearly all chromosomes (Flint, 2003). The present result also shows that there were a large number of chromosomes related to three kinds of emotionality-related tests; the open-field, elevated plus-maze, and social interaction test by using a series of consomic strains established by MSM and C57BL/6J (B6-Chr^{MSM}CSSs). Another groups, using males of CSSs established from A/J and C57BL/6J (B6-Chr$^{A/J}$CSSs), also reported that multiple CSSs are related to the open-field test and light-dark box test (Singer et al., 2005), and their results are comparable to the present results. Interestingly, we found that the number of chromosomes having significant effects on each behavior is larger in B6-Chr^{MSM}CSSs than in B6-Chr$^{A/J}$CSSs. For example, Singer and their colleagues (2005) reported reduced open-field ambulation in three CSSs: males of B6-1$^{A/J}$, B6-6$^{A/J}$, and B6-15$^{A/J}$ strains that have substituted chromosomes 1, 6, and 15 from A/J, respectively. In contrast, seven more chromosomes (chr. 3, 9, 13, 14, 16, and 17) in addition to chromosomes 1 and 6 showed either increased or decreased open-field ambulation in males of our B6-Chr^{MSM}CSSs. Note, however, that I did not find significant effect on chromosome 15 in male but in female. Singer (2005) also reported as novel finding that CSS-11 shows center avoidance in the open-field. The present result confirmed their result; males of B6-11MSM showed center avoidance. In addition, two more CSSs, B6-13AMSM and B6-14MSM, also showed center avoidance. The latter two strains exhibited increased open-field ambulation, thus they are considered as peripheral area runner. The differences of the results between B6-Chr$^{A/J}$CSS and our B6-Chr^{MSM}CSS may possibly be caused by a larger genetic distance between MSM and B6 than between A/J and C57BL/6J. MSM belongs to *Mus musculus*

musculus, while C57BL/6J and A/J were derived from the same small original population belonging largely to *Mus musculus domesticus* (Bonhomme and Guenet, 1996; Ferris et al., 1982; Wade et al., 2002; Yonekawa et al., 1980). In fact, the frequency of SNPs between C57BL/6J and MSM was estimated around 8.2 SNPs per 1 kilo base pairs; much more polymorphic than among usual laboratory strains (Wade et al., 2002). This result indicates that B6-Chr^{MSM}CSS have advantages to detect many QTLs. But also, we can not ignore the methodological differences (e.g. test length) between two studies.

3.4.2 Large phenotypic effects in the CSSs

It have been reported that most QTLs have an average 5% of additive effects on the phenotypes (Flint et al., 2005; Valdar et al., 2006). In contrast, we found that CSSs that had significant differences from B6 showed surprisingly large phenotypic effects. The average phenotypic effect in each CSS became about 45% for the emotionality-related traits. Because this effect is average value of one chromosome, it is possible that many QTLs exist within the chromosome. However, we found that congenic strains of a certain CSS that have shorter segment of a chromosome also showed strong phenotype effect as the intact CSS (Chapter 4). Therefore, CSSs is a method to detect large effect QTLs on the behavioral phenotypes compared to other QTL studies. Shao and colleagues (submitted) was also observed this large phenotypic effect in B6-Chr$^{A/J}$CSSs for blood, bone, and metabolic traits. They reported that an average phenotypic effect in individual CSS was 50%.

One possibility of this large effect is due to the non-additive effect in the CSSs. Most QTL studies using segregating populations (F2, N2, or heterozygote stock) have heterozygote genetic context, and QTLs found in those population have independent and constant phenotype effects regardless of the variant genotype in the other loci. That is, the effects of those QTL are additive, and gene interactions are under-estimated or undetectable. By contrast, CSSs have homozygote genetic context and thus certain kinds of gene interactions are readily detected (Shao et al.,

submitted). Thus, those large effects in CSSs might mostly reflect the interactive, epistatic, effects between the MSM locus/loci and genetic background of C57BL/6J. However, we can not exclude the possibility that there are quite a number of QTLs that have additive effect for both positive and negative direction. Our result showed the large effect in the CSS even for the behavioral phenotypes. This large effect is expected to be advantage for the further step to understand the genetic mechanisms of behavior.

3.4.3 A number of sex dependent QTLs in the CSSs

In this study, I analyzed males and females separately to exclude confounding effect with sex chromosomes in CSS. Many studies have reported sexual dimorphism in gene expressions even if those genes are not on sex chromosomes. Yang and colleagues (2006) recently reported that thousands of genes were estimated to know sexual dimorphisms in peripheral organ and identified hundreds of genes in brain on its expression. Thus, I speculated that many QTLs have different levels of effect for male and for female. The data supported this speculation: about two thirds of total CSSs that showed any behavioral differences are sex-dependent. And females tend to have more QTLs than males. Genotype-sex interactions were reported in several QTL studies for physiological traits, such as obesity, diabetes, or hypertension (Avery et al., 2006; Farber and Medrano, 2006; Herrera et al., 2006), and a few behavioral traits, such as sensitivity toward ethanol or alcohol preference (Downing et al., 2006; Gill et al., 1998). For the emotionality-related behavior, female specific QTLs for the central open-field ambulation were identified in rats (Ramos et al., 1999). However, not many QTL studies focused on the sex-specific QTLs. In contrast, Valder and colleagues (2006) reported that there were not many sex-specific QTLs. They conducted QTL analysis to a variety of physiological and behavioral traits in the heterozygote stock, and estimated that there are in total 843 QTLs and only 20 of them were the sex-specific.

In contrast, my result suggested that there are many CSSs that tend to have prominent effect

in either male or female, named sex-dependent CSSs. Two thirds of significant CSSs showed sex-dependent differences, and females tended to have more chromosomes that have significant effects than males. This was perhaps because females showed larger phenotype variances in many phenotypes. Statistically significant sex-genotype interaction was found in several behavioral measurements, and thus several CSSs for those indices might have QTLs that have sex-specific effect on those phenotypes. Sex differences and sex-genotype interaction have long been reported in the anxiety-related behavior (Blanchard et al., 1991b; Holmes et al., 2000; Ramos et al., 1998), and it was discussed that the quality of anxiety-related behavior is varied between male and female in rats; behavior of male are driven by sexual preference and anxiety while female is characterized primarily by motor activity in rats (Fernandes et al., 1999). These loci we found in this study might relate to the sex differences of anxiety-related behavior or "quality" of emotionality. Theses sex-specific effects might be because of the epistatic effects from sex chromosomes or some other sexual dimorphic genes (Yang et al., 2006).

3.4.4 QTLs for repeated trials of open-field test

The open-field test was performed in two consecutive days, and CSSs that showed significant differences from C57BL/6J were considered to have three different kinds of QTL: first trial specific QTL, second trial specific QTL, and common QTL for both trials. It is expected that the first trial specific QTLs reflect "response to novelty" (Gershenfeld et al., 1997), and the second specific QTLs may reflect "habituation" toward the novel situation (Bolivar et al., 2000). Also, it has been suggested that intersession habituation also reflects memory of the previous session (Muller et al., 1994). I found measurements related to central aversion tended to have large effect from first trial specific QTLs. Thus, the central ambulation may particularly reflect response to novelty. Rearing has contribution mainly from second trial specific QTL, and thus it may be considered to be related to habituation or memory. The temporal changes of rearing support this idea (Chapter 2), and also this behavior has been reported to have relation with the

size of terminal mossy fiber projections to hippocampus (Crusio, 1989a, 1989b, 2001), which is intimately involved in the processing of information about the environment (Schmajuk, 1984) and exploratory learning (Moser et al., 1994). It is hard to explain what is the common QTL for both trials. It may reflect such as aversion toward the light, spontaneous activity, or strong emotionality that persists despite twice exposures in the open-field apparatus. For the ambulation, which has contributions from many common QTLs, we found moderate correlation with the home-cage activity (r= .50) in our behavioral battery.

3.4.5 QTLs for social interaction test

Social interaction test was performed as one of the anxiety-related tests. This test hypothesizes that the novelty of testing apparatus reduces animal's normal social interaction behavior (File and Hyde, 1978), and this behavior is very sensitive to both anxiolytic and anxiogenic effects (for review see File and Seth, 2003). In this study, I found the parental strain MSM showed highly increased social interaction behavior than C57BL/6J. However, as shown so far, MSM shows strongly inhibited ambulation in the novel situation and takes long time to be habituated to the situation than C57BL/6J (Takahashi et al., 2006). This seems to be inconsistent with the first assumption of this test. But again, this test has association with both level of sociality, affiliation or aggression toward other individuals, and emotionality that inhibits the social behavior. Present result may reflect high sociality of MSM that exceeds inhibitory effect of emotionality. Because social interaction was examined in dimly lit open-field in this study, the inhibition of the emotionality to the social behavior was expected to be small (File and Seth, 2003).

I identified two CSSs, B6-6CMSM and B6-17MSM, showing increased duration of social contact. Those two CSSs together accounted almost 100% of total variance in both male and female. Because they exhibited strongly reduced open-field activity, social contact and open-field activity showed negative correlation in the consomic strains (r = -.65, Table 3.3). As in the case of MSM, a hypothesis that high sociality exceeds the inhibitory effect from novelty, can be adopted

in these strains. To verify this hypothesis, it is necessary to examine whether social interaction behavior has contribution from the same genetic loci or different ones from the open-field behaviors. The contact number showed a positive correlation with the open-field activity (r = .62). It is possible that highly active strains in the open-field tended to encounter with other individuals more often. One strain that has substituted chromosome 2 indicated increased contact number but no differences in the activity in the open-field test. It is suggested that the social factor enhances the activity in this strain.

3.4.6 Principal component analysis

To confirm the reliability of multiple factors extracted from wild-derived mouse strains (Chapter 2), I examined the factor structure from the genetic level by using B6-Chr^{MSM}CSSs. As with wild-derived strains, I extracted three factors for the open-field measurements from the CSSs (Table 3.4). The first factor was similar to the previous "locomotor activity" factor because it correlated positively with ambulation and leaning and negatively with pausing. This factor also has loading from grooming and defecation in this analysis. Thus, here I describe this factor as "locomotor activity and autonomic emotionality". Factor 2 has strong loading from % of center ambulation and central stay time. This factor is corresponding to the previous "thigmotaxis" factor. Factor 3 included stretching and rearing in the discrete direction. Factor structure of this factor was similar to the previous "anxious tension state" factor. Although some measurements such as defecation and grooming loaded on different factors, the main three factor structures for the open-field behavior were not different between wild-derived strains and CSSs. Thus I suggest here that there are three distinguished biological and genetic pathways related to these three factors of emotionality. Each factor can be represented by the ambulation for Factor 1, the percentage of central ambulation for Factor 2, and the stretching for Factor 3. Many QTL studies and gene-altered mouse studies examined open-field ambulation and central aversion (Flint, 2002; Crawly, 1999). However, behavioral measurements related to Factor 3 rarely

examined in most of those studies. Ethological significance has been reported for the measurements loaded on Factor 3; for example, stretching has information gathering risk assessment property and it occurs at high levels toward treat (Blanchard et al., 1995; Pinel and Mana, 1989; Molewijk et al., 1995) or to situations with treat potential (Kaesermann, 4986; Blanchard et al., 1991a). Several other principal component analyses of open-field behavior also extracted independent factor related to the stretching behavior from locomotor activity factor (Carola et al., 2002, 2004). Therefore, this factor should include important meaning for the emotionality study.

The elevated plus-maze and open-field test both exploit the natural aversion of rodents to exposed novelty. However, contradictory results between those two tests have been reported. The same strain of mouse has been defined as "anxious" with elevated-plus-maze and "non-anxious" with open-field (Rogers et al., 1999; Trullas and Skolnick, 1993). Factor analyses performed in open-field test and elevated plus-maze test separately reveal a few inter test correlation, strongly between locomotor activity factors in both test, but many factors are independent between the tests (Calora et al., 2002; Ramos et al., 1998). In this study, principal component analysis of all three tests confirmed that the elevated plus-maze and open-field test were contributed mainly by different factors (Table 3.5). Factor 1 and 4 were considered as factors for the elevated plus-maze; Factor 1 was described as "activity in elevated plus-maze" and Factor 4 reflected "open-arm exploration". Some open-field measurements, the locomotion, leaning and grooming also loaded on Factor 1. Therefore, only Factor 1 may be designated as common factor for the open-field and elevated plus-maze. Factors 2, 3, and 5 have strong relation to the open-field test corresponding with previous three factors extracted from the open-field measurements, "locomotor activity and autonomic emotionality", "thigmotaxis", and "anxious tension state" (Chapter 2), respectively. The social interaction test was strongly contributed by Factor 2. This relation of the open-field locomotor activity and social interaction measurements need further consideration, as discussed previously.

3.4.7 Summary

In this chapter, I identified multiple chromosomes that have a QTL or QTLs related to conventional and ethological measurements of open-field behaviors, elevated-plus maze, and social interaction test. I showed here that use of B6-Chr^{MSM}CSSs allows us to identify a large number of QTLs because of the large genetic distance between C57BL/6J and MSM. Many CSSs have substantially large effect QTLs, and thus they are expected to be superior tools for the next step of QTL analysis: identifying the quantitative trait gene, QTG (Hitzemann et al., 2003). This study also revealed that there are sex-dependent QTLs for the emotionality-related behaviors.

The interesting behavioral correlation was also revealed in this chapter. Behaviors in the elevated plus-maze and open-field were suggested to possess contribution from mainly different genetic basis. The social interaction behavior showed unexpected correlation with open-field behavior: low active mouse in the open-field shows prolonged social contact.

By analyzing a series of consomic strains, I confirmed the three factors underlying open-field behavior found in the study of wild-derived mouse strains, “locomotor activity”, “thigmotaxis”, and “anxious tension state”. Because behaviors loaded on “anxious tension state” factor have rarely be analyzed in most recent behavior genetic analyses, I will focus on this factor for further analysis. There were several CSSs that have QTLs for this factor (Figure 3.6). B6-17MSM significantly increased stretching behavior, which is a representative index of “anxious tension state” factor, on both first and second trials in male. They also showed significant differences in many measurements in this study. Furthermore, B6-17MSM exhibited prolonged social contact behavior and reduced open-field activity; this relationship needs further investigation. To identify genetic loci related to those behaviors and confirm these behavioral relationships more precisely, genetic analyses with congenic mouse strains of B6-17MSM have been performed (Chapter 5). Before that, I first characterize B6-17MSM for other aspects of phenotype in more detail (Chapter 4).

CHAPTER 4

Characterization of B6-17MSM Consomic Strain in Behavioral and Brain Morphological Traits.

4.1 Introduction

Chapter 3 provided a behavioral pattern for a panel of B6-Chr^{MSM}CSSs in three kinds of emotionality-related tests. One strain, B6-17MSM, which has substituted chromosome 17 from MSM, showed many interesting behavioral changes in the tests: reduced novelty-induced activity, increased risk assessment behavior, and highly increased social interaction behavior. Because we found no differences in their home-cage activity from C57BL/6J (Nishi, doctoral thesis), the reduction of activity in the novel situation (open-field and elevated plus-maze) may reflect elevated emotional reactivity in B6-17MSM. Other group who studied on consomic strains of A/J and C57BL/6J also reported that B6-Chr17$^{A/J}$, with substituted chromosome 17 from A/J, exhibited elongated latency of the first transition into new chamber in another kind of emotionality related test, light-dark box test (Singer et al., 2004). The same result was confirmed in B6-17MSM in our laboratory (conducted by Dr. Nishi). However, B6-Chr17$^{A/J}$ did not show reduced activity in the open-field (Singer et al., 2005) in contrast to the result that I found in B6-17MSM. This may be because of the differences of genetic variation between A/J and MSM. What it comes down to is that consomic strain with substituted chromosome 17 increased some kinds of emotionality-related behavior. This chapter describes the character of B6-17MSM in other kinds of behavioral and brain morphological traits to understand the emotionality in this strain in more detail.

Animal models of emotionality are conveniently classified as either conditioned or unconditioned responses to stimuli that appear capable of causing anxiety in humans (Rodgers

et al., 1997). The behavioral tests performed in chapter 3 were unconditioned, so-called ethological tests. That is, those tests measure anxiety-like behaviors that elicited spontaneously in a novel situation. In contrast, conditioned fear test is based on Pavlovian conditioning. An initially neutral conditioned stimulus (CS), such as light or tone, is paired with a noxious unconditioned stimulus (e.g. electric shock) several times, and in consequence the CS gains emotion-inducing properties. In this test, CS-induced freezing, a species-typical defensive response, is often measured as a conditioned emotional response that reflects fear memory. For understanding the emotionality of B6-17MSM, combining different aspects of behavioral tests should help to get a better grasp. In this chapter, I first examine fear memory of B6-17MSM in the fear conditioning tests. Neural mechanisms related to this conditioned emotional responses have been progressively understood (Rodriques et al., 2004), and it is considered that neural pathways differ depending on CS, a simple sensory stimulus (a cue) or more complex environmental representation (a context) (Sullivan et al., 2004). Thus, B6-17MSM was characterized in both cue-fear conditioning and context-fear conditioning test. Also, acoustic startle response was measured as another kind of emotional but reflective response toward a sudden loud sound (Plappert and Pilz, 2002).

Behavior can be affected not only by psychological state but also its physical ability. Major physical disability may disrupt the animal's performance. Actually, B6-17MSM has a possibility to have this problem. We empirically found that there is a increased incidence of hydrocephalus in this strain. Mice with hydrocephalus have enlarged skull and comparatively small body size, and it can easily be distinguished from normal individuals. Although, animals with hydrocephalus have been removed from the behavioral experiments, animals of this strain that look normal in appearance may also have a developmental or morphological defect in their brain. Thus, I examined the ventricular size in the brain of normal individuals of B6-17MSM. Their sensorimotor gating ability and motor function were also analyzed to confirm whether they have physical or cognitive defect.

This chapter aimed to characterize B6-17MSM by some kind of behavioral tests related to emotional learning, startle response, sensorimotor gating, and motor function, and also by their brain morphology. All of those traits are considered to affect emotionality of B6-17MSM in some way or another.

4.2 Method

Animals

C57BL/6-Chr17^{MSM} (abbreviated as B6-17MSM) , which has a substituted chromosome 17 from MSM, was established at the NIG (Mishima, Japan), and C57BL/6JJcl (occasionally abbreviated as B6) was purchased from CLEA Japan, Inc (Tokyo, Japan) and bred at NIG. Males aged 10 weeks after birth were used for the tests. Each test used independent population of animals, and thus all animals were naïve to any behavioral test. All animals were maintained at NIG under the 12-h light/dark cycle (light from 8:00 to 20:00) in a temperature-controlled room (23±2ºC). The mice were weaned around 3 weeks of age and housed in same sex groups in standard sized plastic cages on wood chips. Food and water were available *ad libitum*. Mice were maintained according to NIG guidelines, and all procedures were carried out with approval by our institutional animal care and use committee.

Fear conditioning test

10 days before the test, all animals were separated into a isolated cages and kept individually until the test. The chambers used in this study were of two types (Figure 4.1), each housed in sound attenuated cubicles, “Chamber A” (conditioning avoidance apparatus, AA-3202; O’hara & Co. Ltd., Tokyo, Japan) for cue-conditioning and “Chamber B” (passive avoidance apparatus, PA-3202; O’hara & Co. Ltd.) for context-conditioning. The floors consisted of metal grid for the delivery of footshock. Chamber A had a sound generator on the top of the cover. All conditionings and tests were performed between 16:00 and 19:00. Each group in both cue and context conditioning tests consisted of around 10 males of B6-17MSM or C57BL/6J.

Cue-conditioning

On day 1 and 2, animals in the cue-conditioning group received three presentations of a tone-shock pairing in which the tone (55 dB, 1000Hz; 5 s) co-terminated with a footshock (75 V, 1 s). The mouse was placed into the conditioning chamber individually, and after 2 min habituation to the conditioning chamber, tone-shock pairings were presented with 30s interval from the shocker (GT-7705D, O'hara & Co. Ltd.). The no-shock group was treated identically except that the shocker did not deliver electric current. On day 3, each animal of both groups was transfered into the new plastic home-cage (20 × 10 × 10 cm) without bedding material and was left undisturbedly for about 1 hour prior to the test. Then each of them was brought into the test room with the plastic home-cage, and the sound generator was placed on the top of the cage. In the test, each animal was exposed to the tones for 300 s, and their behavior was videotaped for measuring the duration of immobility in a later analysis. Because mice do not show complete freezing behavior like rats, here I measured immobility as a fear response. Immobility was defined as a stationary state without head or bodily movement but whisker motion and body twitching.

Context conditioning

The conditioning procedure was identical to the cue-conditioning except for tone presentation. Briefly, after habituation for 2 min in the conditioning chamber, animals received three presentations of footshock (50 V, 2 s) with 30 s intervals from the shocker (PA-2010, O'hara & Co. Ltd.) for two straight days. On day 3, each animal was placed into the same conditioning chamber for 300 s without foot shock, and their behavior was videotaped to measure the immobility behavior.

Sensitivity toward electrical stimuli

An animal was placed in the chamber B and was presented gradually increased electrical shock

(5, 10, 15, 20, 25, 30, 35, 40, 45, 50 V, for 2 s each) with 30 s interval. The response of the animals was observed and recorded as three levels: Level 1 (brief stop of the movement and backward moving), level 2 (licking paw, shaking or stepping the hind paw, and brief chipping), level 3 (running around and jumping). Each 5 males were used for both strains.

Acoustic startle response and Prepulse inhibition

Acoustic startle response (ASR) is a contraction reflex of skeletal and facial muscles in response to an abrupt, intense auditory stimulus. Test sessions began by placing the mouse in a clear Plexiglas holding cylinder placed on a piezoelectric accelerometer, which detected the vibrations caused by startle reflection to the sound of the mouse (Figure 4.1C; SR-Laboratory, San Diego, CA, USA). A 65 dB background noise was presented throughout the test session. ASR was measured as a part of the standard prepulse inhibition (PPI) procedure (programmed by A. Nishi, doctoral thesis). PPI is the suppression of the normal startle response to an abrupt startling stimulus when that stimulus is immediately preceded by a weak prestimulation, and used to measure sensorimotor gating in the brain (Paylor and Crawly, 1997). Briefly, animal was acclimated for 2 min and then it was presented with startle trials (120 dB, 40 ms sound pulse) and prepulse + startle trials (20 ms noise prepulse sound followed by a 30 ms 120 dB sound pulse with 70 ms interval). There were 3 different prepulse intensities (70, 75, 80 dB). Each trial was presented 6 times in pseudo-random order with a variable interval (range 5 to 10 s). ASR was defined as average voltage across the entire responses in the startle trials. PPI was defined as inhibition rate of the startle response by an adjacent prepulse which itself does not enough to induce startle response. Percentage of PPI was calculated by following equation:

$$PPI_x = [1 - (SR_{xm} / ASR)] \times 100$$

Where PPI_x represents the inhibition rate of startle response by x dB prepulse, and SR_{xm} represents the mean startle amplitude of prepulse trial with x dB prepuls. Thirteen males of B6-17MSM and ten males of C57BL/6J were used in this test.

Rotarod test

Animal's motor function was determined by using rotarod test. This apparatus has been used to assess motor coordination in the rodent (Jones and Roberts, 1968). The apparatus consisted of a black striated rod (3 cm in diameter), 20 cm height from the floor (Figure 4.1D; O'hara & Co. Ltd.). Animals were placed on the rod with a constant low speed rotation (5 rpm), and few seconds later when they adjusted to walking on the rod, the test was started. In the test, rotation speed of the rod was gradually accelerated from 5 to 40 rpm over 5-min period. Latency at which mice fell off the rotating cylinder was automatically measured. Trials were repeated twice with 30 min interval for each individual, and better score (longer latency) was adopted as the score of the animal.

Brain morphology

Histological analysis

The mice were deeply anesthetized by intraperitoneal injection of pentobarbital (10 ml/kg), and transcardially perfused first with saline and followed by 4% paraformaldehyde with 0.5% picric acid dissolved in phosphate-buffered saline (PBS). The brains were removed, and after immersion-fixation overnight in the same fixative at 4˚C, coronal sections 70 um thick were prepared on a Vibratome. Every two sections were collected for immunostaining. The sections were kept in PBS at 4˚C.

In this study, I picked up calbindin antibody for observing the brain morphology because calbindin is known to express in the entire brain (The Gene Expression Database, MGI). The immunostaining procedure was performed following VECTASTAIN ABC system (Vector Laboratories, Burlingae, CA, USA). Briefly, sections were incubated with 10 % normal goat serum in PBS for 1 hour at 37˚C and then with anti-calbindin D-28k rabbit monoclonal antiserum (CB-38; SWant, 1:8000 dilution) in PBS overnight at 4˚C. Sections were then washed and exposed to biotinylated goat anti-rabbit IgG for 1 h followed by incubation with horseradish

peroxidase (HRP)-conjugated avidin-biotin complex for 1 h. Color reaction was carried out by incubating the sections in a chromagen solution containing diaminobenzidine and nickel sulphate intensification (DAB Substrate kit; Vector) for 2 min. The reaction was stopped by PBS washing. Sections were mounted on silane coating micro slides (Muto Pure Chemicals Co., Ltd, Japan), air-dried, dehydrated and cover-slipped.

Quantification of brain ventricular size

Microscopic images were captured using digital color camera (DP10, OLYMPUS, Tokyo, Japan) connected to a stereoscopic microscope (SZX12, OLYMPUS). Ventricular size in the brain section was measured by using free software package Image J 1.36b (http://rsb.info.nih.gov/ij/ National Institute of Health, MD, USA). In this software, the area was calculated as the number of pixels included in the enclosure. Here, the brain size was defined as the area enclosed by the periphery of coronal section, and the ventricle size was defined as the area of interior cavity in the same coronal section. Because the brain size had a large individual difference, the area of brain ventricle was standardized as follows,

$$sVS_i = (BS_m / BS_i) \times VS_i$$

Where VS_i and BS_i represents the brain ventricular size and the whole brain size for the ith individual, respectively, and BS_m is the mean brain size in all tested individuals.

Statistical analysis

Data analysis was performed using the SPSS version 14.0J software packages and StatView version 5 (SAS Institute Inc). Data in cue conditioning were subjected to two-way analysis of variance (ANOVA) to test for the significant effect of strain and group. Temporal changes of immobility behavior in both fear conditioning tests were analyzed by one-way ANOVA for repeated measures of continuous variable (time). Post hoc comparisons were carried out using the HSD test ($p<.05$). All other data were analyzed using Student's t-test.

4.3 Result

4.3.1 Cue fear conditioning

Both B6-17MSM and C57BL/6J showed increased immobility toward the conditioned tone (Figure 4.2). Two-way ANOVA revealed significant main effects of conditioning [$F(1,33) = 68.93$, $p<.0001$] and interaction between conditioning × strain [$F(1,33) = 14.459$, $p<.001$]. B6-17MSM showed significantly prolonged immobility toward the conditioned stimulus (tone) than C57BL/6J. In the no-shock group, B6-17MSM also exhibited slightly increased immobility but not significant difference from C57BL/6J. Repeated one-way ANOVA revealed significant strain × time interaction [$F(6,102) = 171.24$, $p<.001$], and significantly higher immobility was observed in B6-17MSM for all the tested period except pre-tone presentation.

4.3.2 Context fear conditioning

Figure 4.3 shows the result of context fear conditioning. There was no difference between B6-17MSM and C57BL/6J in the total duration of immobility in the context conditioning ($p=.82$). Repeated one-way ANOVA revealed there was no difference in the temporal change of immobility between two strains [$F(4,76) = 303.8$, $p=.41$].

4.3.3 Sensitivity toward electrical stimuli

To learn whether there is a difference in sensitivity of electrical shock between B6-17MSM and C57BL/6J, the sensitivity toward a range of electrical stimuli in those two strains was examined. Animals in both strains exhibited the level 1 responses (brief stop and backward moving) toward electrical stimuli at 15V to 20V (on average, 16V for B6-17MSM and 17V for C57BL/6J). Level 2 responses, licking paw, shaking, stepping, and chipping, started to show for an average 22V stimuli in both B6-17MSM and C57BL/6J. Level 3 responses started to appear at 30V electrical

stimuli in both strain. Thus, there were no differences in the sensitivity to the electrical stimulus between B6-17MSM and C57BL/6J.

4.3.4 Acoustic startle response and prepuls inhibition

The results of acoustic startle response (ASR) and prepulse inhibition (PPI) are representated in Figure 4.4. There was a slightly higher ASR in C57BL/6J than B6-17MSM, but the difference was not significant (p=.09, Figure 4.3A). PPI were examined by using 3 different intensities of pulses (70, 75, 80 dB) as prepulse. The inhibition rate of startle response was increased with increasing intensity of prepulse in both strains (Figure 4.4B). Again, no significant differences between B6-17MSM and C57BL/6J were observed for any intensity of prepulses (70 dB, p=.28; 75 dB, p=.87; 80 dB, p=.60).

4.3.5 Motor function in rotarod test

To see whether there is difference in the motor coordination ability between two strains, rotarod test was performed. Some mice that fell off at an early period by turn backward in both trials were excluded from the analysis. Thus, data was obtained from 6 animals for each strain. The latency of falling off from the rotarod did not differ between two strains: 191 sec (range 133 to 249 sec) in B6-17MSM and 184 sec (range 145 to 237 sec) in C57BL/6J.

4.3.6 Hydrocephalus-like phenotype in B6-17MSM

For a confirmation of empirical finding in breeders about increased appearance of hydrocephalic individuals in B6-17MSM, I first looked over the past records of B6-17MSM and C57BL/6J. Those records listed information of each litter, such as litter size and presence of any physical defect individuals in each strain. From the records, the total number of individuals and the number of individuals that showed hydrocephalus in the past three years were counted. The litter size, the number of animals in one litter, seemed smaller in B6-17MSM (on average 4.5)

than C57BL/6J (on average 8.1). However, these data lack the accuracy because we crossed one or two females with one male, some litters may include infants from multiple mothers. The incidence of hydrocephalus was 5.7 % in B6-17MSM (26 individuals in total 456 individuals) while 0 % in C57BL/6J (in total 819 individuals). Thus B6-17MSM has a higher incidence of the hydrocephalus phenotypes.

To examine whether there is any difference in the brain morphology for apparently- normal individuals of B6-17MSM, histological analysis was performed. Because the hydrocephalus phenotype is due to the expansion of brain ventricle caused by excess cerebrospinal fluid, I focused on the brain ventricle size in this analysis. The pictures of coronal brain sections of calbindin immunohistochemical staining in B6-17MSM and C57BL/6J are shown in Figure 4.5A, B. The brain size, area enclosed by periphery of coronal section, did not different between B6-17MSM (776144 ± 16457) and C57BL/6J (73938 ± 10783). In contrast, as seen in the picture, there was notable difference between B6-17MSM and C57BL/6J in their ventricle size (Figure 4.5C). The result of t-test revealed the significant differences between two strains for the standardized brain ventricle size ($p<.001$). Scatter plot shows increased variance in B6-17MSM than B6 (Figure 4.5D).

4.4 Discussion

4.4.1 Fear conditioning

Through the fear conditioning an animal learns that a simple sensory stimulus (a cue), or more complex environmental representation (a context), predicts imminent adversity (Sullivan et al., 2004). Amygdala has been focused as the essential brain region related to fear, and studies from many labs have led to the conclusion that damage to the amygdala interferes with the acquisition and expression of conditioned fear (LeDoux, 2000; Maren, 2001). However, it has been also suggested that neural pathways involved in fear conditioning are different dependent on the conditioned stimulus, a cue or a context (Kim and Fanselow, 1992; Sallivan et al., 2004). Sallivan and colleagues (2004) reported that the legions of central nucleus of the amygdala (CE) attenuated both cue and context conditioning, while lesions in the bed nucleus of the stria terminalis (BNST) disrupt only context conditioning but not cue conditioning. Subicular region of hippocampar formation is also considered as key brain region for the context but not cue conditioning (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). It has been reported that contextual fear conditioning emerges later in development than auditory-cue conditioning (Rudy, 1993). In addition, QTL studies also showed distinct genetic regulation of cue and context conditioning (Wehner et al., 1997; Owen et al., 1997). QTL analysis in F2 intercross of C57BL/6J and DBA/2J identified QTLs for both cue and context conditioning on chromosome 1, 10 and 16, and for only context conditioning on chromosome 2 and 3 at a suggestive level (Wehner et al., 1997). Other cross between C57BL/6J and C3H/HeJ showed QTLs for both cue and context conditionings on chromosome 1 at 65cM and chromosome 9, and for context conditioning on chromosome 3, 7, 8, 18, and another locus of chromosome 1 at 29cM. They also analyzed recombinant inbred strains of C57BL/6J and DBA (BXD) and mapped QTLs on chromosome 1 and 19 for both cue and context conditionings, and chromosome 2, 9, 11, and 17 for the

contextual conditioning. These results suggest that in addition to common basic mechanisms for cue and context fear conditioning, there are independent pathways for context conditioning.

In the present analysis, B6-17MSM had different propensities to cue and context fear conditioning. They showed increased fear response toward the conditioned auditory cue, while no differences from C57BL/6J in the context conditioning. This was different from the result of BXD recombinant strains that found QTL for context but not for cue conditioning on chromosome 17 (Owen et al., 1997), and the present result is also inconsistent with previous suggestion: cue conditioning has basic mechanisms common with context conditioning, while only context conditioning has its own mechanisms concurrently. One possibility of this discrepancy is that the result of B6-17MSM can be influenced by sensory processes or levels of excitability. It may be hypothesized that B6-17MSM has higher sensitivity toward sound stimuli than C57BL/6J, because they exhibited slightly higher immobility behavior even when they were not shocked. However, a developmental analysis of auditory brainstem response showed no differences between B6-17MSM and C57BL/6J aged under 7 months (Nemoto et al., 2004). Thus, there may be no difference in the auditory sensitivity between two strains at the tested age. Another possibility is that the increased immobility in the no-shock group of B6-17MSM may be because of elevated tension in this strain. At the test day, each of them was moved to the test room just before the test, and thus the tested environment was very new to them. Because B6-17MSM indicates reduced activity in the novel situation (Chapter 3), the increase of no-moving behavior, immobility, may be induced by the novelty rather than by the auditory stimuli. I checked immobility behavior in no-shock group when they brought into the test room 10 min before. However, B6-17MSM still showed slightly higher immobility toward sound stimulus (preliminary data). Further possibility is that the sound, used as CS, is too loud, and B6-17MSM felt stress or fear toward the CS. In the fear conditioning paradigm, it is preferable that CS is neutral to the animal. This possibility needs further careful examination.

In spite of these possibilities, I consider that B6-17MSM had increased fear memory in the cue

fear conditioning from the temporal change of their immobility behavior. B6-17MSM showed prolonged immobility over the test period, while C57BL/6J did not show immobility in the later session. Because animals in no-shock group showed immobility just at the beginning, immobility behavior in the later session may be independent from the quality of the tone. In contrast, B6-17MSM and C57BL/6J indicated the same temporal pattern in the context conditioning. Thus, B6-17MSM is expected to have differences in their cue- specific brain region and/or genetic pathway.

4.4.2 Acoustic startle response and Prepulse inhibition

The acoustic startle reflex (ASR) is a protective response found in all mammalian species (Landis and Hunt, 1939). The neurons involved in this reflex are located in the brainstem (Lee et al., 1996). It is known that there are large between strain differences in ASR (Marks et al., 1989), and QTL analysis of ASR mapped genetic loci on chromose 4 and 7 (Le Roy et al., 1999). It was reported that ASR has positive correlation with anxiety-related measures in elevated plus-maze (Trullas and Skolinck, 1993). It is also reported that ASR is enhanced by stress or anxiogenic drugs, and thus proposed as a non-conflict model to study fear that does not depend on response inhibition (Davis, 1990, 1992). However, the present result showed no differences in ASR between B6-17MSM and C57BL/6J. Rather, C57BL/6J showed slightly higher ASR than B6-17MSM. Similar result was obtained with B6-6CMSM, which had reduced novelty induced activity and significantly diminished ASR than C57BL/6J (Nishi, doctoral thesis). Our results suggested that there may be different biological mechanisms underlying ASR and novelty induced activity or conditioned fear response.

Prepulse Inhibition (PPI) of acoustic startle is a model of sensorimotor gating and information processing in the brain. Sensorimotor gating is a neural filtering process that allows attention to be focused on a given stimulus, and is affected in patients with neuropsychiatric disorders (Paylor and Crawly, 1997). Present data shows that B6-17MSM has normal sensorimotor gating

as C57BL/6J.

4.4.3 Hydrocephalus-like phenotype in B6-17MSM

When an animal shows changed emotionality-related behavior, we behavioral scientists hope to identify neurological, hormonal, or genetic mechanisms related to the "emotionality" by using that animal. However, behavior is strongly affected by their physical conditions, and thus we need to consistently beware of this possibility.

B6-17MSM has higher incidence of hydrocephalic individuals. Actually, hydrocephalus rarely or never appears in C57BL/6J and MSM. Furthermore, apparently-normal individuals of B6-17MSM also showed enlarged brain ventricle size than C57BL/6J. It is considered that this underlying phenotype in normal-looking individual may be related to the hydrocephalus phenotype.

No heritability of brain ventricular size has been reported in human (Reveley et al., 1984). However, there is a large individual variability in the ventricular size, and abnormal enlargement of the ventricles has been genetically associated with schizophrenia (Marsh et al., 1994; Shihabuddin et al., 1996). Hydrocephalus is also associated with many inherited disorders such as ciliary dyskinesia, Dandy-Walker malformation, and a number of X-linked disorders. In mice, several genes have been reported to be related to the hydrocephalus phenotype; seven of them were mapped on chromosome 4 (*Tgfb1*, *Nfia*), 7 (*hyh*, *bh*), 8 (*hy3*), 13 (*Foxc1*), and X (*L1*). QTL analysis for the normal variation of ventricular size in AXB and BXA recombinant inbred strains identified several QTLs, where QTL on chromosome 8 was the major QTL contributing 53% of the variance and QTLs on chromosome 4 and 7 showed strong epistatic interaction for the phenotype (Zygourakis and Rosen, 2003). So far, there has been no report about genes or genetic loci related to hydrocephalus or ventricular size on chromosome 17 in the mouse.

4.4.4 Summary

In this chapter, several behavioral characterizations of B6-17MSM were conducted. Fear conditioning tests revealed interesting phenotype of B6-17MSM. This strain had increased fear memory in the cue-fear conditioning but not in the context-fear conditioning. Thus, it was expected there are genetic loci related to cue-specific fear learning on the chromosome 17.

Our empirical notation of the increased incidence of hydrocephalus in this strain was confirmed by examining the past records of B6-17MSM, and I found that apparently-normal individuals of B6-17MSM had enlarged brain ventricle size than C57BL/6J. Despite the hydrocephalus phenotype, B6-17MSM showed normal sensorimotor gating and motor coordination as C57BL/6J. The present result of B6-17MSM does not show the link between emotionality-related behavior and the hydrocephalus phenotype. To analyze relationships among several behavioral phenotypes and the hydrocephalus phenotype in B6-17MSM, genetic link was examined by making congenic mouse strains in the next Chapter.

CHAPTER 5

Genetic Dissection of Behavioral and Brain Morphological Phenotypes By Using Multiple Congenic Mouse Strains of B6-17MSM

5.1 Introduction

So far, B6-17MSM has been behaviorally characterized as reduced novelty induced activity, increased risk assessment behavior (stretching), prolonged fear response in cue fear conditioning, and elongated social interaction behavior compared to C57BL/6J. Meanwhile, B6-17MSM showed increased incidence of hydrocephalus and enlargement of brain ventricle size in all individuals. However, their home-cage activity, sensorimotor gating, and motor function did not differ from those of C57BL/6J. To identify genetic loci related to those phenotypes on chromosome 17, this chapter describes about establishment and analysis of congenic mouse strains of B6-17MSM.

It has been reported that there are several genes that alter anxiety-like phenotypes when it was deleted or mutated on chromosome 17 (PosMed, RIKEN, Tokyo, Japan http://omicspace.riken.jp/PosMed/). Also, some QTLs related to behavior have been mapped on this chromosome so far. One study with B6-Chr$^{A/J}$CSSs performed QTL analysis on an F2 intercross between B6-17$^{A/J}$ and C57BL/6J, and they mapped a QTL between D17Mit39 and D17Mit221 at 51.2cM that was related to total time in the lighted chamber in the light-dark box test (Singer et al., 2005). Other groups using recombinant inbred strains (RI) of mouse, developed from long-sleep (LS) and short-sleep (SS) selected lines, identified QTLs related to open-field locomotor activity, and one QTL was mapped around D17Mit39 at 45.3cM (Radcliffe et al., 1998). Other RI cross established by BALB/cBy and C57BL/6By found a QTL that affected activity in novel environment (runway) on chromosome 17 around 19cM to 25cM (Neiderhiser et

al., 1992). In forced swim test, QTLs were identified around D17Mit185 at 35cM by analyzing F2 intercross between C57BL/6J and C3H/He (Yoshikawa, 2002), and contextual fear conditioning at the same 35cM interval by analyzing BXD recombinant inbred strains (Owen et al., 1997). Analyzing B6-17MSM in detail will allow us to confirm some of those reported QTLs and to identify several genetic loci that have yet to be found.

To identify genetic loci related to those phenotypes, QTL analysis is usually performed at first to figure out the candidate genetic locus/loci. Then, congenic strains are established to confirm the effect of the candidate loci on the relevant phenotype. Congenic strains are produced by repeated backcrosses to an inbred (background) strain, with selection for a particular marker from the donor strain (Snell, 1978). It is required to backcross over 10 generations to a donor strain in order to match the genetic background, and then intercross them for making homozygous congenic strain (Rules for Nomenclature of Mouse and Rat Strains, MGI). Here, one of the advantages of using CSS is the "speed" for making subsequent congenic strains, because of their matched genetic background (Nadeu et al., 2000). In the case of CSSs, 10-generation backcross is unnecessary, and thus it just needs a few generations of backcross to make a congenic strain. By taking this advantage, I decided to establish multiple congenic strains to cover the entire chromosome 17 without mapping the candidate loci on the chromosome by QTL analysis beforehand.

Congenic strains have advantages not only to narrow down the genetic loci related to a particular phenotype, but also to figure out the genetic relationships among phenotypes precisely. This chapter aimed to confirm the relationships within open-field indices and between other phenotypes including social interaction and brain ventricle size by using congenic strains of B6-17MSM.

5.2 Method

F1 intercross

F1 progeny (named as (17×B6)F1) was generated by crossing B6-17MSM females and C57BL/6J males. Reciprocal intercross (named as (B6×17)F1) was also performed by crossing C57BL/6J males and B6-17MSM females. Animals were first tested in the open-field and then elevated plus-maze in two days. The number of animals used in this experiment is shown in Table 5.1. Males and females at the age 10 weeks were used in each intercross.

Congenic mouse strains of B6-17MSM

Establishment of Congenic strains

Both groups of F1 individuals were then backcrossed to C57BL/6J, and the offspring that carried desirable recombination within chromosome 17 were used for the subsequent cross. Those individuals were backcrossed to C57BL/6J one more time to obtain the cohort with same recombined segment of MSM chromosome. They were then intercrossed to make homozygotes for the substituted segment. A panel of congenic strains established in this study are listed in Figure 5.1. They are formally named B6.MSM-(D17MitAA-D17MitBB)/Ms, where AA and BB show the genetic markers used during the establishment of congenic strains and those markers positioned at the outer ends of both substituted MSM region on the chromosome 17. For the sake of simplify, here I named sixteen congenic strains as C1 to C15 tentatively (Figure 5.1). To indicate the position of the substituted MSM region, I will describe the name of the congenic strains with the rough physical distance, e.g. C1(c-31Mb), in the text. Where “c” and “t” represent the ends of the centromere and the telomere, respectively.

Genotyping of the markers in chromosome 17

The following MIT microsatellite markers, which differ between C57BL/6J and MSM, were chosen for the genotyping to construct the congenic strains (Mouse Microsatellite DataBase of Japan, MMDBJ, NIG http://shigen.lab.nig.ac.jp/mouse/mmdbj/top.jsp), D17Mit164 (1.1cM), D17Mit58 (1.1cM), D17Mit165 (3.3cM), D17Mit81 (5.6cM), D17Mit34 (10.0cM), D17Mit168 (14.5cM), D17Mit36 (20.2cM), D17Mit9 (26.2cM), D17Mit20 (30.6cM), D17Mit253 (31.8cM), D17Mit217 (35.1cM), D17Mit53 (35.1cM), D17Mit189 (36.2cM), D17Mit3 (36.7cM), D17Mit220 (41.8cM), D17Mit74 (44.0cM), D17Mit128 (44.0cM), D17Mit258 (45.2cM), D17Mit122 (47.4cM), D17Mit41 (48.5cM), D17Mit189 (48.5cM), D17Mit129 (50.7cM), D17Mit1 (51.8cM). Several new markers were found by using MSM BAC-ends sequence database (The MSM-B6 Comparative Map, RIKEN http://stt.gsc.riken.jp/msm/). D17C101 (20.05Mb), D17T403 (64.57Mb), D17T405 (65.95Mb), D17T406 (66.42Mb), D17T407 (69.73Mb), D17T302 (71.98Mb), D17T301 (73.90Mb), D17T308 (78.26Mb) in Figure 5.1 are part of those custom made markers.

Genomic DNA of each animal was prepared from the tail or ear. Polymerase chain reaction (PCR) was used to detect sequence length polymorphisms. PCR primer information of the above MIT microsatellite markers were obtained from the database (BROAD Institute of MIT and Harvard, http://www-genome.wi.mit.edu/), and those primers were obtained from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). Length polymorphisms were detected by agarose gel electrophoresis with 3% agarose in 1 x TAE buffer, visualized by ethidium bromide staining.

Behavioral and Brain morphological analysis

A panel of congenic mouse strains were characterized in open-field test (OF), elevated plus-maze test (EPM), and social interaction test (SI). An animal experienced OF and EPM in the same order on the two separate days. A new set of animals was used for the SI. Several strains were picked up for the histological analysis. Figure 5.1 shows congenic strains subjected to each test and number of animals tested in each strain. All animals were tested at the age 9 to

10 weeks. Detailed methods for each behavioral test and for histological analysis were described in Chapter 3 and Chapter 4, respectively.

Further behavioral characterization was performed in two selected congenic strains, C10 and C15, in the home-cage activity test and two kind of fear conditioning tests. In the home-cage test, the movements of an animal in the home-cage were recorded by infrared sensor ACTIVITY SENSOR (O'hara Co Ltd., Tokyo). Total activity for three days after one-day habituation to the new home-cage was measured as home-cage activity. Males and females aged 9 to 12 weeks were used in this analysis (for the number of animals used in this analysis, see Figure 5.5). For the method of cue and context fear conditioning, see Chapter 4. Males aged 10 weeks were used in this analysis (for the number of animals, see Figure 5.6).

Statistical Analysis

Data analysis was performed using the SPSS version 14.0J software packages. ANOVA techniques were used for the comparisons of tested strains and sex differences. For the strain comparison, two kinds of t-test were adopted depending on the data: t-test with Bonferroni correction and Dunnet's t-test. A principal component analysis was also performed using SPSS version 14.0J software packages.

5.3 Result

5.3.1 Behavioral analysis of F1 progeny made from C57BL/6J and B6-17MSM

Because 2-way ANOVA revealed no significant strain×sex interaction in all measurements [$F(3,196) \leq 1.83$, $p>.14$], I combined the data of both sexes for this analysis. Two reciprocal F1 crosses were compared by student's t-test to examine the effect of the cross, and I found significant differences in the OF ambulation, central ambulation, stretching, and EPM ambulation ($p<.05$). Thus, these reciprocal crosses were considered as separated group for this analysis. One-way ANOVA showed that there are significant effects of strain in all measurements [$F(3,203) \geq 6.79$, $p<.01$] except jumping [$F(3,203) = 2.101$, $p=.09$] and % of open-arm time [$F(3,126) = 0.543$, $p=.65$]. Parental C57BL/6J and B6-17MSM showed significant differences in most measurements. Two F1 crosses indicated complicated results, some measurements showed similarity to C57BL/6J while others are similar to B6-17MSM (Table 5.1). In the ambulatory activity in OF and EPM, (17×B6)F1 showed lower activity as B6-17MSM, while (B6×17)MSM exhibited slightly reduced but similar activity to C57BL/6J. Stretching was increased in (17×B6)F1 ($p=.07$), while (B6×17)MSM showed no differences from C57BL/6J. In contrast, pausing behavior was low in (17×B6)F1 as C57BL/6J and higher in (B6×17)F1 than C57BL/6J. Defecation was high in both reciprocal F1 crosses as B6-17MSM. In addition to these indices, I noticed that animals squeak as distress call when they were picked up by its tail with tweezers before or after the open-field test, and it seemed there are strain differences in this reaction. Parental C57BL/6J and B6-17MSM showed significant difference in this reaction; B6-17MSM shows higher squeaking (60% of animals) than C57BL/6J (20% of animals). In F1 hybrids, 50% of animals squeaking in the (B6×17)F1, while 30 % in (17×B6)F1.

5.3.2 Identification of the genetic loci related to brain ventricle size.

Figure 5.1 presents a panel of congenic strains established in this study and the number of animals used for the analyses. The number of hydrocephalus animals in each strain was also shown in this list. The appearance of hydrocephalus was observed in C1(c-31Mb) to C6(4-44Mb). The result of brain ventricle size in congenic strains is presented in Figure 5.2. Six congenic strains that cover whole chromosome were picked up for this analysis; C1(c-31Mb), C6(4-44Mb), C7(31-57Mb), C8(43-66Mb), C11(65-84Mb), and C14(80Mb-t). Dunnet's T-test revealed that C6 has larger ventricle size than C57BL/6J. Thus, there are genetic loci related to the ventricle size in the C6(4-44Mb) region. However, scatter plot of each individual seems to show increased distribution not only in C6(4-44Mb) but also in C1(c-31Mb) (Figure 5.2B). F-test for examination of equality of variances revealed significant differences of the distribution in C1(c-31Mb), C6(4-44Mb), and B6-17MSM from C57BL/6J ($p<.01$). Thus, there may be genetic loci related to the ventricle size in the C1(c-31Mb) region.

5.3.3 Behavioral analysis of congenic strains of B6-17MSM

Two-way ANOVA revealed that there was significant effect of strain in all behavioral measurements [OF indices: $F(15,480) \geq 2.01$, $p<.02$; EPM indices: $F(15,469) \geq 2.17$, $p< .01$; SI indices: $F(14,281) \geq 1.919$, $p<.03$]. The effect of sex was significant in OF stretching, rearing, face-washing, and contact number [OF indices: $F(1,480) \geq 4.13$, $p< .05$; SI index: $F(1,281) = 6.15$, $p<.02$] but no strain×sex interaction was found in any behaviors except EPM ambulation [$F(15,471) = 2.53$, $p<.01$]. Thus, I combined both sexes for the post-hoc comparison in this analysis, and Dunnet's t-test was performed to compare each congenic strain with C57BL/6J. Figure 5.3 shows the result of open-field ambulation and stretching behavior. Almost all congenic strains showed reduced ambulation than C57BL/6J. Ten congenic strains exhibited significantly lower ambulation than C57BL/6J ($p<.01$), and only C2(c-34Mb), C3(c-44Mb), C4(c-57Mb) and C13(78Mb-t) did not show significant differences from C57BL/6J. In stretching,

significant increase was observed in C6(4-44Mb), C12(65Mb-t), C13(78Mb-t), and C15(83Mb-t) and suggestive in C14(80Mb-t).

The result of all behavioral measurements in a panel of congenic strains was summarized in Table 5.2. Significant decreases of EPM ambulation were also found in the almost all strains except C2(c-34Mb). Percentage of central OF ambulation was increased in C2(c-34Mb) and C6(4-44Mb). C6(4-44Mb) also showed increased open-arm entry in the EPM. Pausing were significantly higher in the congenic strains with telomeric substitution, C13(78Mb-t), C14(80Mb-t), C15(83Mb-t), and C10(63-70Mb) than C57BL/6J. Squeaking was significantly increased in C4(c-57Mb), C5(c-66Mb), C8(43-66Mb), and C9(44-74Mb). C8(43-66Mb) also showed higher grooming than C57BL/6J. For the social interaction behavior, none of these congenic strains exhibited the extended duration of SI contact as in B6-17MSM. C15(83Mb-t) rather showed significant decrease of the number of SI contact.

5.3.4 Principal component analysis

Table 5.3 shows phenotypic and genetic correlations between each measurement of OF and EPM. To identify the relationship between OF variables in the B6-17MSM congenic strains, principal component analysis was performed on the genetic correlations. Three factors were extracted from the principal component analysis with an eigenvalue higher than 1. However, Factor 3 that explained 9.3 % of variance had strong loading only from one behavioral measurement, leaning. Thus, this factor was considered as unique factor for the leaning behavior, and I decided to exclude Factor 3 from this analysis. Table 5.4 shows the factor loadings obtained from a principal component analysis with Valimax rotation. Two factors accounted for 70.1% of total variance. Factor 1 had positive loadings from the ambulation and center part preference, and negative loadings from the grooming behaviors and pausing. Factor 2 had positive loadings from the leaning and rearing, and negative loadings from the defecation and stretching.

5.3.5 Behavioral analysis of C10(63-70Mb) and C15(83Mb-t) congenic strains: Home-cage activity and fear conditioning

For further analysis, I focused on two congenic strains, C10(63-70Mb) and C15(83Mb-t). These two strains have comparatively short MSM regions and showed reduced ambulation and rearing. C15 also exhibited increased stretching. The MSM regions of C10 and C15 were independent of hydrocephalus-like phenotype, it was thus expected that there are genetic loci directly related to those behaviors. Figure 5.4 shows a list of genes within the C10 and C15 regions (BLAST Build 36.1, NCBI). C10 has a substituted MSM region of 7.10 Mb or smaller, which contains 35 genes, and C15 has a substituted MSM region of around 10.94Mb, which contains 78 genes.

To characterize these two congenic strains, I conducted further behavioral analyses: home-cage activity test and cue fear-conditioning test. Figure 5.5 represents the result of the home-cage activity (this test was done by Dr. Nishi and Ms. Ishii at MGRL). Two-way ANOVA revealed significant effects of strain [$F(3,117) = 13.64$, $p<.001$] and sex [$F(1,117) = 25.66$, $p<.001$]. Strain comparisons by t-test with Bonferroni correction were performed in each sex, and indicated significant increase of home-cage activity in C10 for both sexes compared to other three strains ($p<.01$).

Two kinds of fear conditioning test, cue and context, were also performed. The results of cue-fear conditioning test are presented in Figure 5.6A,B. Summation of immobility behavior for 5 min tests were subjected to T-test with Bonferroni correction, and it revealed that C15 showed significantly higher immobility than C57BL/6J as B6-17MSM, while C10 behaved similarly to C57BL/6J. Repeated one-way ANOVA revealed significant effect of strain × time interaction [$F(18,210) = 2.326$, $p<.01$], and the immobility of C15 was significantly higher than C57BL/6J at the last two minutes. The temporal pattern of C10 was identical to that of C57BL/6J. The result of context-conditioning is indicated in Figure 5.6CD. T-test with Bonferroni correction revealed no strain differences in the summation of immobility behavior for 5 min context fear conditioning tests. Two-way ANOVA showed significant effect of time [$F(4,148) = 22.198$,

p<.0001] but no effect of strain × time interaction [F(12,148) = 1.26, p=.25].

5.3.6 Analysis of sub-lines of C15(83Mb-t) strain

By the further screening of recombinants around the region of break points in C15 during the process of making a panel of congenic strains, I established other three lines of sub-line strains of C15, named C15a, C15b, C15c (Figure 5.4). C15a has a wider substituted MSM region than C15, from D17Mit258 (80.85Mb) to the telomere, and C15b possesses a narrower MSM region than C15, from D17Mit189 (85.10Mb) to the telomere. C15c has a substitution from D17Mit258 (80.85Mb) to D17Mit221 (90.00Mb). Open-field test was conducted in these strains, and Figure 5.7 presents the results of open-field ambulation, rearing, stretching, and pausing. Two-way ANOVA revealed there are significant effect of strain for all measurements [$F(5,217) \geq 5.05$, p<.001]. Sex difference was significant in the rearing [F(5,217) = 7.64, p<.01], but strain×sex interaction was not detected in any measurement [$F(5,217) \leq 1.73$, p>.13]. Thus, sexes were combined in this analysis again. Dunnet's t-test indicated that all the three sub-lines of C15 showed decreased ambulation and pausing like C15 strain as compared with C57BL/6J. Stretching was significantly higher in C15 and C15a. The temporal change of stretching showed drastic reduction of this behavior (Chapter 2), but B6-17MSM showed this behavior later in the session (Figure 5.7). C15 and C15a also showed increased stretching in the later part of the session. C15b had a large variance but tended to show increased stretching. C15c did not show any difference from C57BL/6J. Pausing was significantly increased in C15, C15a, and C15b but not in C15c.

5.4 Discussion

5.4.1 Maternal effect in the reciprocal F1 intercross

By analyzing the reciprocal F1 crosses of C57BL/6J and B6-17MSM, each cross showed discriminating results. (17×B6)F1 was similar to B6-17MSM, whereas (B6×17)F1 showed modest scores between C57BL/6J and B6-17MSM in the most behavioral measurements. As both F1 groups have exactly the same genetic composition, it was considered that having B6-17MSM mother caused to the pups to behave mostly as B6-17MSM, while having B6-17MSM father was less effective. What is the cause of this maternal effect? Was this influenced by genetic or environmental effect?

The effect of early environment is very important factor for the emotional reactivity as genetic effect. There are multiple environmental factors that cause individual differences of emotionality (Lathe, 2004), and one of the important factors for the individuality is the maternal effect. Both prenatal stress (giving stress to the pregnant female) and postnatal stress (giving stress to the neonatal infant) alter the emotionality of offspring when they become adults in many animals including human (Romeo et al., 2003; Thompson, 1957; Vallée et al., 1997). It is known that there are large strain differences in the maternal behavior (Carlier et al., 1982; Shoji and Kato, 2006), and these differences could influence emotional reactivity of the pups in the mouse (Liu et al., 1997; Caldji et al., 1998; Francis et al., 1999; Calatayud and Belzung, 2001). This effect of maternal behavior could continue to affect the behavior of grand offspring (Carola et al., 2006). To examine the effect of maternal behavior for the F1 hybrid of B6-17MSM, further study with cross-fostering between C67BL/6J and B6-17MSM will be required.

An alternative possibility is genomic imprinting. It has been reported that there are a group of genes with genomic imprinting on chromosome 17; three maternally expressed genes (Slc22a2, Slc22a3, and Igf2r) and one paternally expressed non-coding RNA (Air) (Sleutels et al., 2003).

These imprinting genes on chromosome 17 of MSM may express differentially from C57BL/6J, and the expression difference may affect the behavior. This imprinted gene cluster is positioned near the centromere side, from 12.26Mb to 12.61Mb. In this case, cross-fostering procedure will not lead any behavioral change for each reciprocal F1 crosses.

In contrast, both F1 showed the same defecation and rearing behavior as B6-17MSM, and thus QTLs for these behaviors are suggested to be dominant effects and are not affected by the maternal effect. Pausing and squeaking behavior were also exceptional. In these behaviors, (B6×17)F1 consistently showed closer scores to B6-17MSM, while (17×B6)F1 was similar to C57BL/6J. Thus, having B6-17MSM father changed pups to behave as B6-17MSM but B6-17MSM mother does not. This result may suggest the paternal effects on some behaviors.

5.4.2 Identify the genetic loci related to the brain ventricle size

In this study, I identified one significant genetic locus related to the brain ventricle size between D17Mit164 (3.88 Mb) to D17Mit34 (34.34 Mb) region of chromosome 17 from the result of C6(4-44cM) and C7(31-57Mb). Because C1(c-31Mb) also showed increased individual divergence of ventricle size within the strain, there may be genetic loci within the C1 region of MSM. This is only speculation, however there may be two loci related to the ventricle size in the overlapping region between C1(c-31Mb) and C6(4-44Mb) and the C6 specific region around D17Mit81 (30.63 Mb) position, respectively. It is suggested that those two genetic loci have additive effect to the phenotype, and thus C6 showed a stronger phenotype. As hydrocephalus was occasionally observed in the congenic strains of C1 to C6, these two genetic loci relating to ventricle size may also be associated to the hydrocephalus phenotype. Recently, the whole genome sequence of MSM was characterized and SNP density between C57BL/6J and MSM can be explored (Mammalian Genetics Laboratory, NIG). The data indicated that there are regions of high SNP density between C57BL/6J and MSM on chromosome 17 around 31.0 Mb (SNP density: 5%) and 49.5 Mb (3%). The high density of SNPs around 31.0 Mb may have relation

with the causative gene of the hydrocephalus phenotype. The genetic loci for hydrocephalus also overlap with the t-complex region, which comprises the proximal to 20 cM of mouse chromosome 17. T-complex contains many genes affecting spermatogenesis and embryonic development (Silver, 1985). Although it has not been reported that C57BL/6J or MSM carry t-complex region so far, polymorphisms in this region may relate to the spermatogenesis or embryonic development. Thus, high-density of SNPs in this region may lead to some developmental defect and made B6-17MSM to have a hydrocephalus-like phenotype.

5.4.3 Multiple genetic loci related to the behavioral indices on chromosome 17

Behavioral analysis of a panel of congenic strains derived from B6-17MSM revealed several genetic loci related to the behaviors. Some behaviors can be explained by one to two genetic locus/loci; however, other behaviors have multiple genetic contributions even within the chromosome 17. Ambulation in the open-field and elevated plus-maze are the latter case. Almost all congenic strains showed reduced novelty-induced activity. In this kind of case, artificial factor should be taken into consideration, for example tested season, date, order, and experimenter manipulation. However, the tests were done by semi-blind and random order, and several strains including the control C57BL/6J and B6-17MSM were analyzed in one day test. Therefore, this possibility could be rejected, and it is concluded that there are multiple genes related to the novelty-induced activity on the chromosome 17.

For the stretching behavior, I identified two genetic loci on chromosome 17. All strains that have the substituted MSM region around the telomere region increased the stretching, and thus there is a genetic locus related to the stretching behavior between D17Mit122 (82.96Mb) to the end of telomere. Another congenic strain C6(4-44Mb) showed increased stretching. However, this was considered to have complex effect. C6(4-44Mb) exhibited increased stretching, while strains that have longer MSM region including the C6 region, C4(c-57Mb) and C5(c-66Mb), showed no differences from C57BL/6J. C6(4-44Mb) also showed other phenotypes, increased OF

center preference and EPM open-arm entry. These results suggested that there are genetic locus/loci related to those behavioral phenotypes in the C6 region, and also the existence of a suppressive or interactive locus that negates the effect of the C6 locus in the extended MSM region of C4. However, this C6 region also contained the genetic loci associated with hydrocephalus- like phenotype, and there is possibility that their behaviors were affected by the brain defect.

Analysis of congenic strains revealed that there is genetic contribution for the distress call, squeaking, when the animal was picked up by its tail with tweezers, and the genetic locus was identified between D17Mit36 (44.33Mb) and D17Mit20 (56.91Mb) region. The squeaking was measured as one of the indices of wildness in the several mouse strains including wild-derived strains, and there were large strain differences (Wahlsten et al., 2003). However, they found lower squeaking during handling of wild-derived strains than laboratory strains. This behavior thus did not correlate with the wildness, but at least, it may be associated with the reactivity to the handling.

Because B6-17MSM showed more elongated social interaction behavior than C57BL/6J, I expected to be able to identify a genetic locus or loci associated with the social interaction behavior from this study. However, none of congenic strain showed similar level of social contact to B6-17MSM. Although it was not significant, many strains rather showed reduced social contact duration compared with C57BL/6J. Therefore, this result suggests that genetic interactions of two or more genes within the chromosome 17 are required to increase social interaction behavior. Each congenic strains may have only a part of those interacting genes, which have no or very small effect by itself, and thus observable phenotypes may not appear. Genetic correlation indicated moderate positive correlation between OF ambulation and SI contact number ($r = .45$) and also between OF ambulation and contact duration ($r = .30$). The negative correlation between OF ambulation and contact duration found in Chapter 3 is considered as a pseudo-relation from this result, and these behaviors have different genetic

bases rather than sharing a pleiotropic gene for reduced novelty-induced activity and increased social behavior.

5.4.4 Principal component analysis in the congenic strains.

Principal component analysis revealed there were two factors underlying the open-field behavior of B6-17MSM congenic strains. Factor 1 had loadings from both ambulation and central preference, and thus this factor is associated with "locomotor activity" and "thigmotaxis" factors (Chapter 3). Factor 2 had loadings from stretching and rearing, and corresponded to "anxiety tension state" factor. Because B6-17MSM showed significant effects in "locomotor activity" and "anxiety tension state" factors but not in "thigmotaxis" factor (Chapter 3), it was acceptable that principal component analysis on the B6-17MSM derived congenic strains failed to extract "thigmotaxis" factor. Furthermore, the result of congenic strains proved the existence of multiple factors underlying open-field behaviors and the verisimilitude for the factor structure extracted from wild-mouse strains and a panel of consomic strains.

5.4.5 Behavioral characterization of C10(63-70Mb) and C15(83Mb-t) congenic strains

C10 and C15 are congenic strains that have comparatively short MSM region where is independent of the brain ventricle size phenotype. C10 showed reduced novelty induced ambulation and rearing, and increased pausing compared to C57BL/6J. In addition to the same behavioral changes as C10, C15 also showed increased stretching behavior. Thus, it is considered that C10 has the "locomotor activity" factor, and C15 associated with both the "locomotor activity" and "anxious tension state" factors. QTL studies using B6-Chr$^{A/J}$CSSs reported one QTL related to reduced duration in the light chamber of the light-dark box test around 51.2cM on chromosome 17 (Singer et al., 2005). Also, another QTL was reported around 45.3cM of the chromosome 17 which was associated with the open-field activity in LSXSS RI (Radcliffe et al., 1998). The substituted MSM region in C15 congenic strain was 48.5 cM to

telomere, and thus the same factor may be identified in several independent studies. For the C10 region, 31.8 cM to 36.2cM, there are also reports of the existence of the QTL for the forced swim test (Yoshikawa, 2002) and contextual fear conditioning (Owen, 1997).

To characterize C10 and C15 in more detail, home-cage test and fear conditioning tests were performed. The result indicated that C10 and C15 are characterized differently in those behaviors. Increased home-cage activity was observed in C10, while C15 exhibited the same levels of activity as C57BL/6J and B6-17MSM. In contrast, C10 did not change their fear responses in the cue-fear conditioning as C57BL/6J, whereas C15 showed increased fear response as B6-17MSM. I also conducted the context-fear conditioning, and found that the fear responses of both C10 and C15 did not differ from C57BL/6J. It was expected that C10 showed changed fear response due to the same QTL as the one found in the study for the fear conditioning using BXD recombinant strains (Owen, 1997). However, interestingly, the genes on C10 region were not related to the fear conditioning in the MSM background. In contrast, C15 showed increased fear response in cue-fear conditioning but not in context-conditioning; the same pattern as parental B6-17MSM (Chapter 4). Thus, it is suggested that this region contains the gene/genes related to cue- specific fear response.

This result revealed that the C10 and C15 have different effects on the behavioral factors, "locomotor activity" and "anxious tension state". Thus, these factors on these strains may be associated with the different genetic and biological pathways. Further analysis of these congenic strains will give us an idea to understand the genetic and biological mechanisms related to this multiple dimension of emotionality. Ongoing study is examining the change of the stress hormone, corticosterone, upon the stress on C10 and C15.

5.4.6 Analysis of sub-lines of C15(83Mb-t) congenic strains

Because all of the C15 sub-lines showed reduced ambulation and rearing, it was expected that at least single genetic locus for these behaviors were positioned between D17Mit189 (85.10Mb)

and D17Mit221 (90.00Mb). In contrast, stretching behavior was significantly higher in C15 and C15a than C57BL/6J. The stretching in C15b had large variance, but they also tended to show increase of this behavior. I thus suppose that there is a genetic locus related to the stretching behavior between D17Mit1 (88.13Mb) to the end of telomere. The pausing may have affected by the same genetic locus. This result indicated that the genetic loci related to the open-field ambulation and stretching were separated even in the C15 region. Thus, this result confirmed that the "locomotor activity" and "anxious tension state" are independent trait, and suggested that those factors have different genetic and biological pathways. It must be interesting to examine for example by using microarray technique whether they have common genetic pathway in part or totally distinct pathways,.

5.4.7 Summary

B6-17MSM showed many interesting phenotypes related to emotionality, and also hydrocephalus-like phenotypes. By analyzing a panel of congenic strains of B6-17MSM, I successfully revealed novel genetic loci associated with the brain ventricle size on chromosome 17. Behavioral analysis also identified several genetic loci related to each behavior. Social interaction behavior was prominently high in B6-17MSM, however, no congenic strains showed increased duration of social contact. It was supposed that there are interacting epistatic genes to induce social interaction on this chromosome.

The analysis of reciprocal F1 intercross revealed maternal effects on their behavior. It is very difficult to determine whether the genetic loci are related to the maternal behavior or individual emotionality. However, the effect of maternal behavior on the pups may give us a hint to understand the effect of the genetic loci found in this study. The cross-fostering analysis will be required for the future study.

So far, I conducted the factor analyses of open-field measurements in the wild-derived strains and consomic mouse strains, and confirmed that there are "locomotor activity", "thigmotaxis",

and “anxious tension state” factors related to their behaviors. Behavioral analysis of congenic strains also revealed the existence and independences of those factors. Differences of home-cage activity and fear conditioning between C10 and C15 suggest that the “locomotor activity” and “anxious tension state” are independent traits and under different genetic and biological pathways.

CHAPTER 6

Genetic analysis of aggressive behavior in B6-15MSM consomic strain of mouse

6.1 Introduction

Aggression is also very important emotion and evolutionarily ancient behavior. Aggressive behaviors are separated into two types, offense and defense, which can be distinguished by the motor pattern, function, and severity (Adams, 1979, Blanchard and Blanchard, 1988). Offensive behavior occurs between members of the same species, and it is regarded as behaviors for acquisition and defense of territory, social status, and vital resources such as food, shelter, or mates. In contrast, defensive behavior elicits toward the opponent, including the same species and other predators that harm the life of one's self or one's progeny, and thus causes serious injury than offensive attack (Masxon and Canastar, 2003). In laboratory tests of aggression, mice show predominantly offense behaviors (Didier-Erickson et al., 1989).

Genetic contribution to the offensive aggressive behavior has been confirmed in many species (Sluyter and Schalkwyk, 2003). In mouse, large strain differences have long been reported (Scott, 1942, Ginsburg and Alee, 1942). Selective breeding studies have established the high and low aggression lines of mouse for intermale aggression and also interfemale aggression in wild mice (Lagerspetz and Lagerspetz, 1971; van Oortmerssen and Bakker, 1981; Hyde and Sawyer, 1980). Recent studies with gene-altered mice have successfully elucidated several genes related to aggressive behavior (Miczek et al., 2001; Maxson and Canastar 2003; Ogawa et al., 2004). However, the attempts to identify naturally occurring genetic variation related to aggressive behavior have not been sufficiently done yet. Several studies showed importance of Y chromosome in intermale aggression in reciprocal F1 intercross or by making Y chromosome

congenic strain in mouse and rat (Guillot et al., 1995; Maxon et al., 1989; Roubertoux et al., 1994; Toot et al., 2004). Haplotypes in *t* region of chromosome 17 have also been reported for association to aggressive behavior (Lennington et al., 1996). There are only one QTL study with whole genome scanning has been reported QTL for mouse aggressive behavior, and they identified two loci on chromosome 10 and X are identified by using N2 backcross of NXB/B1 and A/J mouse (Brodkin et al., 2002). Limitation of QTL studies for aggressive behavior is due to its cost of time and enormous effort to assess aggressive behavior, as QTL study requires many, at least several hundred, animals for the statistical significance. Again, there are advantages of consomic strains (CSSs) for the forward genetics approach to aggressive behavior (Brodkin, 2005).

Mouse is a typical colonizing species (Micheck et al., 2001; Sluyter et al., 1996 for review). In the natural circumstances, they form social units that have been described as demes (breeding units) (Lewontin and Dunn, 1960). Deme is composed of a dominant male, several females, pre-pubertal juveniles, pups, and subordinate males (Reimer and Petras, 1967). When males mature, it begins to leave their parental deme, and these young adults and subadults form an itinerant population. Breeding males mark, patrol, and defend their territories, and exclude or dominate other males (Crowcroft and Rowe, 1962; Hurst, 1987: Poole and Morgan, 1976). Aggressive behaviors in the wild animals are extremely frequent and ranged wider in the behavioral variation compared to laboratory animal (de Boer et al., 2003). Wild mice are pugnacious by its nature, but laboratory mice reduced the aggression during the course of domestication. It is also reported that housing condition causes the inhibition of aggressive behavior in laboratory mouse. In laboratory, several male mice were forced to cohabit, and thus male learned inhibition against intermale fighting (Scott, 1966; O'Donnell, 1981).

MSM/Ms was derived from Japanese wild mice and inbred at NIG since 1978. Although it has been inbred in laboratory for more than 60 generations, the mice still retains considerable aggression. When we keep males of littermate of MSM in the same cage after weaning, one

aggressive despot often attacks and sometimes kills the other males (personal observation). In Chapter 3, I conducted social interaction test for B6-Chr^{MSM}CSSs and parental C57BL/6 and MSM. In the analysis, I only measured the duration and number of social contact between animals with an automatic test apparatus. However, during the experiments, males of MSM actually showed aggressive behavior while C57BL/6 merely or never showed that kind of behavior in that test. Ongoing collaborative study for behavioral components during this test with Dr. Kazuya Tomihara at Kagoshima University showed increased aggressive behavior in MSM and also in some CSSs. Therefore, B6-Chr^{MSM}CSSs offers the possibility to identify genetic loci related to the aggressive behavior.

In this chapter, I focused on one CSS, B6-15MSM, which showed aggressive behavior during the social interaction test. To investigate the aggression of B6-15MSM properly, I conducted the resident-intruder test that is more specialized test for aggressive behavior. Resident-intruder test uses animal's nature to defense their territory toward an intruder male of same species. By performing the reciprocal choice of intruder in this procedure, the effect of opponent on their aggressive behavior was also examined in this study. Genetic analysis was conducted in F1 hybrid. Congenic strains of B6-15MSM were also established to identify the genetic loci related to aggressive behavior of B6-15MSM. This behavioral analysis of congenic strains is based on preliminarily data. However, I will introduce the data to show the possibility of identification of the genetic locus/loci associated with aggressive behavior on chromosome 15.

6.2 Method

Animals

C57BL/6-Chr15^{MSM} (abbreviated as B6-15MSM), which has a substituted chromosome 15 from MSM, was established at the NIG (Mishima, Japan), and C57BL/6J (occasionally abbreviated as B6) was purchased from CLEA Japan, Inc (Tokyo, Japan) and bred at NIG. The mice were weaned around 3 weeks of age and housed in same sex groups in standard sized plastic cages on wood chips. Ten days before the test, they were weighed and separated into isolated cage. One testing set consists of 4 animals, one resident and three intruders, and the heaviest animal was chosen as resident. The resident animal was housed in a large-sized home-cage (22 × 32 × 13.5 cm) and the intruder animals were kept in small-sized home-cages (14 × 35 × 13.6 cm) individually until the test. Males that were naïve to any behavioral test were tested at the age of 10 weeks. All animals were maintained at NIG under the 12-h light/dark cycle (light from 8:00 to 20:00) in a temperature-controlled room (23±2°C). Food and water were available *ad libitum*. Mice were maintained according to NIG guidelines, and all procedures were carried out with approval by our institutional animal care and use committee.

Resident-intruder test

Resident-intruder test was conducted for three times every other day. The resident mouse was tested in his home-cage against an intruder animal for 15 min. Each resident mouse was tested against different intruder mice in three trials. Behaviors between resident and intruder were videotaped during the test, and behavioral observation was performed by recorded video data. All tests were carried out during the light period (16:00-20:00). The following test groups were analyzed in this study.

Homogenous pair test

The resident and intruder mouse consisted of the littermates of the same strain. One resident and three intruders made up one set, and 14 sets of B6-15MSM (abbreviate to 15*15) and 13 sets of C57BL/6J (abbrev. B6*B6) were tested.

Reciprocal pair test

The resident mouse and intruder mouse had different genotypes. Pairs consisted of C57BL/6J resident versus B6-15MSM intruder (abbrev. B6*15), and other pairs consisted of B6-15MSM resident versus C57BL/6J intruder (abbrev. 15*B6). Nine sets of animals were tested in each group.

Urination effect test

To examine the effect of chemical signal of B6-15MSM, the effect of urine in aggressive behavior of C57BL/6J was examined. Urine was collected from 10 animals per strain and mixed for each B6-15MSM and C57BL/6J. The mixed urine was stored at 4°C up to 1 month. Animals for urine collection were different from animals for the behavioral tests. 60 µl of the urine mixture was applied to cotton, and put on the neck and the upper base of the tail of the intruder mouse. C57BL/6J mouse was used for both resident and intruder, and 8 sets of animals for each of the B6-15MSM urine and C57BL/6J urine groups were tested. In the urine test, one testing set consisted of two intruders toward one resident mouse.

Analysis of F1 progeny and congenic strains of B6-15MSM

F1 mice were generated by crossing a B6-15MSM female and a C57BL/6J male (named (15×B6)F1). Reciprocal F1 mice were also made by crossing a C57BL/6J male and a B6-15MSM female (named (B6×15)F1). Homogenous pair test was performed, and 10 sets of animals were tested for each cross. In the F1 analysis, each resident mouse was tested against different

intruder mice in three trials. Congenic strains of B6-15MSM were also subjected to homogenous pair test. This is just beginning of analysis, and sufficient data have not collected yet. Here, I will show preliminary data in relation to possibility of identification of genetic loci by using B6-15MSM congenic strains. The number of animals analyzed is shown in Figure 6.7. In the analysis of congenic strains, one testing set consisted of two intruders toward one resident mouse.

Behavioral analysis

Time sampling method for one second interval was adopted, and presence or absence of each behavior was recorded as 1/0 for each 1-sec period. The behaviors collected included the following 7 behavioral items by van Abeelen (1963).

[Non-aggressive social behavior]

Sniffing: sniffing several body parts of other mice, especially around the nose and anogenital region.

Grooming: grooming fur of other mice. Behavior in which an animal mounts the other individual was also included in this item.

[Aggressive behavior]

Tail-rattling: flicking its tail often with the pounding sound.

Attack: biting, lunge, wrestling, and offensive lateral attack were included.

Chasing: one mouse races after the other and bites it at the lower back, the tail, or the hind-legs

Upright: offensive upright posture displayed in both mice concurrently, including boxing behavior.

Submission: rears on its hind legs, draws one fore-leg close to the body, extends the other stiffly, remains motionless, and squeals when touched by the other mouse.

Non-aggressive behaviors were exclusively counted in each bin (1 second) but were not counted when they co-occurred with aggressive behavior in the same bin. On the other hand, items of aggressive behavior were counted nonexclusively, and multiple items were put in the same time bin. Latency of attack (time to the first attack) was also measured from this data.

Congenic mouse strains of B6-15MSM

Detail of establishment of congenic strains from B6-15MSM were almost the same as the case of B6-17MSM (Chapter 5). A panel of congenic strains established in this study are listed in Figure 6.6. The following MIT microsatellite markers, which differ between C57BL/6J and MSM (MMDBJ, NIG), were chosen for the genotyping in constructing the congenic strains; D15Mit174 (0cM), D15Mit224 (4.4cM), D15Mit111 (13.1cM), D15Mit5 (17.5cM), D15Mit121 (24.0cM), D15Mit104 (29.5cM), D15Mit105 (38.3cM), D15Mit261 (42.6cM), D15Mit73 (50.3cM), D15Mit244 (56.8cM), D15Mit77 (61.2cM), D15Mit40 (65.6cM).

Statistical Analysis

Data analysis was performed using the SPSS version 14.0J software packages and StatView version 5 (SAS Institute Inc). Repeated one-way ANOVA was performed to examine the trial-by-trial change of aggressive behavior and it's interaction with strain. To consider the effect of the genotype of resident and intruder, the result of the homogenous and reciprocal pairs were subjected to Two-way ANOVA. Two-way ANOVA was also performed to examine the behavior of each type of animals (resident and intruder) and its interaction with the pair (two homogenous and two reciprocal pairs). Strain comparison was performed by Student's t-test, and multiple comparisons were conducted by either t-test with Bonferroni correction or Dunnet's t-test depending on the data.

6.3 Result

6.3.1 Homogenous pair test in B6-15MSM and C57BL/6J

Pairs that showed the attack in B6-15MSM were 14, 12 and 11 pairs for trial 1, 2 and 3, respectively. Whereas, C57BL/6J exhibited the attack behavior in 4, 5, and 9 pairs for trial 1, 2 and 3, respectively. Figure 6.1A-C shows the changes in the frequency of attack, tail-rattling, and latency of the first attack during three trials. Repeated one-way ANOVA revealed that the effect of strain×trial interaction was significant in the frequency and the latency of attack [$F(2,46) \geq 3.85$, $p<.05$], and suggestive in the tail-rattling [$F(2,46) = 2.85$, $p=.07$]. B6-15MSM showed trial by trial reduction of the attack and tail-rattling, whereas C57BL/6J indicated increase in the attack and tail-rattle behavior. Attack latency showed the opposite pattern in both strains. Frequency of the attack was significantly higher in B6-15MSM than C57BL/6 at the first trial, but did not differ in trial 2 and 3. The tail-rattling was also more frequent in B6-15MSM than C57BL/6 at the first and second trial, but no significant differences in last trial. The attack latency was significantly longer in C57BL/6 than B6-15MSM in trial 1 and 2. Other indices also exhibited similar pattern, significant differences in the trial 1 and 2. Therefore, behaviors in trial 1 and 2, but not trial 3, were suitable to represent differences of B6-15MSM and C57BL/6J. Figure 6.1 D-F shows combined data of trial 1 and 2 for the all measurements. Student's t-test revealed significant effect of the strain in all measurements ($p<.01$) except the upright ($p=.06$) and chasing ($p=.66$). B6-15MSM showed high frequency of aggressive behaviors, whereas C57BL/6 exhibited higher frequency of non-aggressive social behaviors than B6-15MSM.

6.3.2 Reciprocal heterogeneous pair test

To examine the effect of intruders, reciprocal pair test was conducted. Because the result of

trial 3 in homogenous test was not informative for the comparison of two strains, I used the data of trial 1 and 2 in this statistical analysis. Both homogenous and reciprocal pairs were subjected to two-way ANOVA to examine the effect of the genotype of the resident and intruder, and revealed significant effect of the intruder in all measurements [$F(1,86) \geq 5.52$, $p<.03$] except the chasing [$F(1,86) = 0.07$, $p=.79$]. Effect of the resident was significant in the sniffing [$F(1,86) = 4.44$, $p<.05$] and suggestive in the attack frequency [$F(1,86) = 3.22$, $p=.08$] but not significant in other indices [$F(1,86) \leq 2.52$, $p>.12$]. Resident×intruder interaction was significant in the attack latency [$F(1,86) = 6.74$, $p<.02$] and suggestive in the attack frequency [$F(1,86) = 3.125$, $p=.08$], but not significant in other measurements [$F(1,86) \leq 2.06$, $p>.16$]. This result indicates the significance of the effect of intruder but small or no effect of resident in most measurements. The attack observed in 3 pairs of 15*B6 for both trials, and in 5 and 7 pairs of B6*15 for trial 1 and 2, respectively.

Figure 6.2 shows the comparison of the results for homogenous and reciprocal pairs. T test with Bonferroni correction showed significantly increased tail-rattling in 15*15 and B6*15 compared to 15*B6 and B6*B6 ($p<.02$). A similar pattern was observed in the upright and submission, but the differences in reciprocal crosses were not significant. Frequency and latency of the attack was significantly different only in 15*15 compared to other three pairs, and 15*15 showed increased attack in short latency. Sniffing had a similar pattern as attack latency.

For investigating the behavioral differences between the resident and intruder in the homogenous pairs and reciprocal pairs, behaviors of each resident and intruder were separately analyzed (Figure 6.3). Two-way ANOVA was performed to examine the effect of the type (resident or intruder) and its interaction with the pairs in the grooming, submission, and tail-rattling (Figure 6.3A-C). Significant effect of type was observed only in the grooming [$F(1,172) = 11.18$, $p<.001$] and the resident showed higher grooming toward the intruder. Type×pair interaction was not significant in all the measurements [$F(3,172) \leq 0.15$, $p>.14$]. I also examined which animal started the first attack (Figure 6.3D). First attack was defined from

which type of animal, resident or intruder, showed aggressive behavior adjacent the first attack. If it was indistinguishable, I gave a point to both animals. The resident tended to start the first attack in both homogenous pairs. In contrast, the intruder tended to start the first attack in both reciprocal pairs. The resident of C57BL/6J showed the first attack at almost the same frequency irrespective of the intruder, while the resident of B6-15MSM greatly changed the tendency of starting the attack depending on the intruder.

6.3.3 Urination effect test

Because the effect of the intruder was apparent in some aggressive behaviors, especially tail-rattling, I next examined the cause for the effect of the intruder. The mouse mostly uses chemical signals for the social communication, and such social odors are largely produced in urine (Vosshall, 2005). Thus, I examined role of the chemosensory signals contained in urine for the effect of the intruder. Urine was collected from 10 animals of each C57BL/6J (B6-urine) and B6-15MSM (15-urine), and was applied to the C57BL/6J intruder. As this study has been only done with C57BL/6J, I report here the effect of urine on the aggressive behavior of C57BL/6J (Figure 6.4). Student t-test was performed to compare the effect of B6-urine and 15-urine on the intruder, but no significant effect was detected on any behavioral measurements ($p \geq .23$).

6.3.4 Aggressive behavior in F1 progeny made from C57BL/6 and B6-15MSM

Figure 6.5AB shows the changes of tail-rattling and attack latency during three trials in (15×B6)F1 and (B6×15)F1. Repeated 1-way ANOVA revealed that the effect of trial was significant in attack latency [$F(2,32) = 4.57$, $p<.02$] and suggestive in tail-rattle [$F(2,32) = 2.80$, $p=.08$]. There were no significant effects of trial×strain interaction [$F(2,32) \leq 2.32$, $p \geq .11$]. Both F1 intercrosses showed trial by trial reduction of tail-rattling and increase of attack latency; that is similarly to B6-15MSM.

Combined data of trial 1 and 2 were subjected to t-test with Bonferroni correction for

comparing the two types of F1 from reciprocal crosses, B6-15MSM, and C57BL/6J (Figure 6.5C-E). (15×B6)F1 showed significantly reduced sniffing, and increased tail-rattling, upright posture, and submission compared to C57BL/6J. This F1 also had significantly shorter attack latency than C57BL/6J. On the other hand, (B6×15)F1 showed more frequent tail-rattling and submission posture than C57BL/6J at the suggestive level, but as long attack latency as C57BL/6J and significantly longer than B6-15MSM.

6.3.5 Aggressive behavior in congenic strains of B6-15MSM

A panel of congenic strains established in this study are shown in Figure 6.6. The behavioral analysis is still ongoing, and 6 congenic strains that have been analyzed over 5 sets of animals are shown here as preliminary data; C4(c-58cM), C5(c-80cM), C7(58-80cM), C9(58-61cM), C10(43cM-t), and C11(73cM-t). The cumulative numbers of animals that showed the attack were 26 (0.93%) and 9 (0.35%) for B6-15MSM and C57BL/6J, respectively. The results of congenic strains were as follows; C4: 1 (0.07%), C5: 8 (0.57%), C7: 10 (0.63%), C9: 7 (0.70%), C10: 12 (1.00%), C11: 5 (0.42%). The results of the tail-rattling and attack latency are shown in Figure 6.7. Dunnet's T-test revealed that, as B6-15MSM, C10 showed shorter attack latency than C57BL/6J ($p<.001$). The tail-rattling was significantly higher in C9 ($p<.02$) and C10 ($p<.001$) and suggestively higher in C7 ($p=.09$) compared to C57BL/6J. Though it was not significant, C4 exhibited longer attack latency and fewer tail-rattling than C57BL/6J.

6.4 Discussion

6.4.1 Increased aggressive behavior in B6-15MSM

Several transgenic or knockout mice that showed altered aggressive behavior have been reported (Maxson and Canastar 2003; Miczek et al., 2001; Entrez Gene, NCBI). These genes include those for neurotransmitters, hormones, cytokines, enzymes, growth factors, and signaling molecules. However, no gene on chromosome 15 has been reported to be associated with intermale aggression until now.

Homogenous pair tests showed increased aggression in B6-15MSM compared to C57BL/6J. The former strain showed increased frequency of attack, tail-rattling, and submission as well as shortened attack latency. As a laboratory mice infrequently expresses aggressive behavior at the first encounter (King, 1957), I conducted three trials of encounter in this test. As expected, C57BL/6J showed gradual increase of aggressive behavior over trials, and gradual decrease of latency for the first attack. By contrast, B6-15MSM exhibited the strongest aggression at the first encounter, and the aggressive behavior gradually reduced trial by trial. At the third trial, B6-15MSM and C57BL/6J had no differences in their aggressive behavior any more. One possibility of this observed pattern in B6-15MSM is that B6-15MSM showed strong reactivity at the first encounter, and this emotional reactivity may connect to aggression. After habituation over three trials, their reactivity reduced, and aggressive behavior came down to normal level. The relationship between aggression and emotional reactivity has been discussed (Clement and Chapouthier, 1998). However, the conclusions were very controversial among the studies. Some studies offered the positive correlations between aggression and anxiety: more attacking males have a higher level of anxiety (Guillot and Chapouthier, 1996; Palanza et al 2001). Other studies suggested the negative correlations (Nyberg et al., 2003; Miczek and O'Donnell, 1980). B6-15MSM showed highly increased defecation in the open-field tests (Chapter 3), which

reflects elevated autonomic reactivity (Hall, 1934b). This strain also showed significant reduction in "locomotor activity" compared to C57BL/6J (Chapter 3). It is interesting to explore the relation between aggressive behavior and autonomic reactivity and/or "locomotor activity" in B6-15MSM. Congenic strains may give us further understanding of this relationship. In contrast, when parental MSM was examined in this test, they showed immobility and jumping over 15 min test period, and did not show any encounter and aggressive behavior between two animals (preliminary examination only in 2 pairs). This suggests that MSM evokes extremely strong emotional reactivity at the encounter which, in turn, interrupts the social behavior in MSM.

6.4.2 Effect of intruder for the increased aggression in B6-15MSM

The nature of opponent is also one of the important factors for the aggressive behavior (Maxson and Canastar 2003; Ogawa et al., 2004). The consomic strain DBA/1-$Y^{C57BL/10}$, which has substituted chromosome Y from C57BL/10 strain in the DBA/1 strain background, showed less aggressive behavior than parental DBA/1 when aggression was measured between the same genotype mice (Selmanoff et al., 1976). By contrast, when they encountered to a different strain, BALB/c, which experienced repeated defeat, aggressive behavior of DBA/1 was reduced below that of DBA/1-Chr $Y^{C57BL/10}$ (Didier-Erikson et al., 1989). Dyadic encounter design between DBA/1 and DBA/1-Chr $Y^{C57BL/10}$ revealed only DBA/1-DBA/1 pair showed enhanced aggressive behavior, and no differences was found in reciprocal pairs of DBA/1 and DBA/1-Chr $Y^{C57BL/10}$ (Maxon et al., 1989). The effect of the opponent was also reported in gene altered mice. Females of ER-α knockout mouse, but not males, display high levels of aggressive behavior in the resident-intruder test (Ogawa et al., 1998a,b). This increase of aggression in the resident ER-α KO female was observed toward the wild-type littermate control intruder male. However, if the intruder was an olfactory-bulb ectomized male, aggression reduced to a very low, normal level as the wild-type female (Ogawa et al, 2004).

The present result also indicated the strong effect of intruder. B6-15MSM showed increased aggressive behavior when the resident and intruder had same genotype (15*15). On the other hand, aggressive behavior was largely inhibited between the B6-15MSM resident and the C57BL/6J intruder (15*B6). This aggressive behavior was almost the same as the result of the C57BL/6J homogenous pair (B6*B6). In contrast, another reciprocal pair of the C57BL/6J resident and B6-15MSM intruder (B6*15) exhibited increased tail-rattling and submissive posture, but no differences in their attack behavior from B6*B6 pair. Thus, it was expected that the B6-15MSM intruder induce some kind of aggressive behavior, especially tail-rattling, in any genotype residents. But attack behavior was increased only when both resident and intruder were B6-15MSM.

The “intruder effect” leads to the expectation of some differences in the B6-15MSM intruder. Does the B6-15MSM intruder act rudely toward the resident? For example, does the B6-15MSM intruder only get excited and show aggressive behavior toward the resident? Or, does it have chemical signals that activate resident animals to induce aggressive behavior? To examine the first possibility, behavior of each resident and intruder was examined separately (Figure 6.3). However, I could not found any evidence that only the B6-15MSM intruder showed elevated aggressive behaviors, the tail-rattling and submission posture. This result denies the possibility that only the B6-15MSM intruder shows aggressive behavior toward the resident. But still, there remains the possibility that the B6-15MSM intruder is different in the interactive behavior with the resident before leading to the aggressive behavior. When I examined which animal started the first attack (Figure 6.3D), I found that the B6-15MSM intruder showed higher tendency to start the first attack than the C57BL/6J resident in B6*15 pair. However, in case of 15*15 pair, the B6-15MSM resident has higher tendency to start the attack than the B6-15MSM intruder. Therefore, even though the B6-15MSM intruder does not always start the attack toward the resident, it has increased potential to induce aggressive behavior than the C57BL/6J intruder. When the resident is peaceful C57BL/6J, the increased potential of

B6-15MSM may invoke the attack behavior for B6-15MSM intruder. On the other hand, when the resident is B6-15MSM, some kind of pre-attack interaction with the B6-15MSM intruder ignites the attack of the resident. Once the attack phase started, the pattern of aggressive behavior conducted by both animals was more affected by which animals dominated in the fights rather than by that the animal was resident or intruder. Because I conducted very rough behavioral observation in this analysis, more detailed observation of animal's interaction during the pre-attack phase may be required to detect behavioral differences of the B6-15MSM intruder.

To explore the second possibility, the effect of urination was examined. The importance of odor in intermale aggressive behavior has been amply documented (see Guillot and Chapouthier, 1996, for review). Olfactory cue is primary for rodents to gather information and social recognition. Animals that suffered artificial anosia by bilateral ablation of the olfactory bulbs or by intranasal irrigation with zinc sulfate lack intermale aggressive behavior (Rowe and Edwards, 1971; Edwards et al., 1993; Ropartz, 1968). Urine contains social information, and it is known that urine odor changes animal's physiological responses and behavior (Guillot and Chapoutheir, 1996). Vigorous aggression of resident toward unfamiliar intruder was reduced when the intruder was swabbed with the urine of a familiar cage-mate (Nakamura et al., 2006). If the chemical signals included in the urine of B6-15MSM made the resident "upset" and induced aggressive behavior in the resident, it is expected that applying the urine of B6-15MSM to the C57BL/6J intruder would also cause the increase of resident's aggression. As the C57BL/6J resident increased tail-rattle and submission behavior toward the B6-15MSM intruder, a similar behavioral change was expected for the C57BL/6J resident toward the C57BL/6J intruder with the urine of B6-15MSM but not with the urine of C57BL/6J. However, I failed to find any differences between the intruder groups of C57BL/6 with urines of C57BL/6J and B6-15MSM, and both groups showed slightly increased tail-rattling. It seems that swabbing with urine was stress to the intruder mouse, as some mice squeaked, and this agitation in

intruders may cause the increase of tail-rattling. Thus, I may need to improve this method. To examine the effect of the odor of the intruder precisely, I am planning to castrate the intruder in the future analysis. It is well known that there is a class relationship between the testicular hormone testosterone and aggressive behavior (Soma, 2006). Castration leads to abolish the secretion of testosterone in male mice, and castrated males rarely or never show aggressive behavior (Beeman, 1947). By castrating intruder mouse, it stops to show aggressive behavior toward resident mouse. Then, aggressive behavior of the resident mouse can be purely examined in this urine effect test.

6.4.3 Aggressive behavior in reciprocal F1 progeny of C57BL/6J and B6-15MSM

Analysis of reciprocal F1 intercross revealed similarity and differences between the two crosses. Both F1s showed higher frequencies of tail-rattling and submission posture than C57BL/6J. This result indicates that tail-rattling and submission behaviors in B6-15MSM are dominant traits. The trial by trial changes of tail-rattling and attack latency in both F1 progeny were also similar to those in B6-15MSM. Thus, the genetic factor associated with the reactivity to the first encounter may also have dominant effect.

In contrast, (15×B6)F1 showed shortened attack latency as B6-15MSM, while (B6×15)F1 exhibited longer attack latency and no differences from B6. This result suggested the maternal effect for the aggression of B6-15MSM: F1 having the B6-15MSM mother causes offspring to develop higher aggression than having the B6-15MSM father. As discussed, it was suggested that B6-15MSM has strong reactivity toward the novel encounter and the novel situation, and it is known that the maternal behavior influences the emotional reactivity of pups (for more discussion, see Chapter 5). Therefore, maternal behavior in B6-15MSM may be different from C57BL/6J and that causes increased aggression, or emotional reactivity, of pups. The other possibility of maternal effect was genomic imprinting, and two imprinted genes has been reported on chromosome 15; Ata3 (Mizuno et al., 2002) and Peg13 (Smith et al., 2003). However,

both are paternally expressed genes, and mother-derived genes are inactivated. It is possible that an unidentified maternally expressed imprinting gene may exist on this chromosome and may be involved in the aggression.

6.4.4 An approach to identify the genetic locus of aggressive behavior on chromosome 15 using congenic strains of B6-15MSM

In this study, I established a panel of congenic strains of B6-15MSM to identify the genetic loci associated with the aggression of B6-15MSM, and behavioral analysis has been started with those congenic strains. Although the data is preliminary, I found some congenic strains that showed changes of aggressive behavior from C57BL/6J. Especially, C10(43cM-t) had increased aggression; all pairs of C10 showed the attack behavior and thus shortened attack latency. The tail-rattling of C10 was higher than B6-15MSM so far. Thus, it is expected that there is/are a genetic locus/loci related to the attack behavior and tail-rattling in the C10 region, D15Mit5 (43.39cM) to the telomere. In contrast, shortened attack latency was not observed in any other congenic strains, although some of them have a part of the C10 region. Thus, the C10 region may contain multiple genes that have epistatic effect for the attack behavior. Increased tail-rattling was also observed in C7(58-80cM) and C9(58-61cM). Therefore, there is a single genetic locus related to the tail-rattling exists between D15Mit121 (58.17cM) to D15Mit261 (80.29cM). The number of animals that showed the attack was also increased in these congenic strains. On the other hand, C4(c-58cM) showed an opposite change to B6-15MSM. Although it is not significant, only one of 14 pairs showed the attack in this strain, and the tail-rattling occurred very infrequently compared to C57BL/6J. This result suggests that there may be genetic loci in the C4 region that suppress aggression, from centromere to D15Mit121 (58.17cM). It was presumed that because of its counteractive effect, C5(c-80cM), which include both the C4 and C7 region exhibited the same level of tail-rattling as C57BL/6J. To confirm this result, enough data sets need to be analyzed in the near future.

6.4.5 Summary

Aggression has considerable importance for animal's living and is evolutionally ancient behavior. On the other hand, maladaptive aggression is perceived as a serious social issue, and biological mechanisms of aggressive behavior are getting a large concern. In this chapter, I performed genetic analysis of aggressive behavior in B6-15MSM. By analyzing both homogenous pairs and reciprocal heterogeneous pairs in the resident-intruder test, I found a prominent effect of the opponent (intruder) in their aggressive behavior. The analysis of F1 indicated that there are dominant effects on the tail-rattling and submission behavior, and also maternal effect on attack behavior. It is desirable with near future to explore the "intruder effect" and "maternal effect" by using castrated males and cross-fostering analysis, respectively. Preliminary analysis of congenic strains showed the possibility to identify the genetic loci associated with the aggressive behavior of B6-15MSM, and suggested that there are multiple genetic loci related to the aggressive behavior on this chromosome 15.

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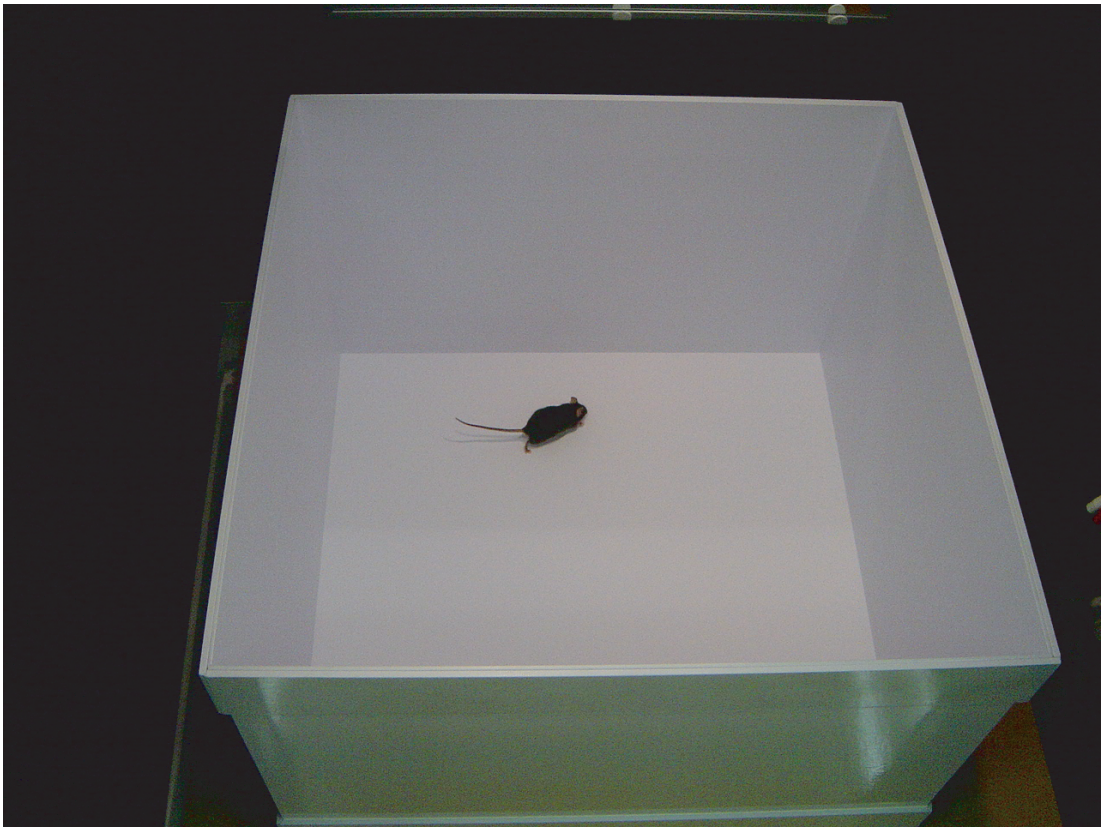


Figure 1.1 Open-field apparatus used in this study (60 x 60 x 40 cm)

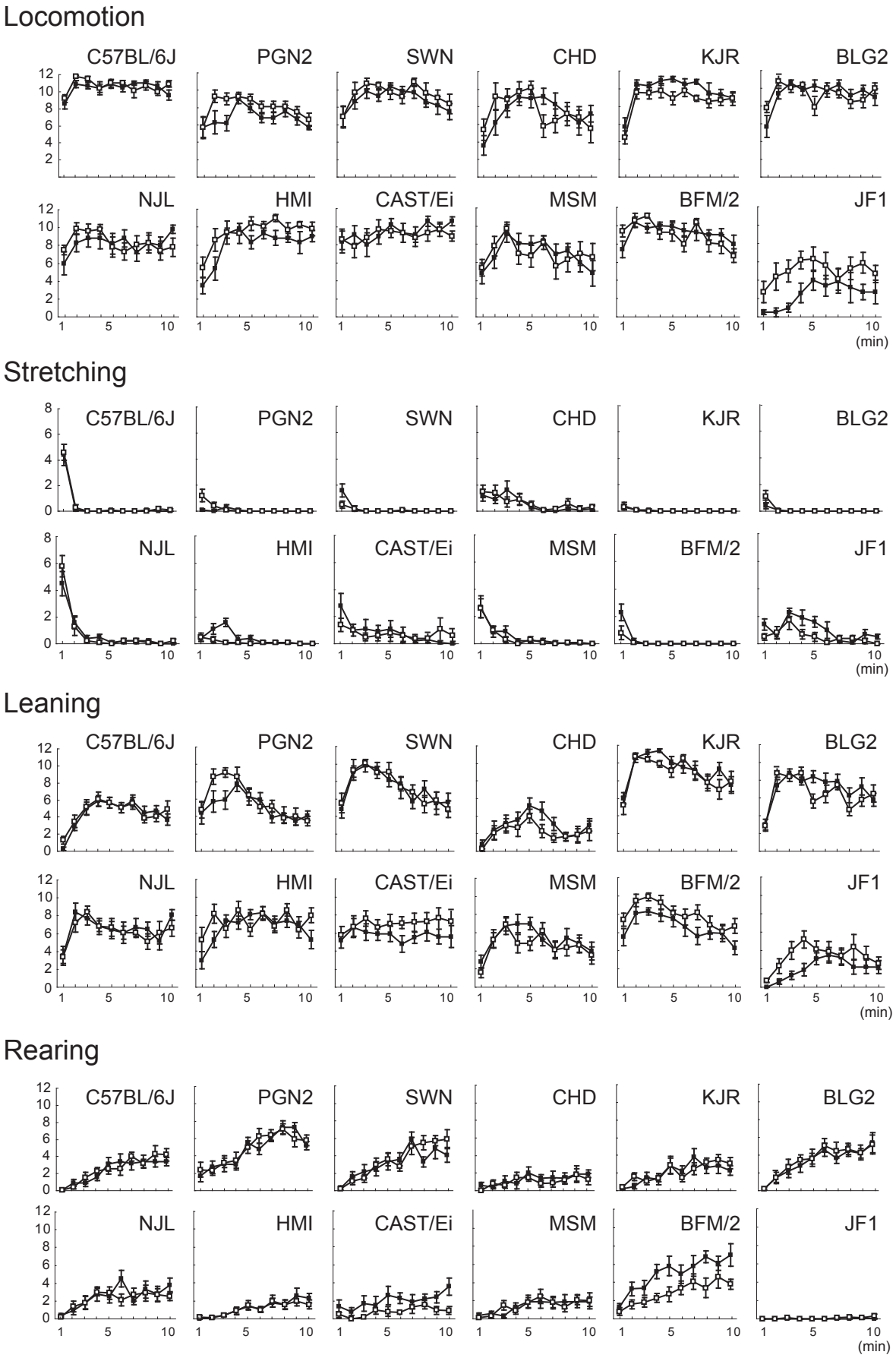
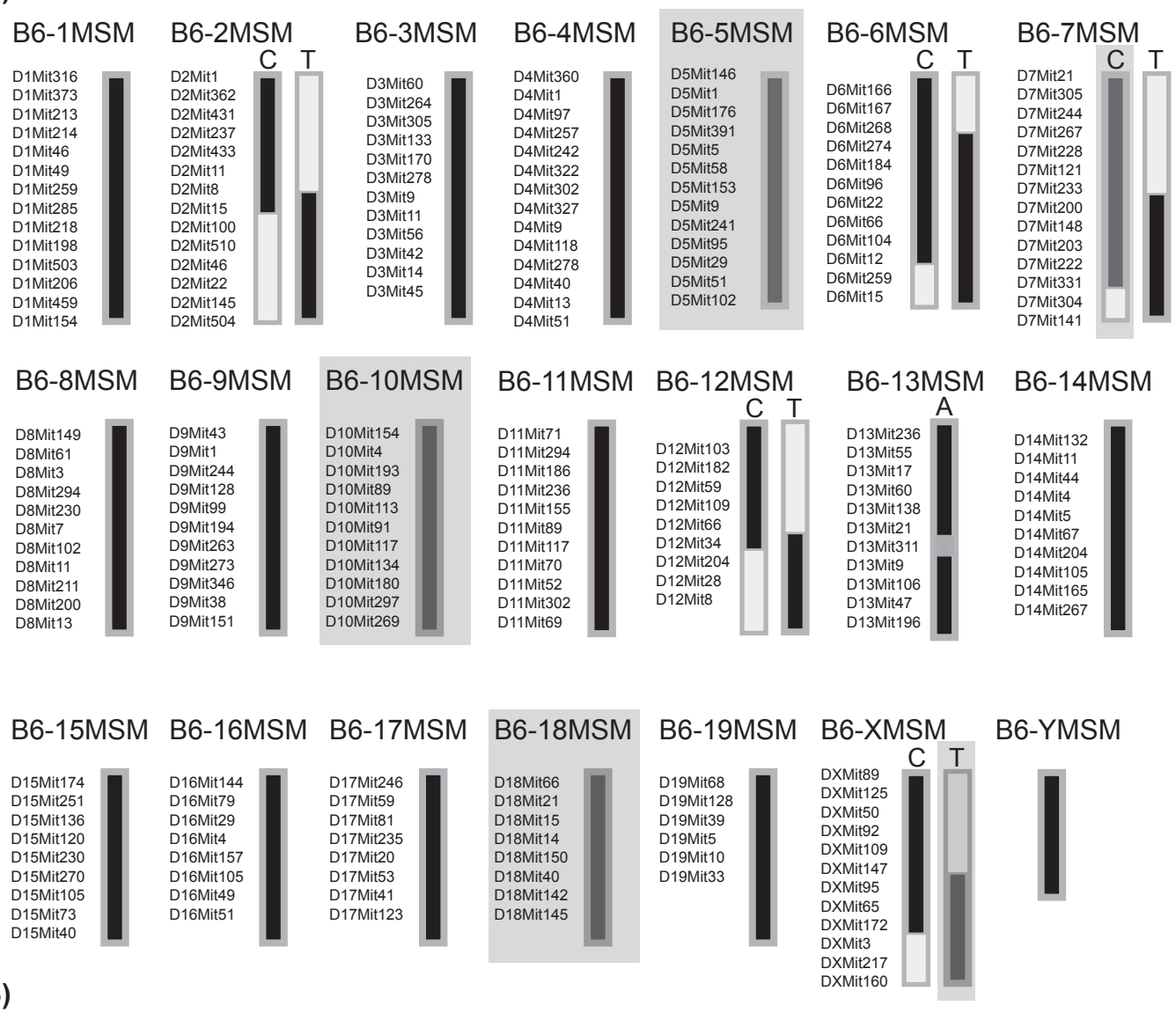


Figure 2.1 The temporal changes of each behavioral component. For calculating the frequency, the presence or absence of each behavior was recorded as 1/0 in each 5-sec period, and summed for each minute. Filled boxes and open boxes indicate the frequency of each one-minute period of behavior in males and females, respectively.

(A)

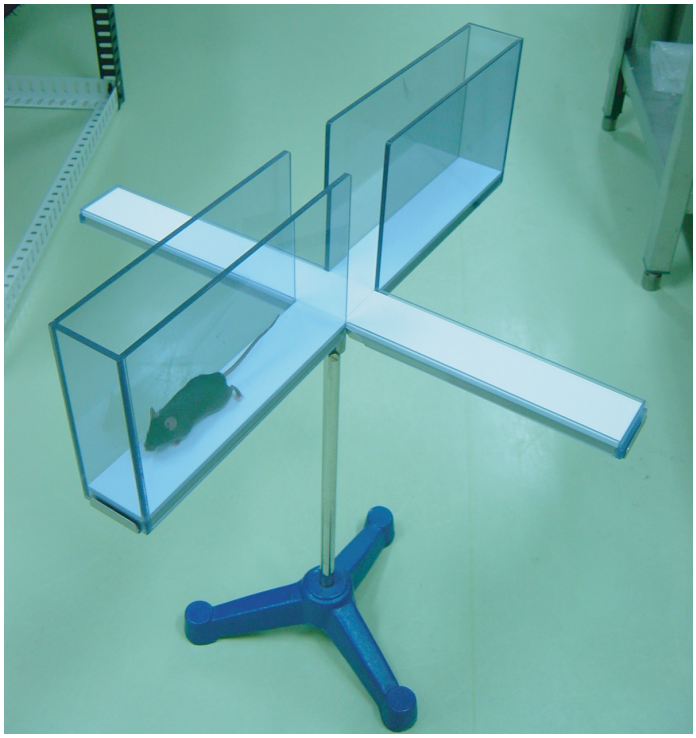


(B)

	Number of animals				Weight	
	OF, EPM		SI (pairs)			
	M	F	M	F	M	F
C57BL/6J	20	20	14	14	24.4	19.5
MSM	16	16	10	10	12.8	10.5
B6-1MSM	17	18	13	11	25.0	19.4
B6-2CMSM	16	16	10	11	21.4	17.7
B6-2TMSM	17	16	11	10	22.4	18.9
B6-3MSM	17	15	11	10	19.9	16.4
B6-4MSM	16	19	-	-	20.9	17.4
B6-6CMSM	16	15	10	11	25.2	20.6
B6-6TMSM	15	15	12	11	25.4	20.9
B6-7TMSM	19	16	13	11	23.0	19.0
B6-8MSM	16	15	11	10	24.9	18.4
B6-9MSM	16	15	11	10	24.9	19.4
B6-11MSM	15	16	10	7	22.6	18.8
B6-12CMSM	17	15	11	11	24.6	19.4
B6-12TMSM	18	16	11	10	23.3	18.5
B6-13AMSM	20	19	11	10	23.5	19.3
B6-14MSM	16	16	11	11	22.5	18.1
B6-15MSM	20	16	16	12	25.7	19.6
B6-16MSM	15	15	11	11	22.6	20.2
B6-17MSM	15	17	11	11	27.2	22.2
B6-19MSM	15	15	12	10	25.4	21.3
B6-XCMSM	11	11	-	-	28.0	22.3
B6-YMSM	16		11		24.8	

Figure 3.1 Consomic strains (CSSs) used in this study. (A) A panel of consomic strains established from C57BL/6J and MSM. The MIT microsatelite markers used for the establishment of each CSS were listed. Note that B6-13AMSM strain possesses heterozygote locus around D13Mit311 region. Strains that have not completed in the behavioral characterization were covered with gray color. (B) Number of animals used in open-field test (OF) and elevated plus-maze (EPM), and the number of pairs used for social interaction test (SI). Mean weight in each sex of each strain were also described.

(A)



(B)

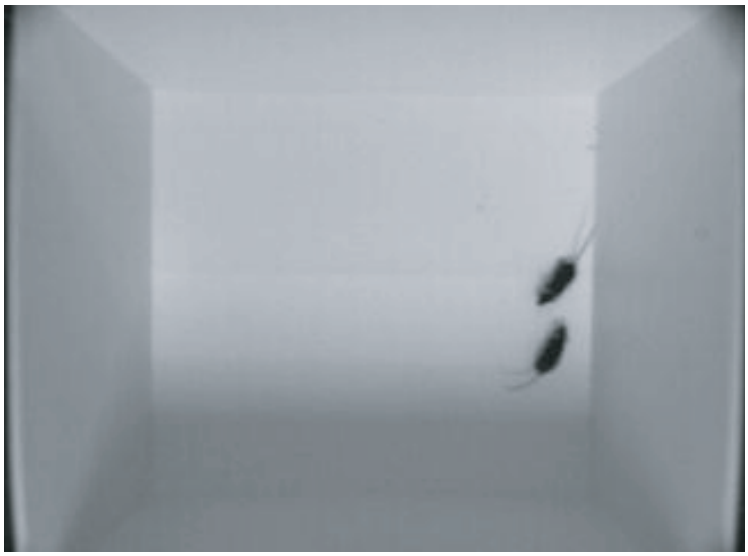


Figure 3.2 Two kind of emotionality-related behavioral tests. (A) Elevated plus-maze apparatus, (B) Social interaction test in the open-field apparatus.

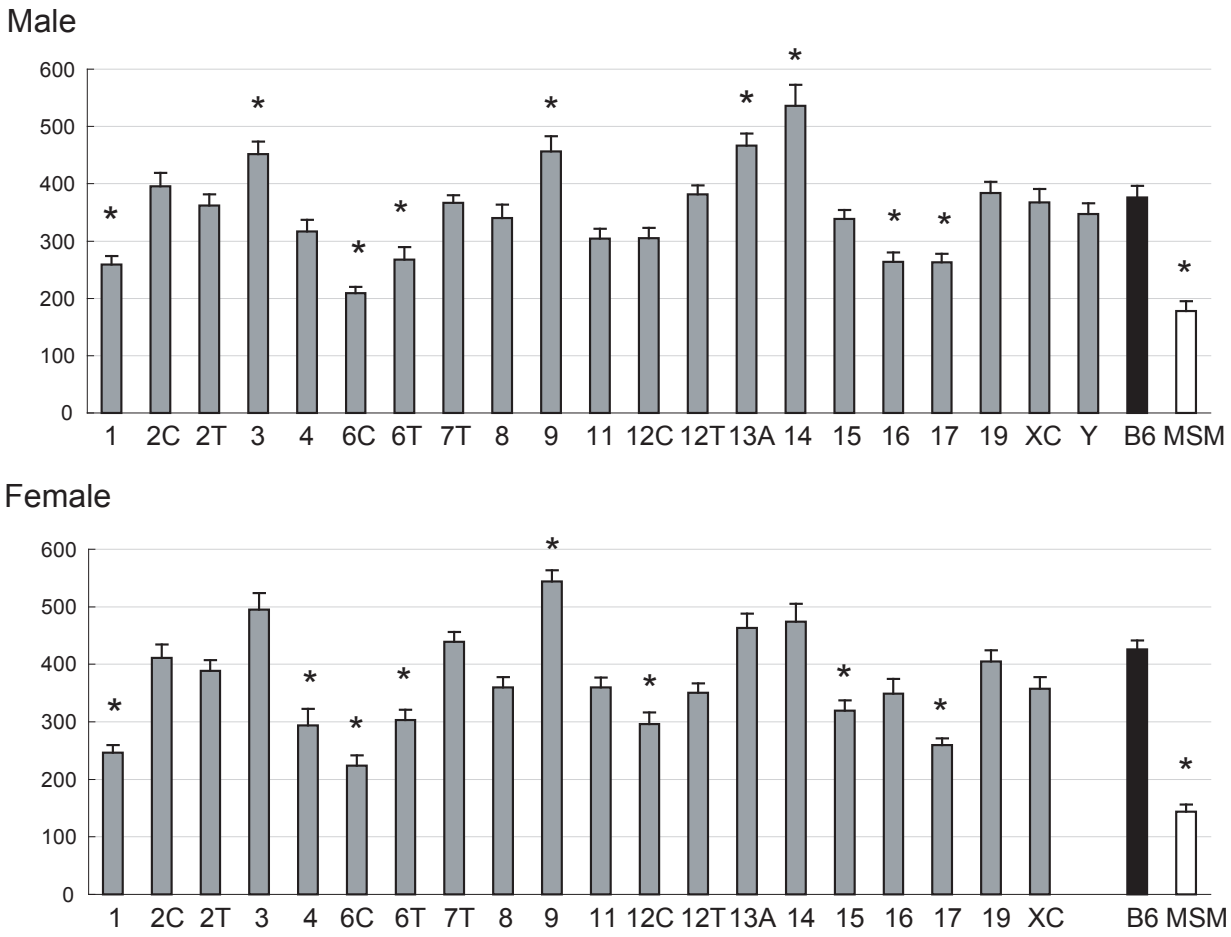


Figure 3.3 Open-field ambulation in the CSSs. The name of each CSS was abbreviated as the number of substituted chromosome. * indicated the CSS that showed significant difference compared to C57BL/6J (B6) in Dunnet's t-test ($p<.05$).

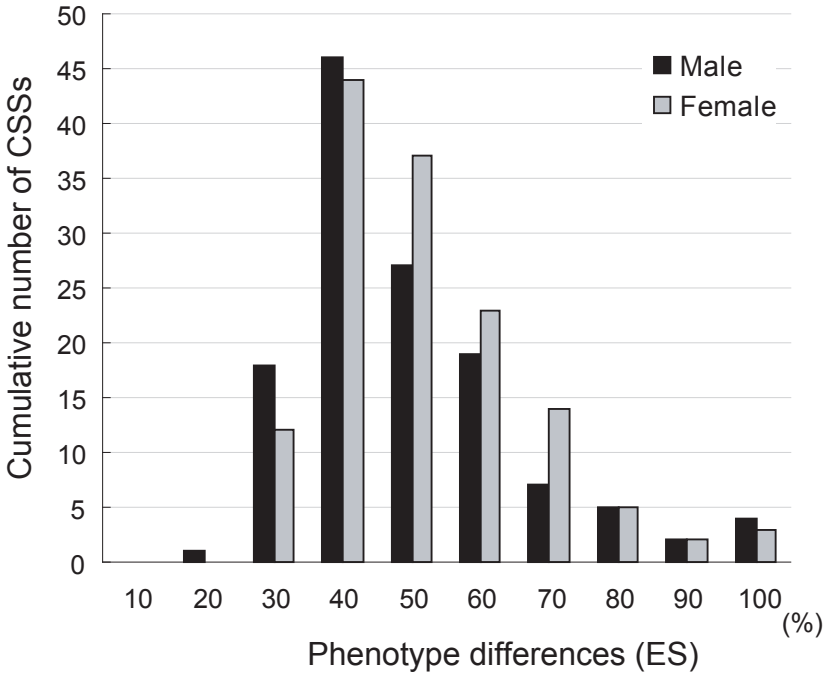


Figure 3.4 Distribution of phenotype differences (ES) for the emotionality-related behaviors in B6-Chr^{MSM} CSSs.
ES represented the effect size of hypothesized-QTL for each CSS, and were calculated by following equation

$$ES_i = 100*|(CSSi - B6) / (Hi - Lo)|$$

Where *Hi* is the highest mean phenotypic value among the progenitor strains and the CSS panel, and *Lo* is the lowest mean phenotypic value.

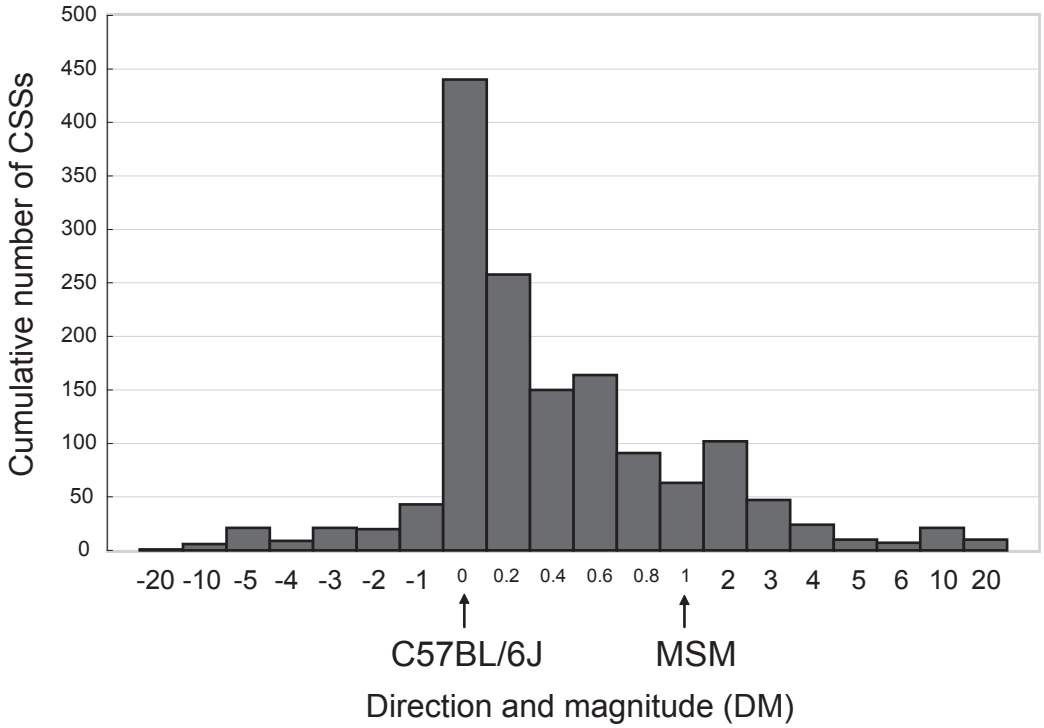


Figure 3.5 Frequency distribution of direction and magnitude (DM) of hypothesized QTL effect on each CSS. Vertical axis indicated the DM of each CSSs. DM between C57BL/6J and MSM are considered as 1, and the effect of DM in each CSS was calculated for each behavioral measurements. DM values were calculated by following equation: DM*i* = (B6-CSS*i*) / (B6 -MSM)
When the hypothesized QTLs on *i*th CSS have actions in the same direction as MSM related to C57BL/6J, DM*i* become positive. And if it has effect in inversed direction, DMi become negative. DMi is zero if there is no QTL action on a CSS, and 1 if direction and magnitude of the hypothesized-QTL action on a CSS equal to MSM. Note that the scale of horizontal axis was not constant and epitomized.

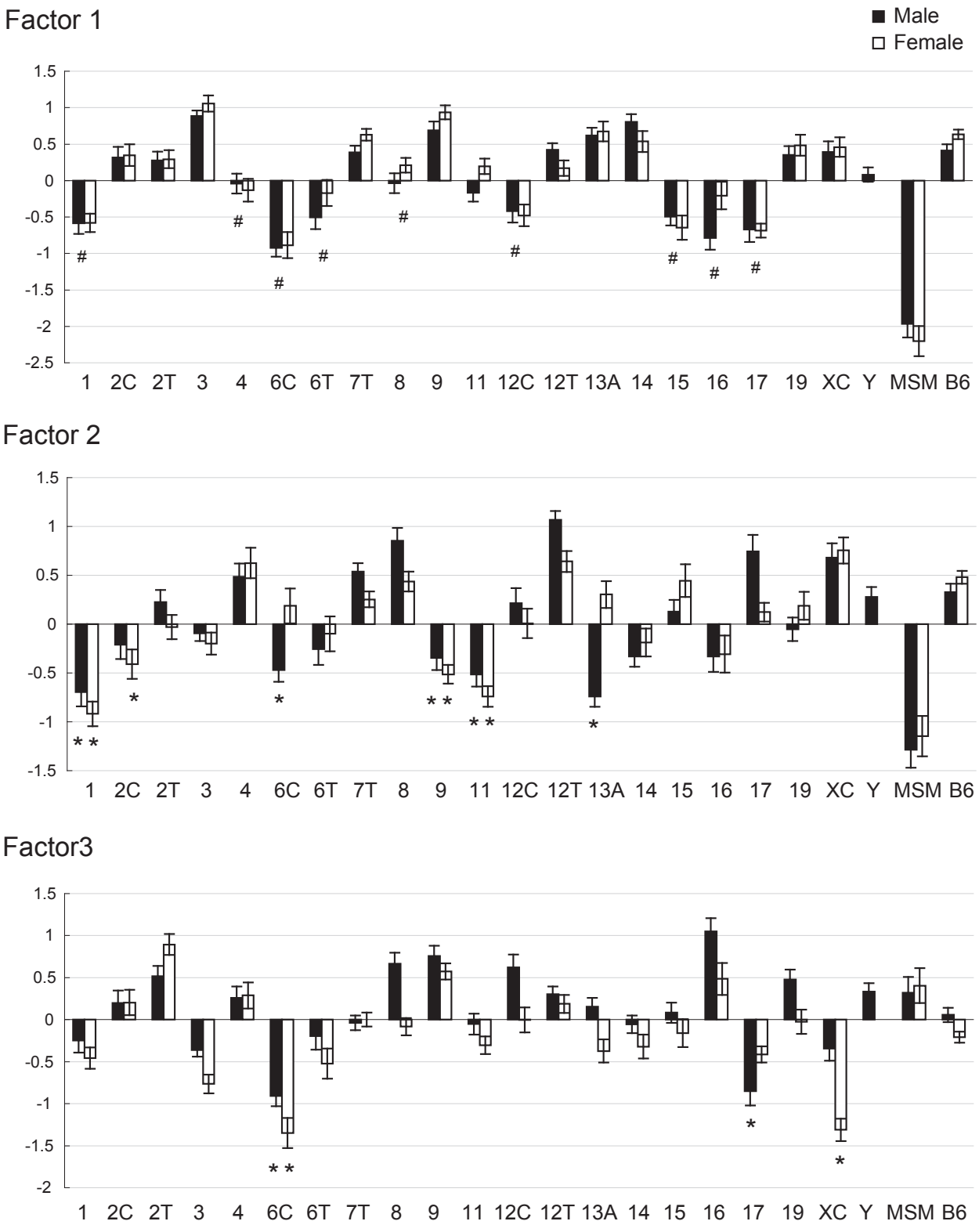


Figure 3.6 Representation of each CSS in the three factors extracted from factor analysis of open-field behaviors. The name of each CSS was abbreviated as the number of substituted chromosome.Dunnet's t-test was performed to compare each CSS with C57BL/6J (B6). # significant differences in the combined data of sexes ($p<.05$). * significant differences in the t-test performed for each sex ($p<.05$)

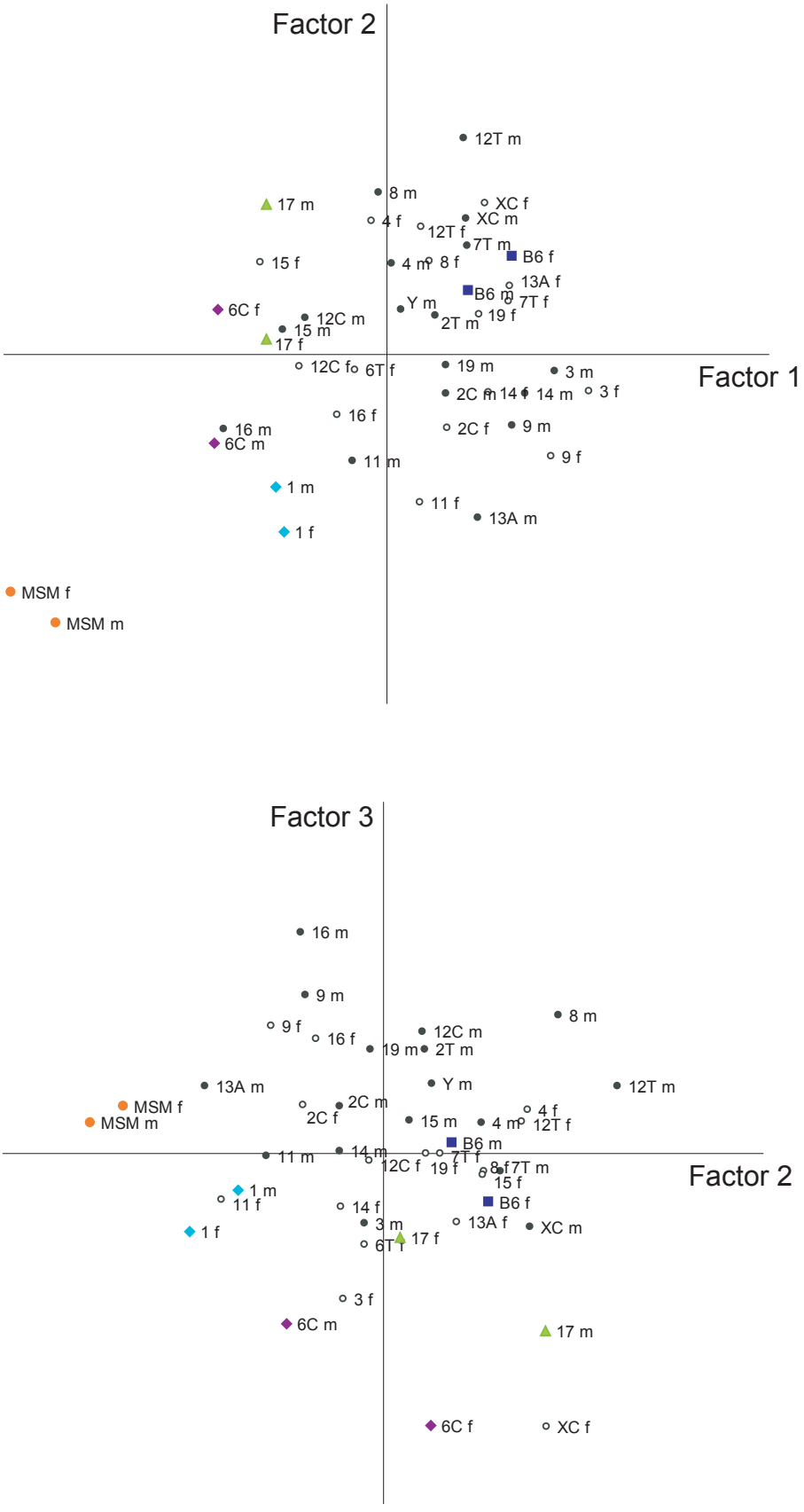
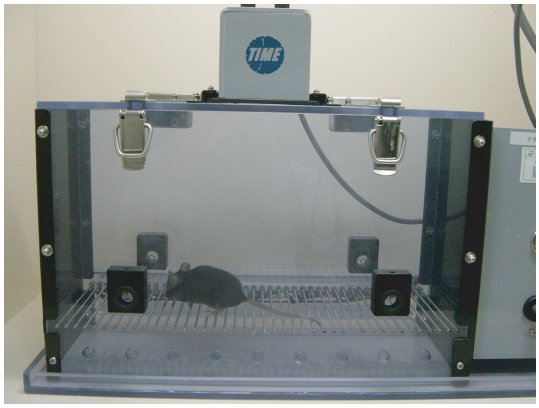
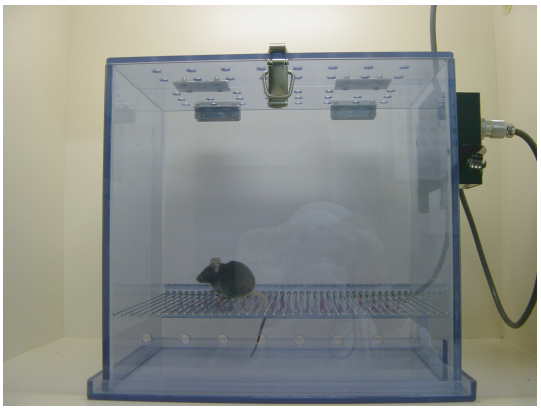


Figure 3.7 Two-dimensional representation of open-field behavior in the CSS. Each axis was derived by principal component analysis (Table 3.4). (A) x: factor 1, y: factor 2. (B) x: factor 2, y: factor 3. Each point represents mean of the factor scores of each sex of each CSS. C57BL/6 (■), MSM (●), and three CSSs B6-1MSM (◆), B6-6CMSM (◆), B6-17MSM(▲) were represented as colored points. The name of each CSS was abbreviated as the number of substituted chromosome.

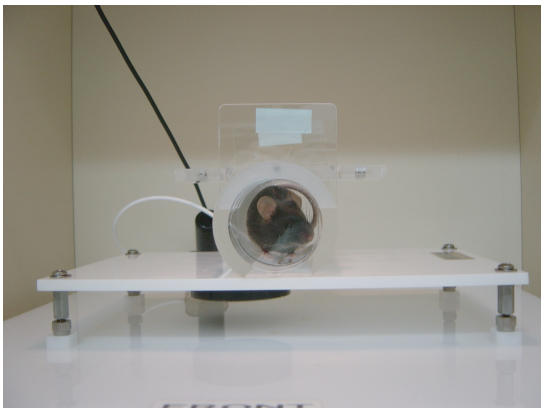
(A)



(B)



(C)



(D)

Figure 4.1 Behavioral Apparatus used in this study. (A) Cue-fear conditioniong test. (B) context fear-conditioning test. (C) Acousit statle response and prepulse inhivition test. (D) Rotarod test.

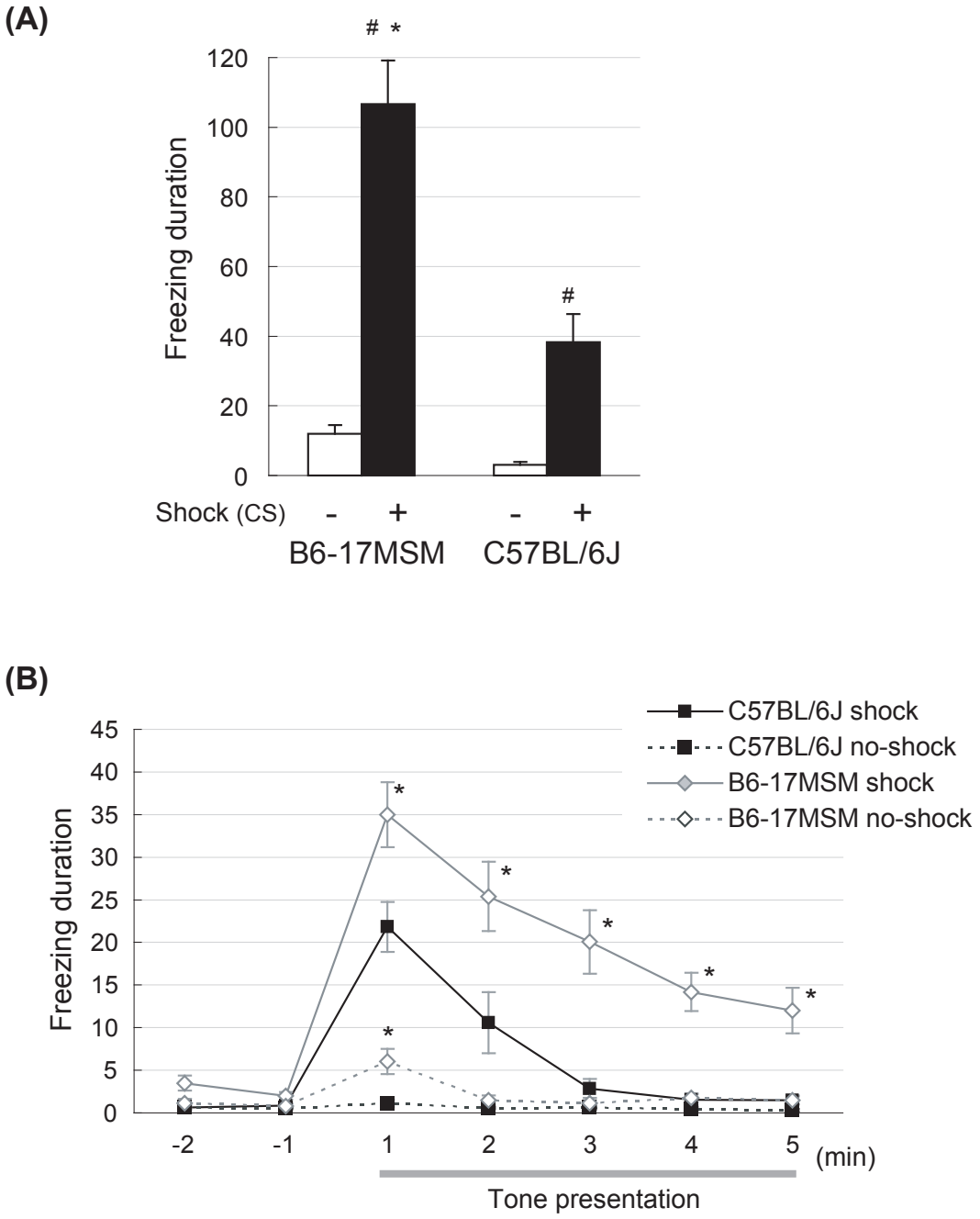


Figure 4.2 Fear responses of B6-17MSM and C57BL/6J in cue-fear conditioning. (A) Total freezing response during the tone presentation for 5 min in shocked group (+) and non-shocked group (-). # significant differences compared to no-shock group of the same strain ($p<.05$). * significant differences compared to the corresponding C57BL/6J groups ($p<.05$). (B) Temporal change of fear response. Observation of freezing behavior was started 2 min before the tone presentation. * significant differences compared to corresponding C57BL/6J groups ($p<.05$).

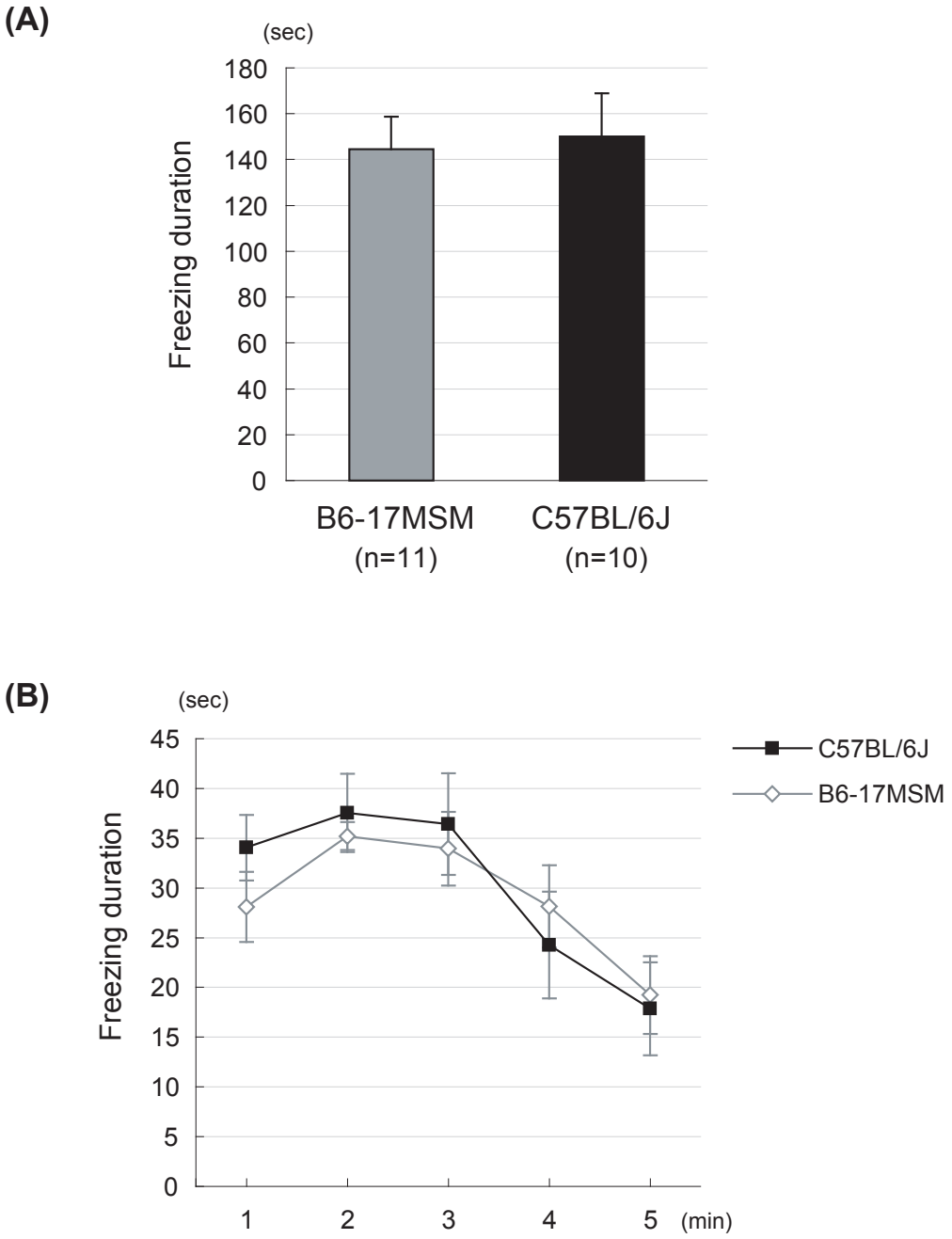


Figure 4.3 Fear responses of B6-17MSM and C57BL/6J in context-fear conditioning. (A) Total freezing response during 5 min exposure to the conditioned chamber. (B) Temporal change of fear response in the B6-17MSM and C57BL/6J. No significant difference was observed between B6-17MSM and C57BL/6J in the context-conditioning paradigm.

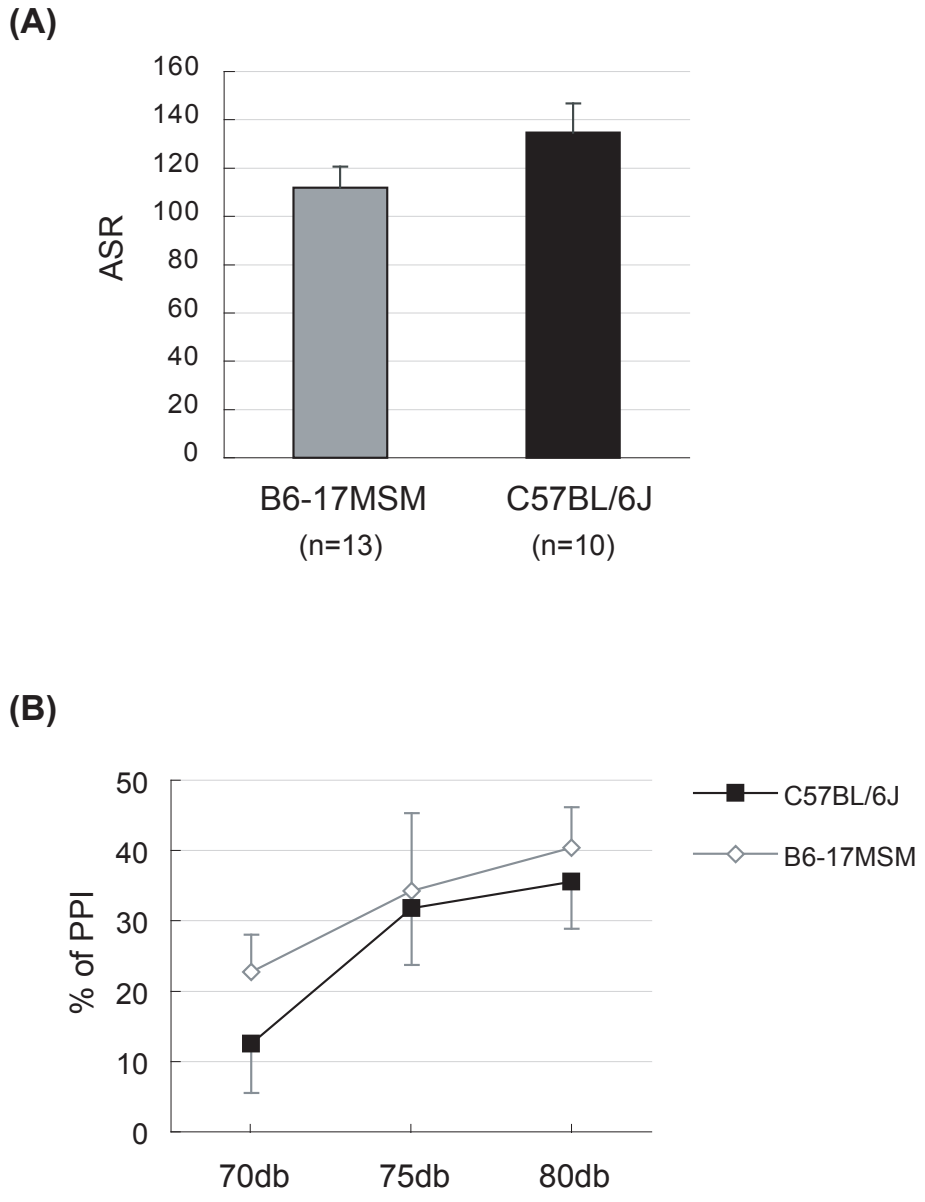


Figure 4.4 Acoustic startle response (ASR) and prepulse inhibition (PPI) in B6-17MSM and C57BL/6J. (A) ASR toward 40 ms of 12 dB sound stimulus. (B) Percentage of the prepulse inhibition of ASR. No significant difference was observed between B6-17MSM and C57BL/6J in the ASR and PPI.

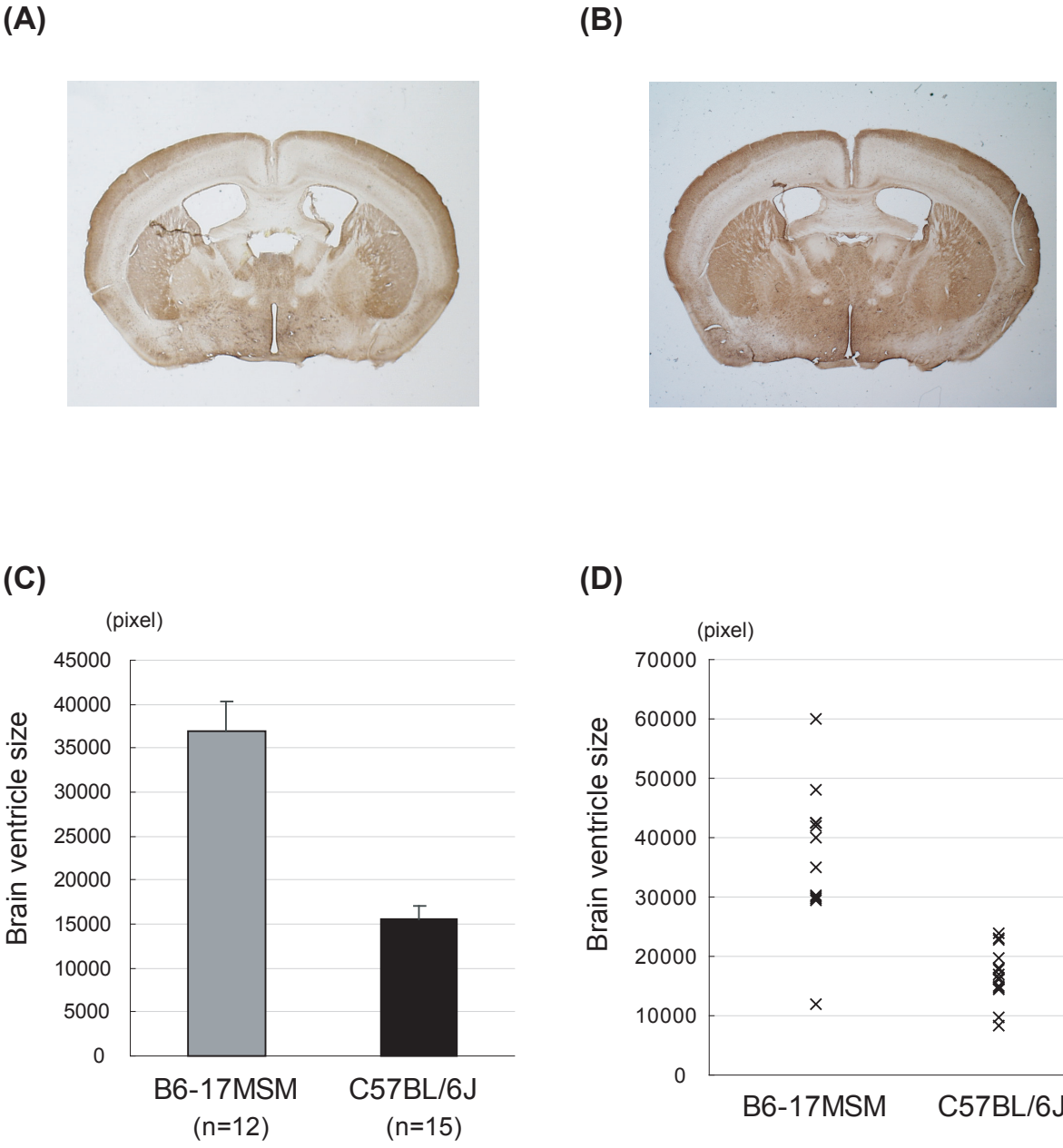
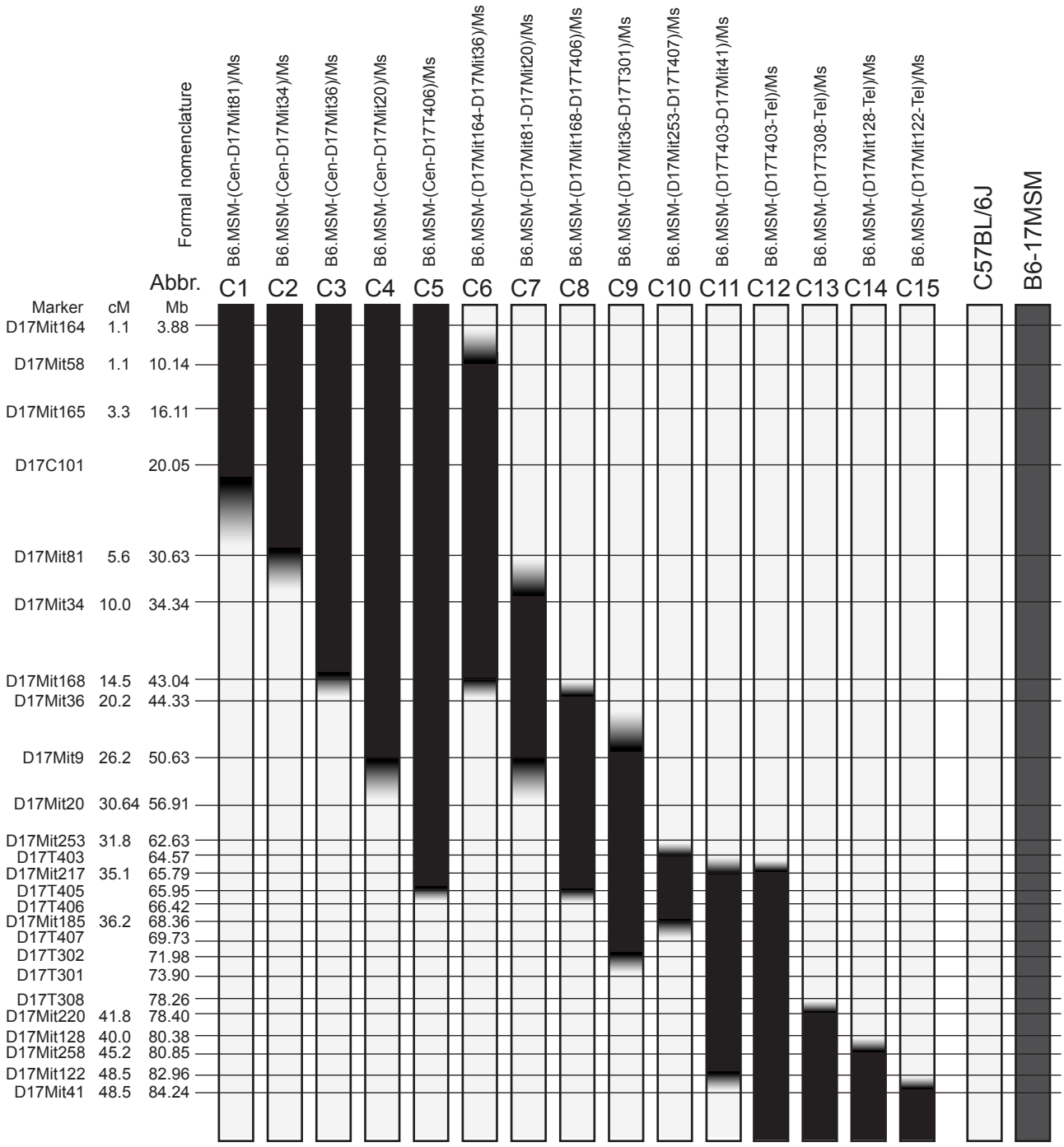


Figure 4.5 Differences of brain ventricle size (VS) between B6-17MSM and C57BL/6J. Picture shows the brain sections with calbindin staining of B6-17MSM (A) and C57BL/6J (B). (C) Mean VS calculated by using Inage J software. * significant differences from C57BL/6J ($p<.001$) (D) Individual variation of VS are larger in B6-17MSM than C57BL/6J.



		C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C57BL/6J	B6-17MSM
OF	m	21	10	11	12	15	17	-	15	15	19	13	16	15	17	16	23	25
	f	19	14	15	9	16	12	-	15	16	16	13	17	13	18	20	21	19
EPM	m	20	9	11	12	15	15	-	15	15	18	13	16	15	17	16	23	23
	f	19	14	15	9	16	12	-	15	16	16	13	17	13	18	20	20	17
SI	m	11	5	4	-	12	13	-	10	8	8	8	9	10	10	9	21	21
	f	11	5	10	-	10	7	-	9	9	8	6	9	9	10	8	22	19
Histology	m	16	-	-	-	-	14	6	7	-	-	6	-	-	6	-	15	12
Hydrocephalus		1	2	2	5	1	3	0	0	0	0	0	0	0	0	0	0	26
Total		110	28	44	46	63	72	20	42	45	125	22	57	49	49	142	819	456

Figure 5.1 A series of congenic strains derived from B6-17MSM. For the simplicity, their name was abbreviated C1 to C15. Substituted chromosomal regions from MSM were represented as Black color. The number of males and females in each strain used for open-field test, elevated plus maze test, social interaction test and historogical analysis for examin ventricle size were listed under the diagram. The number of hydrocephalus and total number of animals obtained so far were also described in each strain.

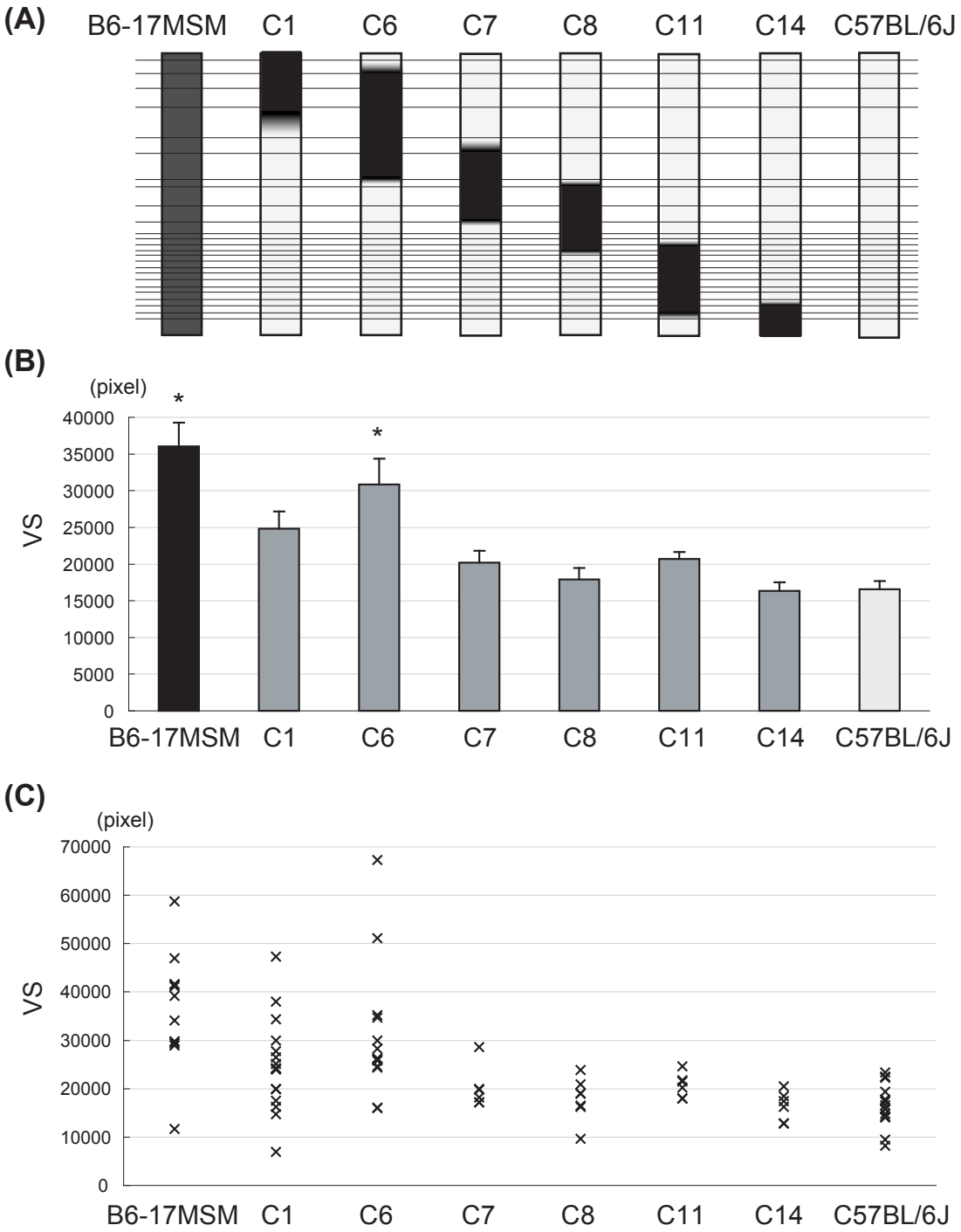


Figure 5.2 Brain ventricle size (VS) in the B6-17MSM congenic mouse strains.
(A) Diagram of congenic strains used for the analysis. Substituted chromosomal regions from MSM were represented as Black color. (B) Mean VS calculated by using Inage J software. * significant differences from C57BL/6J ($p<.01$) (C) Individual variation of VS.

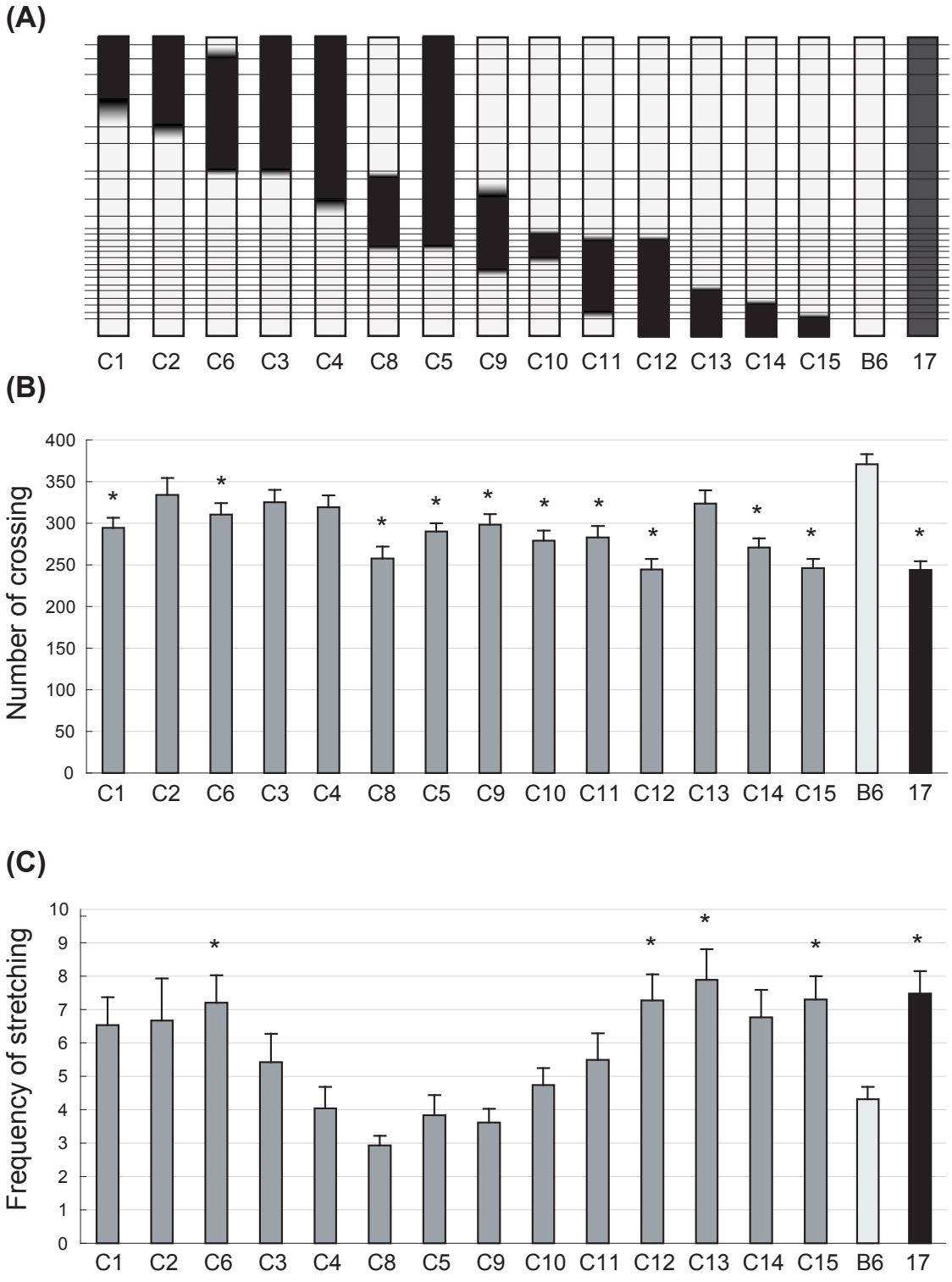


Figure 5.3 Open-field ambulation (B) and stretching (C) in the congenic strains of B6-17MSM. Sex was combined in this analysis.(A) Diagram of congenic strains used for the analysis. Substituted chromosomal regions from MSM were represented as Black color. * significant differences from C57BL/6J ($p<.05$, Dunnet's)

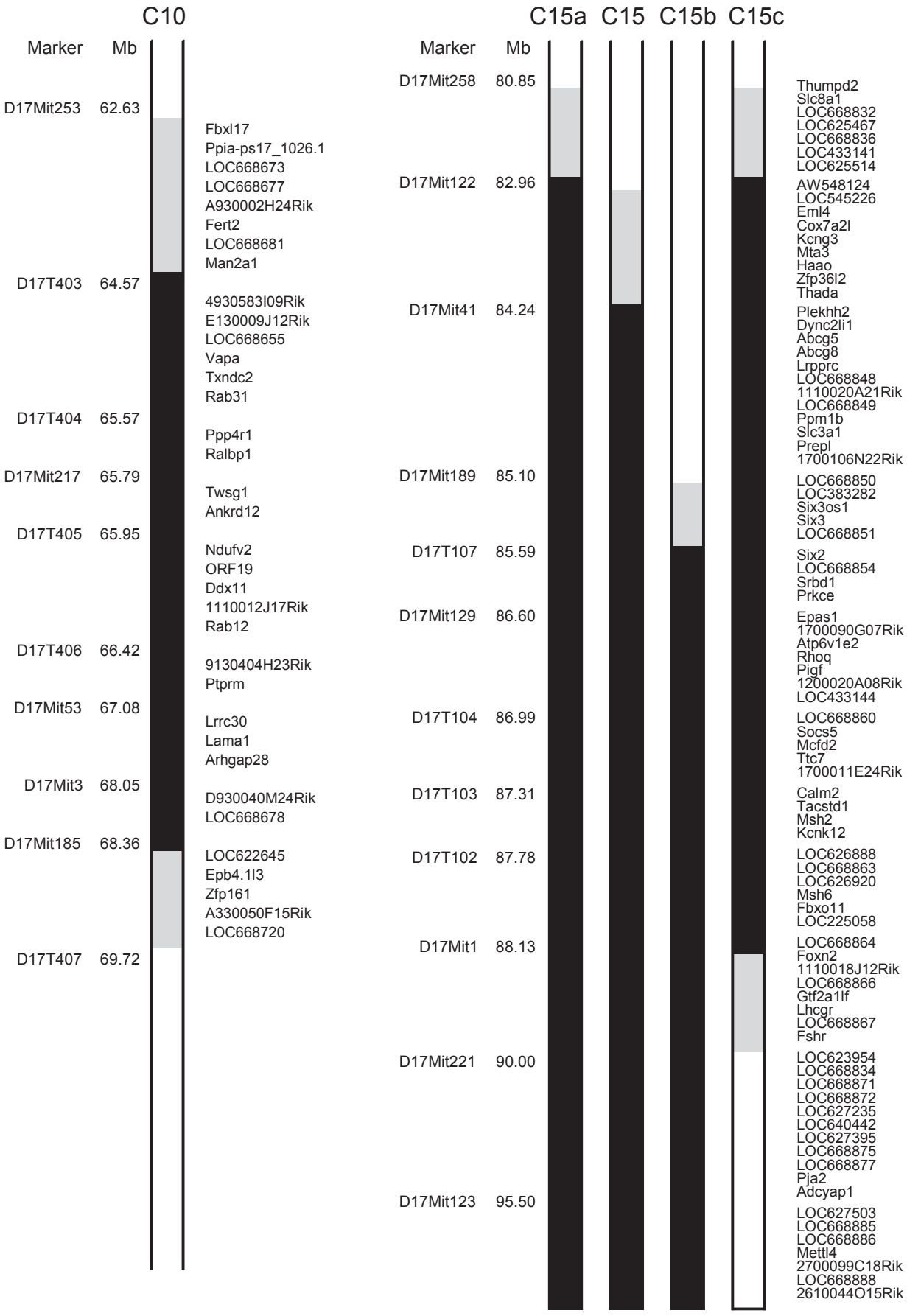


Figure 5.4 Genes on C10 and C15 congenic strains. C10 has substituted MSM region of 7.10 Mb or smaller which includes 35 genes, and C15 has substituted MSM region of around 10.94Mb which contains 78 genes. Three sub-lines of C15 strain,C15a, C15b and C15c, were also represented.

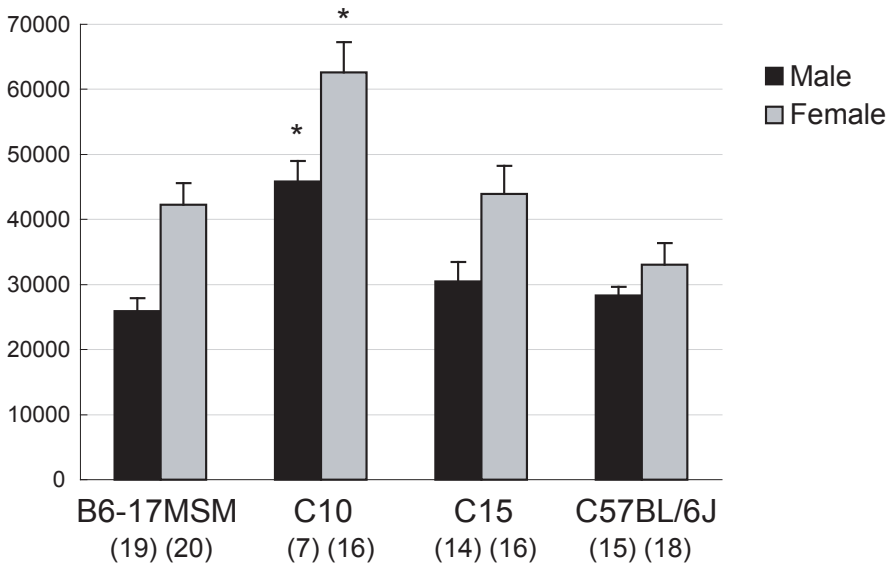


Figure 5.5 Home-cage activity for 3 days in C10 and C15 congenic strains. * significant differences compared to C57BL/6J. Numbers in parenthesis indicate the number of animals analyzed.

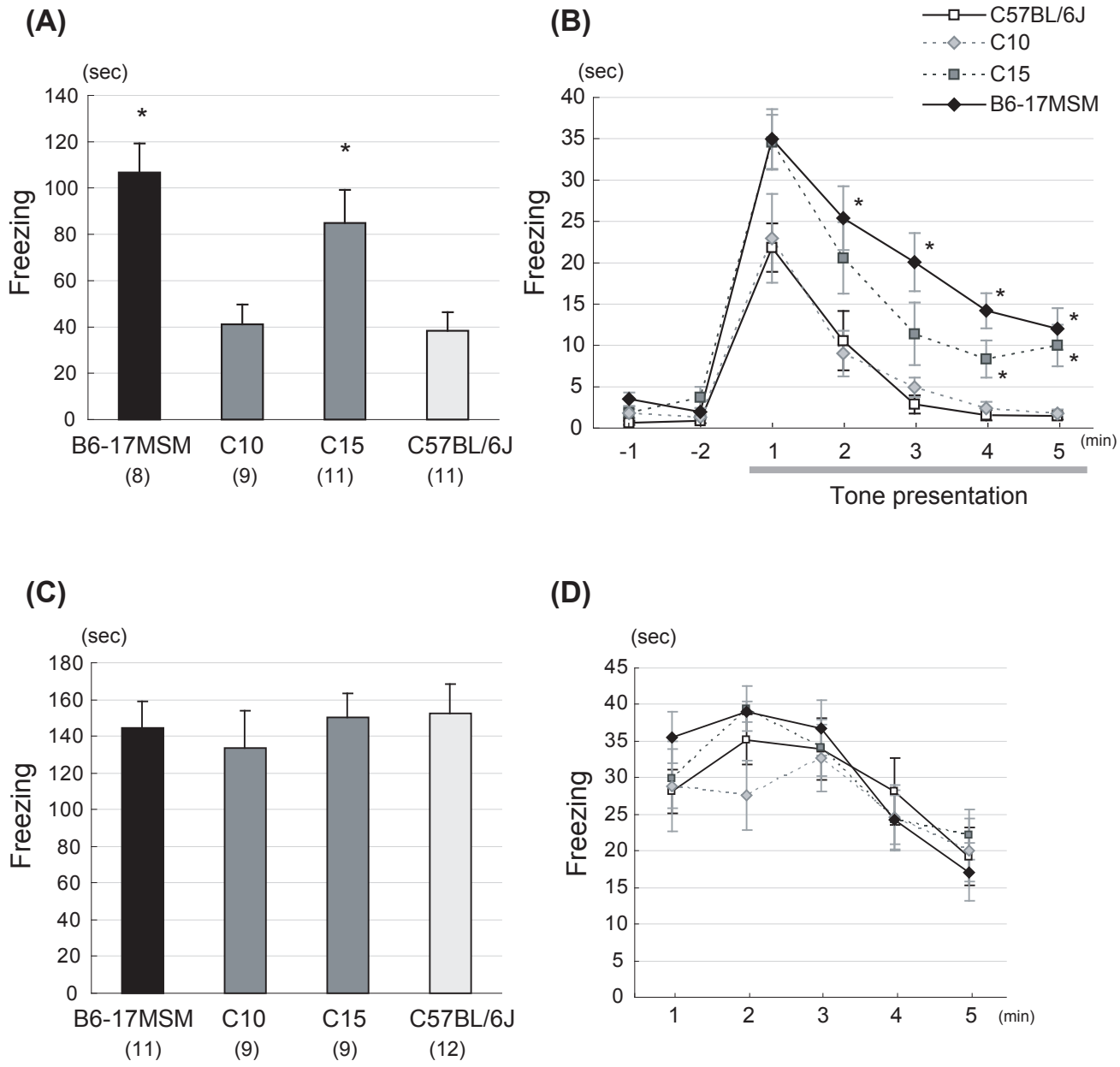


Figure 5.6 Fear responses of C10 and C15 congenic strains in cue-fear conditioning (A,B) and context-fear conditioning (C,D). (A) Total freezing response during the tone presentation for 5 min. * significant differences compared to C57BL/6J ($p<.05$). (B) Temporal change of fear response. Observation of freezing behavior was started 2 min before the tone presentation. * significant differences compared to C57BL/6J ($p<.05$). (C)Total freezing response during 5 min exposure to the conditioned chamber. (B) Temporal change of fear response in C10 and C15. Numbers in parenthesis indicate the number of animals analyzed.

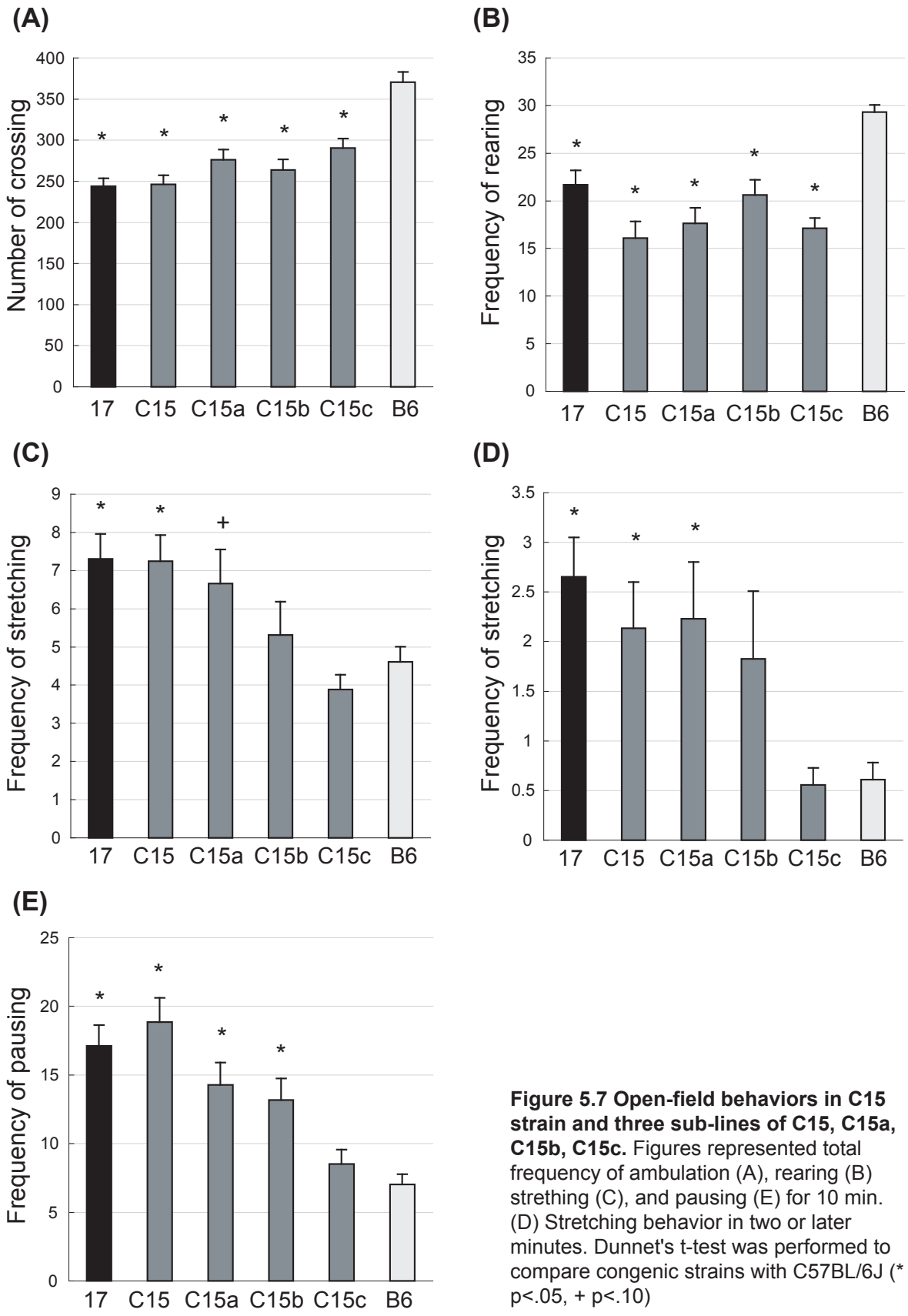


Figure 5.7 Open-field behaviors in C15 strain and three sub-lines of C15, C15a, C15b, C15c. Figures represented total frequency of ambulation (A), rearing (B) strething (C), and pausing (E) for 10 min. (D) Stretching behavior in two or later minutes. Dunnet's t-test was performed to compare congenic strains with C57BL/6J (* p<.05, + p<.10)

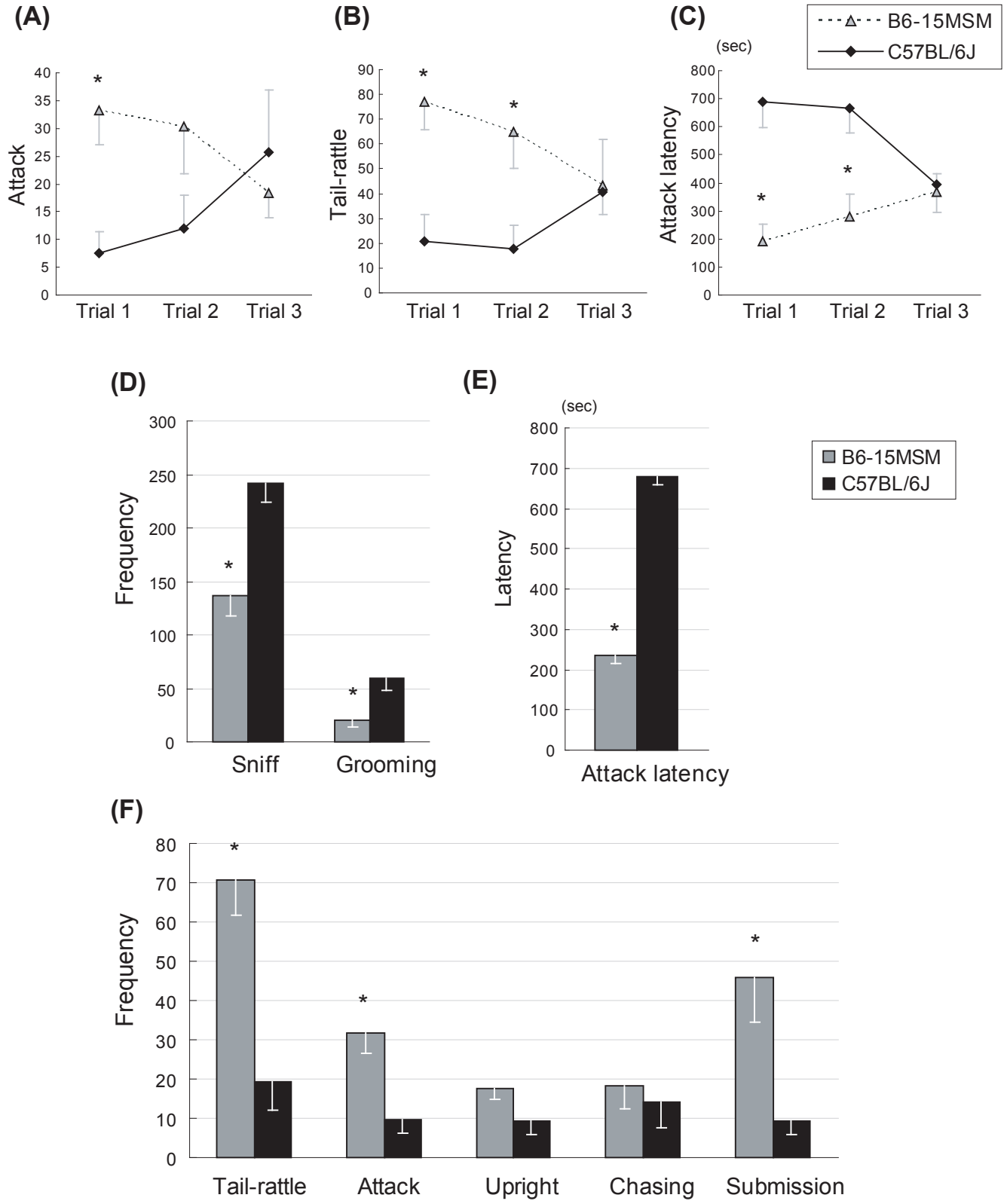


Figure 6.1 Aggressive behavior of B6-15MSM and C57BL/6J in the resident-intruder paradigm. Trial-by-trial change of the frequency of attack (A), tail-rattling (B) and latency of the first attack (C). Because strain difference was prominent on trial 1 and 2, the data of trial 1 and 2 were combined to compare B6-15MSM and C57BL/6J in Non-aggressive behaviors (D), latency of the first attack (E), and aggressive behaviors (F). * Significant differences from C57BL/6J ($p<.01$)

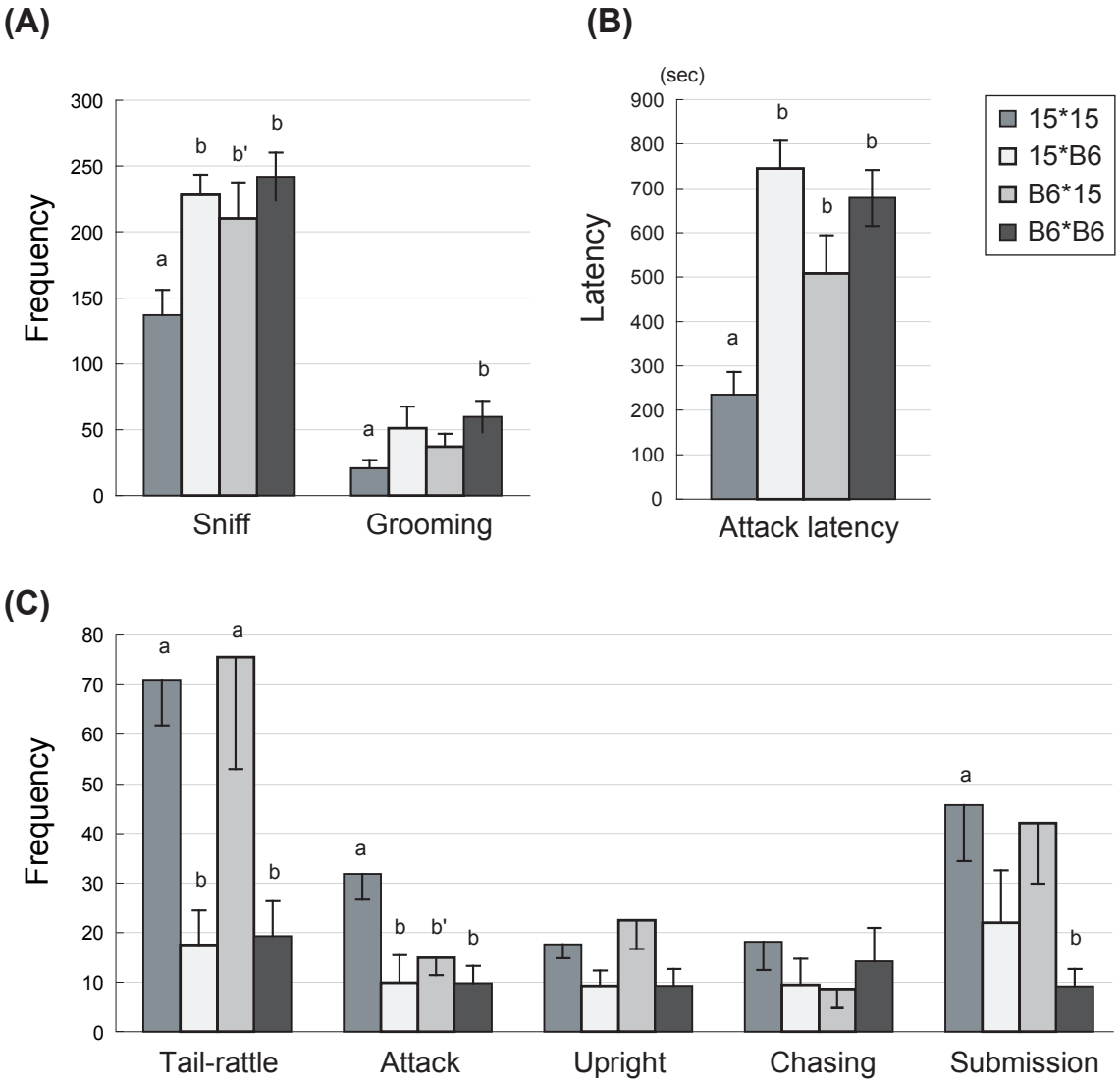


Figure 6.2 Aggressive behavior in homogenous pairs and resiprocal pairs. Combined data of trial 1 and 2 for Non-aggressive behaviors (A), latency of the first attack (B), and aggressive behaviors (C). a: significant differences compared to C57BL/6J ($p<.05$, Bonferroni), b: significant differences from B6-15MSM ($p<.05$). Suggestive differences compared to C57BL/6J (a') and B6-15MSM (b') was also indicated ($p<.10$).15*15: both resident and indtuder were B6-15MSM. 15*B6: B6-15MSM resident vs C57BL/6J intruder. B6*15: C57BL/6J resident vs B6-15MSM intruder. B6*B6: both resident and intruder were C57BL/6J.

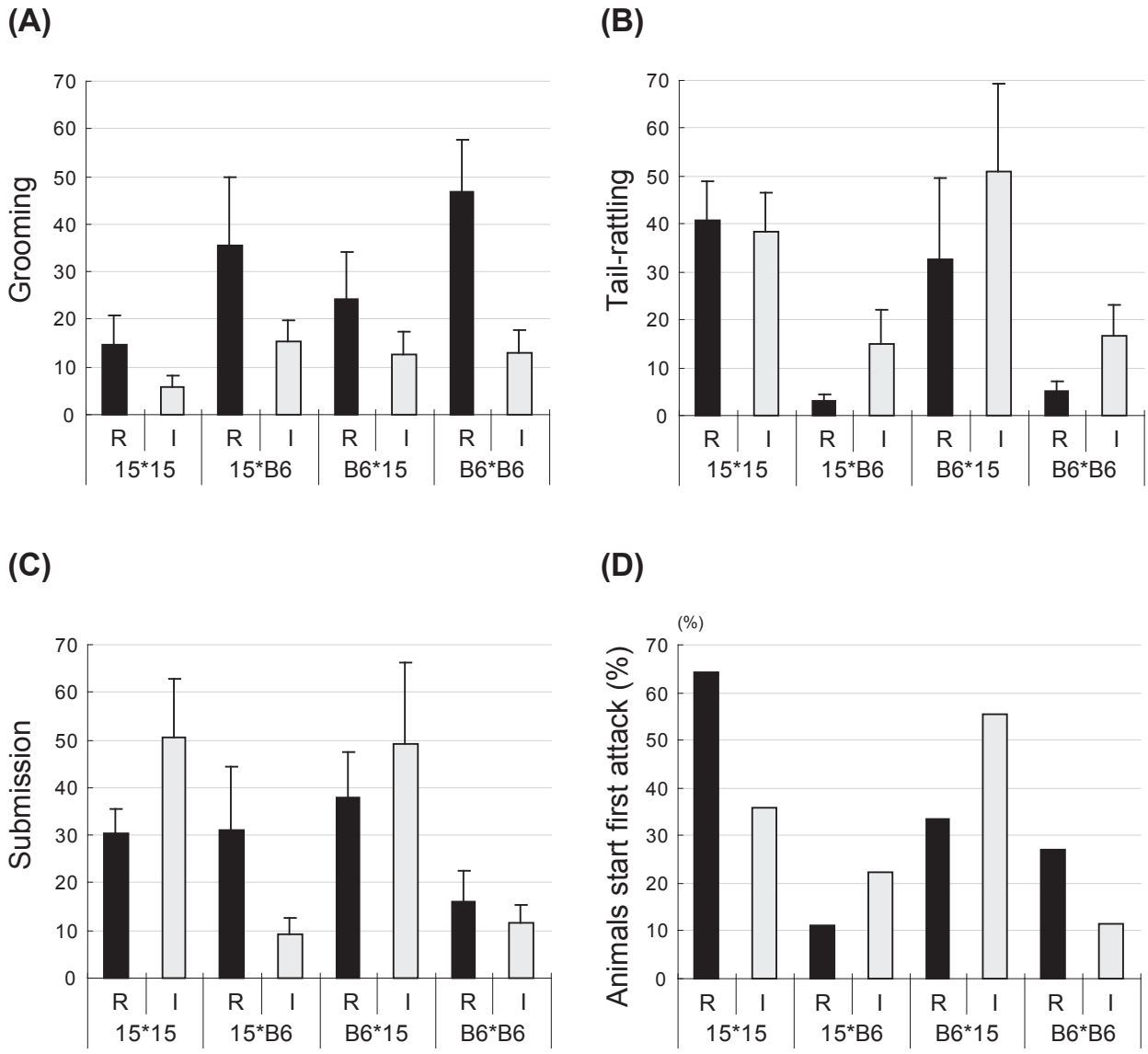


Figure 6.3 Behavior of resident and intruder in homogenous and reciprocal pairs. The result showed the combined data for trial 1 and 2 in the grooming (A), tail-rattling (B), and submission (C) in each resident (abbr. R) and intruder (abbr. I). (D) Percentage of animals that started first attack in trial 1 and 2. First attack was defined from which type of animal, resident or intruder, showed aggressive behavior just before the first attack. If it was indistinguishable, both resident and intruder get 1 point. 15*15: both resident and indtuder were B6-15MSM. 15*B6: B6-15MSM resident vs C57BL/6J intruder. B6*15: C57BL/6J resident vs B6-15MSM intruder. B6*B6: both resident and intruder were C57BL/6J.

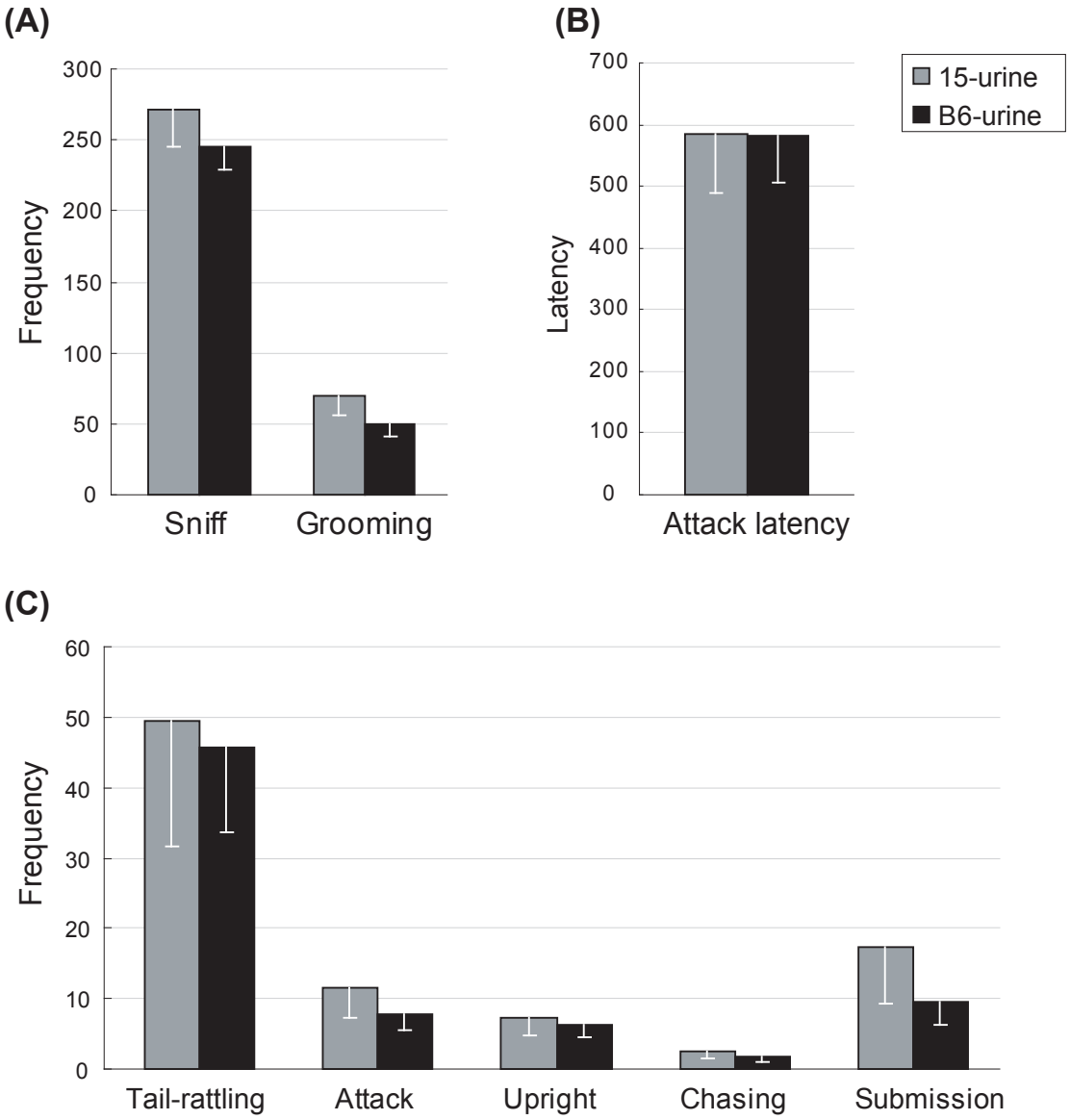


Figure 6.4 Effect of urine on the Aggressive behavior of C57BL/6J. C57BL/6J Intruder was scrubbed with the urin of either B6-15MSM (15-ruine) or C57BL/6J (B6-urine). Combined data of trial 1 and 2 was shown for non-aggressive behaviors (A), latency of the first attack (B), and aggressive behaviors (C). * Significant differences from C57BL/6J ($p<.01$)

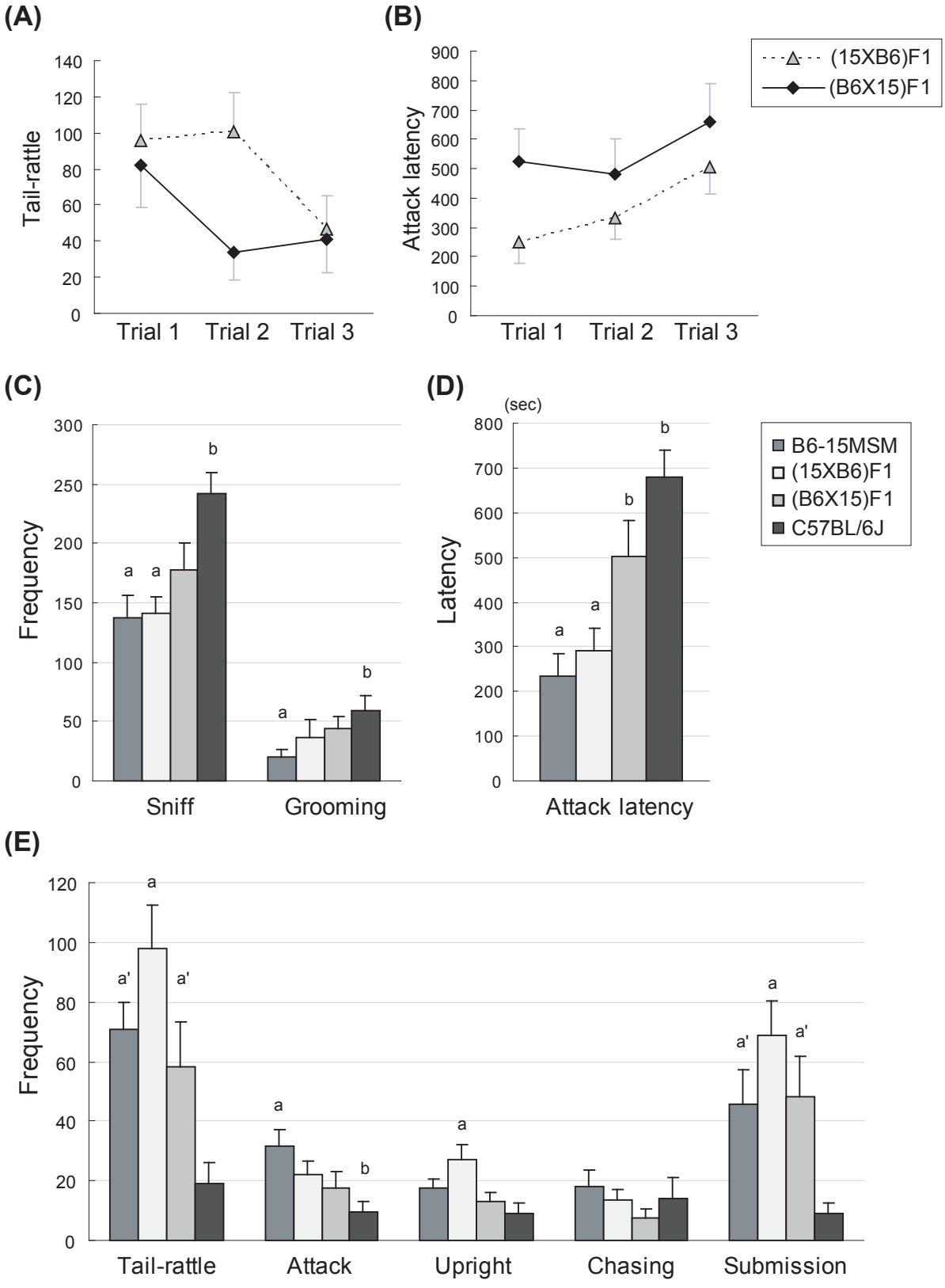


Figure 6.5 Aggressive behavior of reciprocal F1 progeny of B6-15MSM and C57BL/6J. Trial-by-trial change of the frequency of tail-rattling (A) and latency of the first attack (B). Combined data of trial 1 and 2 for Non-aggressive behaviors (C), latency of the first attack (D), and aggressive behaviors (E). a: significant differences compared to C57BL/6J ($p<.05$), b: significant differences from B6-15MSM ($p<.05$). Suggestive differences compared to C57BL/6J (a') and B6-15MSM (b') were also indicated ($p<.10$).

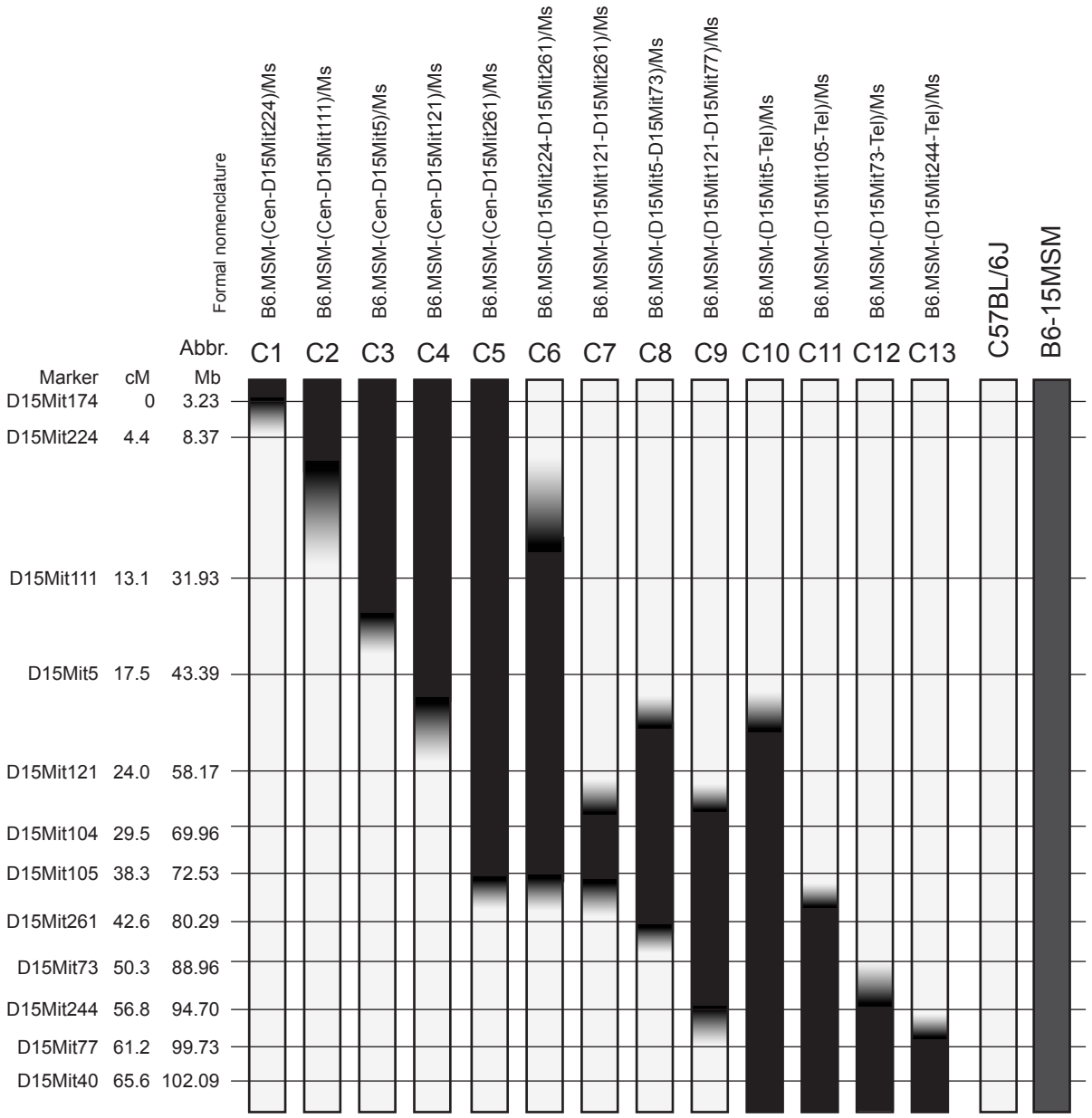


Figure 6.6 A series of congenic strains derived from B6-15MSM. For the simplicity, their name was abbreviated C1 to C13. Substituted chromosomal regions from MSM were represented as Black color.

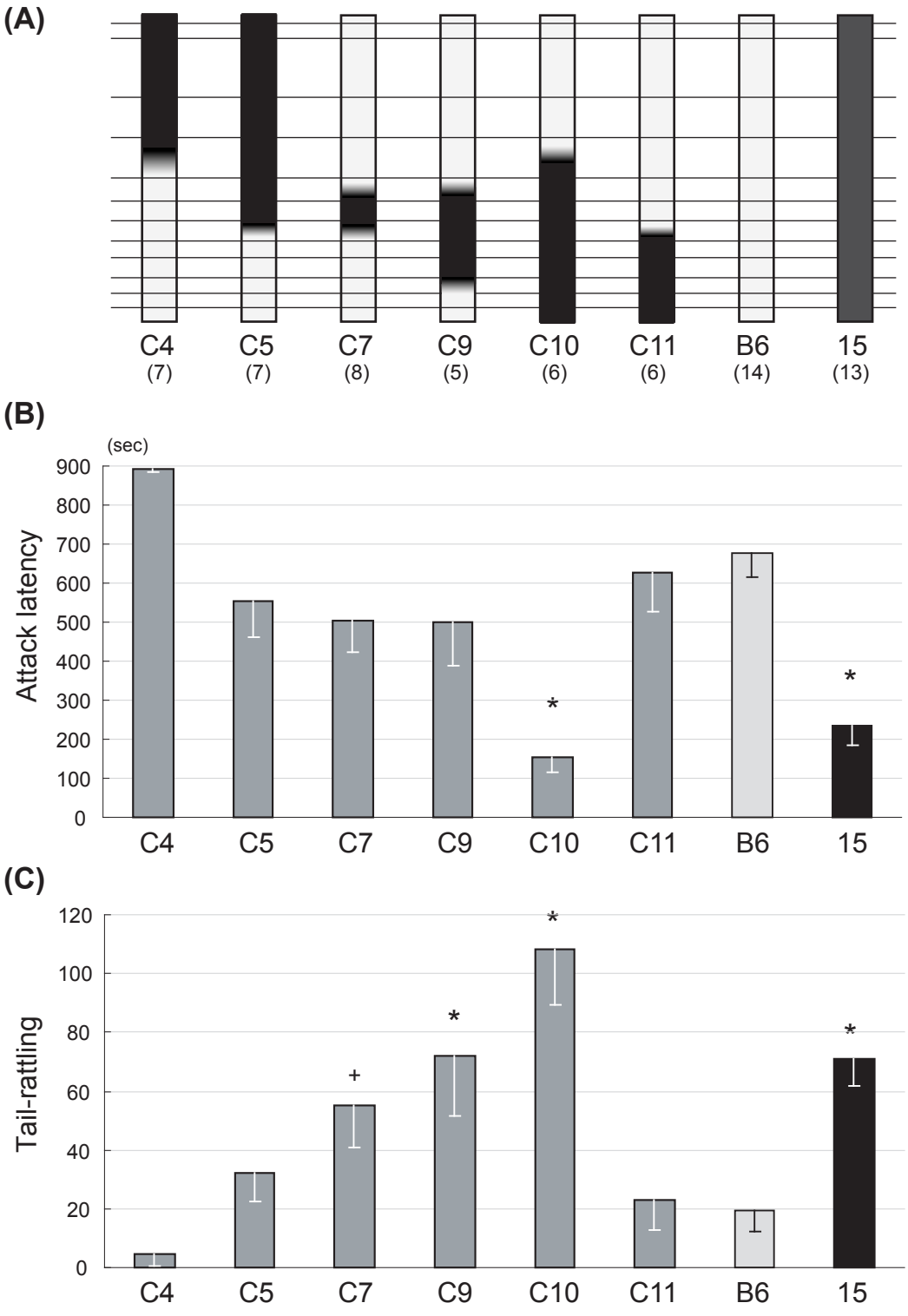


Figure 6.7 Aggressive behavior in congenic strains of B6-15MSM.
(A) Diagram of congenic strains used for the analysis. Substituted chromosomal regions from MSM were represented as Black color. Numbers in parenthesis indicate the set of animals analyzed.(B) Latency of the first attack. (C) Frequency of the tail-rattling in six congenic strains, C57BL/6J (B6), and B6-15MSM (15). Dunnet's t-test were performed to compare congenic strains with B6 (* p<.05, + p<.10).

Table 2.1 Mishima battery of mouse strains

Origin	Strain	Subspecies group	Subspecies	Place of collection
Laboratory	C57BL/6J	Domesticus		
Wild mice	PGN2/Ms	Domesticus	*M. m. domesticus*	Ontario, Canada
	BFM/2Ms	Domesticus	*M. m. brevirostris*	Montepellier, France
	HMI/Ms	Castaneus	*M. m. castaneus*	Heimei, Taiwan
	CAST/Ei	Castaneus	*M. m. castaneus*	Thailand
	NJL/Ms	Musculus	*M. m. musculus*	Northern Jutland, Denmark
	BLG2/Ms	Musculus	*M. m. musculus*	General Toshevo, Bulgaria
	CHD/Ms	Musculus	*M. m. gansuensis*	Chendu, China
	SWN/Ms	Musculus	*M. m. yamasinai*	Suwon, Korea
	KJR/Ms	Musculus	*M. m. yamasinai*	Kojuri, Korea
	MSM/Ms	Musculus	*M. m. molossinus*	Mishima, Japan
Fancy mice	JF1/Ms	Musculus	*M. m. molossinus*	Denmark [a]

[a] JF1 was found in Denmark but characterized as a Japanese fancy mouse by genetic study (Koide et al., 1998).

Table 2.2 Open-field measures of a total of 10 min in the wild-derived strains.

strain	total crossing	central section crossing (%)	defecation	urination	sniffing	locomotion
C57BL/6	378.3±8.8	16.6±0.7	0.5±0.3	2	118.4±0.3	105.5±1.2
PGN2	228.4± 6.3	13.7±1.0	1.1±0.4	1	107.9±2.1	74.7±1.8
BFM/2	324.5± 9.8	19.1±1.4	0.9±0.5	7	114.0±1.8	91.6±1.8
HMI	310.6±11.3	12.0±1.0	1.4±0.4	4	109.0±1.9	87.3±2.2
CAST/Ei	328.6±10.9	12.1±1.6	3.2±0.7	5	117.6±0.4	92.1±2.0
NJL	328.8±12.2	12.3±1.3	5.7±0.8	7	103.6±2.2	82.9±2.1
BLG2	314.8± 8.3	19.9±0.9	4.9±0.6	13	116.2±0.8	92.9±1.7
CHD	247.4±11.3	16.0±2.1	2.2±0.6	8	96.7±3.7	74.5±2.8
SWN	360.9±12.6	14.9±1.6	2.5±0.6	10	110.9±2.0	91.9±2.0
KJR	378.8±12.0	9.4±1.7	9.3±1.0	8	108.5±1.5	92.4±1.8
MSM	200.2± 8.7	10.5±1.0	3.9±0.6	15	100.8±2.8	70.2±2.5
JF1	93.3± 9.1	2.1±0.9	7.1±0.6	9	105.3±2.2	37.4±2.7

strain	stretching	leaning	rearing	grooming	face-washing	digging
C57BL/6	5.0±1.1	44.6±1.8	24.3±1.6	1.7±0.5	5.3±0.7	0.2±0.1
PGN2	1.1±0.4	55.5±2.2	47.3±1.9	1.3±0.4	6.9±0.6	1.7±0.4
BFM/2	1.7±0.5	72.7±2.0	39.5±2.9	1.4±0.7	8.1±0.7	7.2±1.1
HMI	2.7±0.5	69.5±2.1	11.8±1.1	0.2±0.1	9.6±0.8	0.1±0.1
CAST/Ei	7.8±1.1	63.8±2.1	14.2±1.8	2.4±0.7	4.7±0.5	1.7±0.4
NJL	7.9±1.3	64.0±2.1	23.5±1.6	13.9±2.7	3.9±0.5	2.5±0.5
BLG2	0.8±0.3	68.0±0.2	34.1±1.9	2.0±0.6	13.5±1.0	2.7±0.5
CHD	5.9±0.8	25.5±1.9	11.7±1.2	12.5±2.5	4.5±0.6	0.3±0.2
SWN	1.3±0.4	73.1±2.4	33.0±2.0	3.0±0.8	7.1±0.7	4.8±0.8
KJR	0.5±0.2	90.0±2.0	20.6±1.5	4.7±1.0	3.7±0.6	4.3±0.7
MSM	4.9±0.8	49.3±2.1	14.1±1.3	18.8±2.2	3.7±0.5	1.6±0.4
JF1	7.8±0.9	27.1±2.0	0.5±0.2	9.2±1.7	4.3±0.7	0.1±0.1

strain	gnawing	jumping	pausing	freezing
C57BL/6	0.8±0.2	0.1±0.1	3.9±0.5	0.0±0.0
PGN2	3.7±0.5	46.3±2.8	21.6±1.1	10.9±2.3
BFM/2	4.4±0.6	3.7±1.0	7.4±0.8	4.2±1.8
HMI	1.2±0.3	19.4±3.6	15.4±1.2	8.2±2.3
CAST/Ei	4.5±0.6	2.1±0.7	12.9±1.2	0.2±0.1
NJL	4.9±0.7	11.1±2.4	12.4±0.1	1.4±0.6
BLG2	6.3±0.8	3.0±0.8	12.3±1.1	1.0±0.3
CHD	2.5±0.6	1.0±0.5	26.1±1.7	11.0±2.7
SWN	2.4±0.5	9.0±1.5	14.5±1.1	5.5±1.5
KJR	3.0±0.5	19.9±2.9	12.2±1.1	1.8±0.5
MSM	1.8±0.4	0.2±0.1	2.86±1.7	5.0±1.5
JF1	0.1±0.1	0.0±0.0	2.82±1.4	16.3±3.7

All data, without urination, presented as mean values±SEM. For urination, the digit shows the number of animals urinating. For calculating the frequency of the behavioral items, presence or absence of each behavior was recorded as 1/0 in each 5-sec period.
Number of animals = 10 male and 10 female in each colums.

Table 2.3 Factor analysis in the wild-derived mouse strains

items	Factor		
	1	2	3
Ambulation	**0.92**	0.20	
Central amb	**0.67**		**0.70**
Central amb %	0.35		**0.86**
Defecation			**-0.78**
Locomotion	**0.89**	0.20	0.34
Stretching	-0.25	**-0.80**	
Leaning	**0.69**	**0.59**	-0.26
Rearing	0.24	**0.63**	0.48
Grooming	-0.31	**-0.62**	-0.32
Face-washing		0.50	**0.55**
Jumping		**0.82**	
Pausing	**-0.84**		-0.23
Freezing	**-0.91**		
Variance explained %	34.210	21.732	21.323

Factor loadings over 0.5 are boldfaced and below 0.2 are omitted.

Table 3.1 Behavioral analysis of consomic mouse strains established from C57BL/6J and MSM

			Consomic mouse strains																				MSM	# CSS	CSS * sex
			1	2C	2T	3	4	6C	6T	7T	8	9	11	12C	12T	13A	14	15	16	17	19	XC	Y			
Open-field [1st trial]	Ambulation	m	32.6			21.2		46.5	30.1			22.6				25.3	44.8		31.3	31.5					8	
		f	45.0				33.1	50.5	30.7			29.4		32.5				26.7		41.7					7	0.003
	Center time	m	37.3					30.6					32.2			33.8	17.9								5	
		f	55.3																						1	0.057
	Central amb	m	38.9					44.5	26.8				34.8		24.9				30.8						5	
		f	60.6					44.1					47.0	40.1						38.9					5	0.034
	Central amb %	m	21.2										26.2			37.2	28.9								4	
		f	33.4									33.4	39.6												3	0.002
	Defecation	m	57.0					80.6						49.0				98.2	86.1	98.2					6	
		f		29.9				97.5										75.0		33.1					4	0.056
	Stretching	m										44.1								55.9					2	
		f						40.2														58.9			2	0.027
	Leaning	m		31.0		49.1					50.9	42.6			36.9	30.9				33.1					7	
		f		51.7	38.4	71.1						50.8													3	0.134
	Rearing	m						72.5	51.0									34.7							2	
		f	29.3		39.3			55.9	40.0																3	0.511
	Grooming	m																							0	
		f																							0	0.107
	Face-washing	m														59.9			79.3						2	
		f	56.6		63.1				42.7		44.1	64.4	73.3	51.4			46.9		100.0						9	0.296
	Jumping	m				90.0						34.5													2	
		f				81.7																			1	0.995
	Pausing	m	43.2					29.8	36.6							20.0		38.1		40.2					5	
		f	59.5					32.7	29.8									61.3		45.2					4	0.422
Open-field [2nd trial]	Ambulation	m				40.6	31.5	35.3	28.0			29.6				56.1	54.9		33.1	31.8					8	
		f	37.3			35.3		47.4	31.2		31.3	33.4		38.5		31.1		34.0	36.0	37.7					10	0.021
	Center time	m																							0	
		f	49.1											44.6											2	0.259
	Central amb	m					41.2									53.6	43.4					44.9			4	
		f	39.1					35.9								46.4									3	0.452
	Central amb %	m																							0	
		f	42.3																						1	0.521
	Defecation	m						69.2	34.1											58.8					2	
		f						83.1						28.9				49.9		25.4					4	0.215
	Stretching	m											77.3							71.9					2	
		f						76.7																	1	0.480
	Leaning	m	33.9			64.3					35.7	35.0			28.9	43.7		26.3							7	
		f		42.0		68.7		29.2						31.3											4	0.021
	Rearing	m			36.5			50.7	46.0		32.0	44.2						34.9							5	
		f	49.6		41.8			56.0	44.9		38.1		29.3	37.3	34.2			45.8	36.4	38.5		34.7			10	0.054
	Grooming	m																							0	
		f																							0	0.015
	Face-washing	m	64.9													70.7	56.9		62.6						4	
		f				64.2						64.3	60.7						76.1						4	0.062
	Jumping	m				98.8																			1	
		f				99.6																			1	0.999
	Pausing	m	51.8								29.5					48.2		51.2				32.1			5	
		f	69.2						39.1							30.8		58.3	39.1	39.7					6	0.051
Elevated plus-maze	total distance (cm)	m				47.4		39.8	29.3		32.0	41.6		26.4			35.0								6	
		f	42.8	27.4		27.2		41.4			36.1	35.5	34.0	52.0		33.5		29.8	34.7	40.0		33.4			13	0.011
	Closed-arm entry	m				39.0		46.8	40.7																2	
		f	53.2	42.0				51.2	34.6		45.6		37.6	62.6		61.3		34.9	46.5	55.8					10	0.002
	Open-arm entry	m	37.9			39.4			37.5			57.2					38.2		42.8						6	
		f	47.2								44.1		48.0	60.9	53.6				55.3	51.4		52.1			7	0.087
	open-arm time %	m																	63.7						1	
		f															60.4								1	0.864
	Protect head dip	m						52.9	46.4				40.9	62.0	42.6					44.2					4	
		f										40.5		59.5	38.3					42.1					3	0.340
	Open head dip	m									31.5	58.6						29.9	41.4	30.4					5	
		f										68.8				57.7	52.0								3	0.861
SI	Contact duration	m						54.0												52.5					2	
		f						49.7												42.5					2	0.343
	Contact number	m		36.2	39.8	55.2		38.3								39.4	61.7								5	
		f		44.8				50.6									49.4								3	0.000

The name of each CSS was abbreviated as the number of substituted chromosome.
Colored cell indicated the CSSs that showed significant differences from C57BL/6J ($p<.05$, Dunnet's).
: significant increase, : significant decrease compared to C57BL/6J.
Numbers in each colored cell indicate the value of phenotype difference.
CSS: the number of CSSs that showed significant differences from C57BL/6J, CSS*sex: interaction between CSSs and sex in two-way ANOVA. SI: social interaction test, m: male, f: female.

Table 3.2 Three types of QTLs related to the repeated open-field trials

	Common QTL		1st trial specific QTL		2nd trial specific QTL	
	Male	Female	Male	Female	Male	Female
Ambulation	7	6	1	1	1	4
Center time	0	1	5	0	0	1
Central amb	0	2	5	3	4	1
Central amb %	0	1	4	2	0	0
Defecation	2	3	4	1	1	1
Stretching	1	1	1	1	1	0
Leaning	5	2	2	2	2	2
Rearing	2	3	0	0	3	7
Grooming	0	0	0	0	0	0
Face-washing	2	3	0	6	2	1
Jumping	1	1	1	0	0	0
Pausing	3	4	2	1	2	2

Common QTL: the number of CSSs that showed significant effect on both trials.
1st trial specific QTL: the number of CSSs that showed significant differences only on 1st trial.
2nd trial specific QTL: the number of CSSs that showed significant differences only on 2nd trial.

Table 3.3 Genetic and phenotypic correlations between measurements of open-field, elevated-plus maze, and social interaction test.

			Phenotypic correlation																															
			Open-field [1st trial]												Open-field [2nd trial]												Elevated plus-maze					
			Amb	C-time	C-amb	C-%	Def	LO	ST	LE	RE	GR	FW	JP	PA	Amb	C-time	C-amb	C-%	Def	LO	ST	LE	RE	GR	FW	JP	PA	Amb	C-ent	O-ent	O-%	P-hd	O-hd
Genetic correlation	Open-field [1st]	Ambulation		0.20	0.62	0.09	-0.29	0.84	-0.16	0.32	0.30	-0.36	-0.01	0.20	-0.62	0.76	0.32	0.57	0.30	-0.32	0.73	-0.15	0.32	0.41	-0.34	0.09	0.12	-0.40	0.58	0.47	0.43	0.15	0.25	0.29
		Center time	-0.06		0.75	0.87	-0.05	0.29	0.33	-0.43	0.24	-0.30	-0.20	-0.06	-0.25	0.17	0.52	0.38	0.56	-0.06	0.40	0.35	-0.20	0.10	-0.32	-0.17	-0.04	-0.14	0.10	0.07	0.11	0.11	-0.02	0.04
		Central amburation	0.63	0.68		0.77	-0.20	0.57	0.12	-0.14	0.29	-0.35	-0.16	0.08	-0.46	0.49	0.50	0.59	0.56	-0.19	0.46	0.13	0.00	0.25	-0.36	-0.09	0.05	-0.28	0.33	0.28	0.25	0.10	0.11	0.13
		Central amburation %	-0.33	0.92	0.51		-0.02	0.31	0.36	-0.44	0.21	-0.34	-0.16	-0.04	-0.21	0.07	0.43	0.30	0.56	-0.03	0.37	0.30	-0.21	0.08	-0.38	-0.14	-0.02	-0.07	0.06	0.05	0.04	0.05	-0.07	-0.03
		Defecation	-0.54	0.05	-0.43	0.15		-0.38	0.02	-0.09	-0.14	0.05	0.04	-0.04	0.29	-0.26	-0.09	-0.17	-0.05	0.38	-0.32	0.07	-0.11	-0.15	0.03	0.04	-0.06	0.19	-0.20	-0.19	-0.13	0.00	-0.10	-0.07
		Locomotion	0.93	0.02	0.69	-0.22	-0.60		-0.16	0.35	0.36	-0.50	0.03	0.15	-0.63	0.60	0.24	0.43	0.18	-0.28	0.71	-0.17	0.30	0.42	-0.46	0.08	0.10	-0.32	0.52	0.45	0.34	0.07	0.18	0.19
		Stretching	-0.30	0.18	-0.09	0.25	0.14	-0.33		-0.40	-0.14	-0.15	-0.17	-0.05	0.02	-0.03	0.22	0.08	0.23	0.00	-0.02	0.57	-0.16	-0.15	-0.16	-0.11	0.06	0.01	-0.05	-0.05	-0.03	0.10	-0.12	0.01
		Leaning	0.55	-0.50	0.03	-0.60	-0.30	0.55	-0.37		0.06	-0.04	0.18	0.41	-0.15	0.25	-0.13	0.07	-0.12	-0.02	0.28	-0.41	0.64	0.30	0.05	0.21	0.26	-0.11	0.28	0.22	0.17	0.01	0.14	0.14
		Rearing	0.38	0.15	0.41	0.07	-0.48	0.41	-0.47	0.17		-0.25	-0.01	0.03	-0.45	0.27	0.21	0.25	0.25	-0.23	0.32	-0.16	0.09	0.67	-0.28	-0.03	-0.06	-0.26	0.25	0.19	0.15	0.09	0.05	0.10
		Grooming	-0.47	0.17	-0.22	0.29	0.34	-0.52	0.09	-0.47	-0.01		-0.08	-0.06	0.35	-0.27	-0.21	-0.23	-0.30	0.16	-0.36	-0.05	-0.09	-0.21	0.72	-0.10	-0.05	0.07	-0.27	-0.22	-0.08	-0.02	-0.01	-0.02
		Face-washing	-0.08	-0.47	-0.47	-0.48	0.10	-0.16	-0.38	0.16	0.05	-0.13		0.03	-0.04	-0.11	-0.12	-0.14	-0.11	0.05	-0.04	-0.13	0.08	0.03	-0.05	0.56	0.01	0.04	-0.01	-0.01	-0.07	-0.09	-0.03	-0.11
		Jumping	0.34	-0.20	0.13	-0.24	-0.23	0.29	-0.02	0.72	-0.04	-0.33	0.06		-0.13	0.19	0.00	0.11	0.02	-0.03	0.18	-0.09	0.39	0.15	-0.02	0.08	0.55	-0.09	0.25	0.20	0.18	0.06	0.06	0.16
		Pausing	-0.71	-0.10	-0.61	0.07	0.70	-0.73	0.10	-0.29	-0.54	0.25	0.08	-0.21		-0.52	-0.26	-0.39	-0.29	0.35	-0.59	0.12	-0.22	-0.45	0.41	-0.04	-0.13	0.60	-0.44	-0.37	-0.25	-0.11	-0.12	-0.17
	Open-field [2nd]	Ambulation	0.89	-0.12	0.52	-0.35	-0.52	0.80	-0.10	0.62	0.34	-0.40	-0.16	0.42	-0.69		0.39	0.81	0.37	-0.35	0.86	-0.13	0.47	0.40	-0.29	0.02	0.17	-0.50	0.56	0.45	0.44	0.20	0.26	0.36
		Center time	0.52	0.31	0.53	0.10	-0.09	0.46	0.00	0.23	0.27	-0.04	-0.15	0.09	-0.42	0.63		0.67	0.86	-0.17	0.18	0.28	0.03	0.28	-0.23	-0.12	0.01	-0.27	0.20	0.17	0.19	0.13	0.05	0.13
		Central amburation	0.79	0.07	0.60	-0.14	-0.41	0.73	-0.02	0.49	0.31	-0.30	-0.20	0.33	-0.66	0.93	0.79		0.73	-0.25	0.64	0.05	0.23	0.37	-0.24	-0.07	0.09	-0.42	0.37	0.28	0.33	0.19	0.16	0.27
		Central amburation %	0.33	0.51	0.59	0.38	-0.04	0.35	0.13	0.07	0.19	-0.01	-0.24	0.05	-0.35	0.44	0.81	0.71		-0.11	0.10	0.25	0.02	0.36	-0.33	-0.12	-0.01	-0.27	0.20	0.15	0.21	0.15	0.03	0.12
		Defecation	-0.64	-0.07	-0.51	0.10	0.78	-0.62	0.10	-0.25	-0.54	0.27	0.08	-0.16	0.72	-0.71	-0.47	-0.65	-0.29		-0.43	0.12	-0.17	-0.22	0.14	0.02	-0.08	0.23	-0.30	-0.24	-0.19	-0.06	-0.06	-0.13
		Locomotion	0.84	-0.10	0.53	-0.30	-0.56	0.83	-0.08	0.60	0.34	-0.45	-0.23	0.36	-0.71	0.97	0.60	0.91	0.44	-0.73		-0.15	0.55	0.49	-0.39	0.04	0.17	-0.53	0.59	0.48	0.42	0.19	0.23	0.33
		Stretching	-0.44	0.04	-0.28	0.16	0.39	-0.43	0.74	-0.41	-0.64	0.17	-0.21	-0.14	0.22	-0.38	-0.21	-0.29	-0.06	0.46	-0.37		-0.32	-0.23	-0.05	-0.12	-0.02	0.08	-0.13	-0.11	-0.02	0.06	-0.06	-0.03
		Leaning	0.53	-0.36	0.14	-0.43	-0.35	0.49	-0.09	0.87	0.10	-0.43	-0.08	0.73	-0.33	0.73	0.31	0.62	0.16	-0.40	0.73	-0.31		0.35	-0.11	0.17	0.40	-0.30	0.41	0.31	0.23	0.07	0.19	0.24
		Rearing	0.60	-0.03	0.39	-0.17	-0.41	0.60	-0.39	0.53	0.80	-0.26	0.05	0.22	-0.61	0.65	0.52	0.63	0.44	-0.54	0.65	-0.60	0.46		-0.23	0.02	0.04	-0.45	0.40	0.30	0.28	0.15	0.14	0.22
		Grooming	-0.40	0.19	-0.13	0.29	0.39	-0.37	0.06	-0.32	-0.14	0.54	0.01	-0.13	0.25	-0.41	-0.08	-0.33	-0.04	0.39	-0.39	0.16	-0.40	-0.25		-0.06	-0.03	0.05	-0.27	-0.21	-0.11	-0.03	0.03	-0.02
		Face-washing	0.10	-0.49	-0.35	-0.52	0.07	-0.02	-0.18	0.33	-0.09	-0.26	0.77	0.35	-0.01	0.07	-0.09	-0.01	-0.21	0.05	0.02	-0.12	0.18	0.08	0.11		0.09	-0.03	0.06	0.03	0.01	0.00	0.01	-0.01
		Jumping	0.35	-0.06	0.23	-0.13	-0.21	0.26	0.17	0.52	-0.13	-0.26	-0.08	0.89	-0.24	0.41	0.15	0.32	0.04	-0.22	0.35	0.01	0.63	0.05	-0.14	0.21		-0.14	0.23	0.20	0.12	0.00	0.02	0.09
		Pausing	-0.57	-0.11	-0.48	0.02	0.45	-0.54	0.01	-0.26	-0.38	0.14	0.16	-0.17	0.83	-0.67	-0.53	-0.70	-0.46	0.54	-0.66	0.10	-0.39	-0.59	0.11	-0.02	-0.20		-0.33	-0.26	-0.22	-0.17	-0.17	-0.24
	EPM	total distance (cm)	0.79	-0.05	0.49	-0.24	-0.47	0.71	-0.23	0.62	0.36	-0.34	-0.11	0.59	-0.58	0.76	0.40	0.61	0.21	-0.55	0.70	-0.41	0.60	0.60	-0.25	0.09	0.51	-0.46		0.86	0.65	0.08	0.27	0.36
		Closed-arm entry	0.67	0.06	0.52	-0.11	-0.50	0.67	-0.20	0.52	0.35	-0.36	-0.15	0.53	-0.56	0.63	0.33	0.49	0.16	-0.54	0.61	-0.40	0.51	0.54	-0.21	0.03	0.50	-0.40	0.93		0.40	-0.23	0.25	0.04
		Open-arm entry	0.77	-0.14	0.39	-0.34	-0.30	0.69	-0.26	0.60	0.27	-0.40	-0.08	0.49	-0.44	0.70	0.36	0.58	0.23	-0.38	0.64	-0.38	0.49	0.58	-0.28	0.11	0.33	-0.32	0.88	0.72		0.44	0.38	0.55
		% of open-arm time	0.47	-0.22	0.12	-0.31	0.00	0.32	0.04	0.25	-0.01	-0.14	-0.14	0.11	-0.31	0.54	0.23	0.52	0.25	-0.11	0.50	-0.02	0.27	0.31	-0.11	0.14	0.02	-0.41	0.34	0.10	0.58		-0.06	0.72
		Head dip (protected)	0.57	-0.26	0.15	-0.42	-0.23	0.57	-0.24	0.60	0.09	-0.51	0.05	0.25	-0.35	0.57	0.30	0.46	0.12	-0.24	0.56	-0.21	0.57	0.41	-0.43	0.13	0.14	-0.43	0.50	0.42	0.51	0.25		0.18
		Head dip (open arm)	0.67	-0.29	0.20	-0.45	-0.22	0.51	-0.10	0.50	0.11	-0.26	-0.06	0.35	-0.47	0.75	0.40	0.70	0.29	-0.32	0.66	-0.14	0.52	0.46	-0.30	0.14	0.23	-0.57	0.64	0.39	0.74	0.79	0.58	
	SI	Contact duration	-0.65	0.14	-0.44	0.27	0.64	-0.68	0.51	-0.43	-0.57	0.43	-0.19	-0.32	0.56	-0.55	-0.21	-0.45	-0.02	0.62	-0.51	0.50	-0.38	-0.48	0.42	-0.12	-0.28	0.34	-0.55	-0.54	-0.39	0.05	-0.41	-0.29
		Contact number	0.62	0.17	0.52	-0.08	-0.44	0.62	-0.20	0.27	0.40	-0.33	-0.17	0.13	-0.41	0.58	0.42	0.52	0.27	-0.63	0.61	-0.47	0.34	0.39	-0.21	-0.01	0.17	-0.24	0.42	0.35	0.42	0.25	0.19	0.24

Correlations exceed 0.70 were represented as red font, and below 0.20 were gray font. SI: social interaction test.

Table 3.4 Factor analysis of the open-field measurements in the consomic mouse strains

items	Factor		
	1	2	3
Ambulation	**0.91**		
Central amb	**0.69**	**0.66**	
Central amb %	-0.20	**0.93**	
Center time		**0.94**	
Defecation	**-0.72**		-0.20
Locomotion	**0.95**		
Stretching		0.27	**-0.79**
Leaning	**0.57**	**-0.60**	
Rearing	0.38	0.21	**0.78**
Grooming	**-0.61**	0.33	0.23
Face-washing	-0.23	**-0.61**	0.44
Pausing	**-0.79**		-0.25
Variance explained %	38.231	26.613	11.164

Factor loadings over 0.5 are boldfaced and below 0.2 are omitted.

Table 3.5 Factor analysis of three kind of emotionality-related tests in the CSSs

	Factor				
	1	2	3	4	5
Open-field					
Ambulation	**0.53**	**0.61**		0.48	0.19
Central amb	0.27	**0.55**	**0.72**		0.17
Central amb %	-0.28		**0.87**	-0.28	
Center time			**0.93**		
Defecation	-0.29	**-0.85**			
Locomotion	**0.55**	**0.65**		0.34	0.26
Stretching					**-0.90**
Leaning	**0.73**		-0.37	0.21	
Rearing		**0.65**			**0.58**
Grooming	**-0.66**	-0.28	0.21		
Face-washing			**-0.63**	-0.26	0.34
Pausing	-0.21	**-0.80**		-0.30	
Elevated plus-maze					
total distance	**0.78**	0.38		0.33	
Closed-arm entry	**0.80**	0.38	0.21		
Open-arm entry	**0.68**	0.25		**0.57**	
% of open-arm time				**0.94**	
Head dip (protect)	**0.66**		-0.27	0.34	0.36
Head dip (open)	0.40			**0.82**	
Social interaction					
Contact duration	-0.42	**-0.63**			-0.39
Contact number		**0.60**		0.25	
Variance explained %	21.943	20.880	15.004	14.355	8.891

Factor loadings over 0.5 are boldfaced and below 0.2 are omitted.

Table 5.1 Behavioral analysis of F1 progeny made from C57BL/6J and B6-17MSM

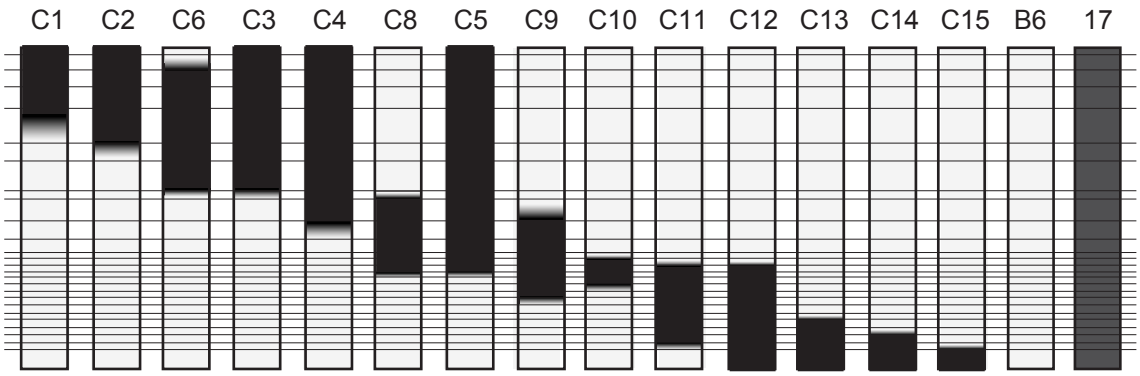
	(B6x17)F1	(17xB6)F1	C57BL/6J	B6-17MSM
Open-Field	n = 30	n = 26	n = 70	n = 68
Ambulation	300.4 ab	253.0 a	354.1 b	255.4 a
Central amb	61.9 b	49.9 a	73.5 b	46.5 a
Center %	20.6	19.9	20.5 b	17.3 a
Defecation	1.5 a	1.5 a	0.4 b	1.5 a
Locomotion	92.6 b	80.8 a	99.4 b	78.3 a
Stretching	5.2	7.1	5.1 b	7.3 a
Leaning	38.3	35.7 a	42.5 b	33.1 a
Rearing	19.4 a	23.2	28.6 b	21.7 a
Grooming	1.2 b	1.7	1.1 b	2.3 a
Face-washing	8.4	6.5 a	11.5	9.3
Pausing	14.7 a	10.8 b	9.3 b	19.2 a
Squeaking	0.5	0.3 b	0.2 b	0.6 a
Elevated Plus-Maze	n = 16	n = 16	n = 40	n = 55
Total distance	1139.3	927.6 a	1363.6 b	910.0 a
Closed-arm entry	9.1	6.3 a	12.7 b	4.8 a
Open-arm entry	7.9	5.9 a	9.9	8.0

T-test with Bonferroni correction was performed,

a: significant differences compared to C57BL/6J ($p<.05$)

b: significant differences compared to B6-17MSM ($p<.05$)

Table 5.2 Behaviors of congenic mouse strains of B6-17MSM in open-field (OF), elevated plus-maze (EPM) and social interaction test (SI)



		Congenic strains														B6	17
		C1	C2	C6	C3	C4	C8	C5	C9	C10	C11	C12	C13	C14	C15		
OF	Ambulation	294.6	334.2	310.6	325.3	319.0	257.9	290.1	298.5	279.1	283.2	244.5	323.9	270.7	246.1	370.6	243.9
	Center amb	65.4	81.4	76.6	80.8	64.0	47.0	62.3	64.3	48.8	61.4	48.1	69.1	48.4	46.1	75.6	44.6
	Center %	22.0	24.8	24.4	24.4	19.9	17.5	21.6	21.5	16.7	21.9	19.1	21.3	17.6	18.2	20.2	17.2
	Center time	70.5	83.3	82.4	84.7	68.3	55.1	70.2	79.1	51.3	72.5	57.0	64.4	56.2	51.8	71.5	48.3
	Defecation	0.8	0.3	0.8	0.3	0.2	0.7	0.6	0.5	0.8	0.4	1.5	0.4	1.1	1.3	0.4	1.6
	Locomotion	89.5	96.0	92.3	98.8	101.4	84.1	91.0	92.3	87.4	91.0	78.5	90.6	86.3	79.0	101.3	77.5
	Stretching	6.5	6.7	7.2	5.4	4.0	2.9	3.8	3.6	4.7	5.5	7.3	7.9	6.8	7.3	4.3	7.5
	ST2-10min	2.1	2.3	3.0	1.5	0.7	0.4	0.9	0.5	1.0	1.2	2.3	2.5	2.4	2.2	0.4	2.8
	Leaning	39.2	34.6	32.7	35.6	47.4	33.5	37.1	33.1	39.8	39.0	34.6	33.9	36.1	34.4	44.9	32.7
	Rearing	21.5	29.2	23.6	25.7	27.0	24.1	26.7	25.4	19.0	19.9	13.6	17.8	20.6	15.9	29.4	22.0
	Grooming	1.3	1.4	0.8	1.5	1.4	2.8	2.4	1.6	2.3	1.2	2.0	1.7	1.7	2.0	1.1	2.3
	Face-wash	8.8	6.5	5.3	4.7	9.2	11.6	7.6	8.3	11.8	8.5	10.4	12.1	11.7	11.9	12.8	8.5
	Jumping	1.0	0.1	0.0	0.2	1.6	0.6	0.5	0.4	0.4	0.6	0.4	0.1	0.1	0.2	0.6	0.3
	Pausing	8.8	6.5	10.0	4.6	9.5	12.3	9.0	6.9	12.3	6.9	12.1	13.5	12.4	19.2	7.1	16.8
	Squeaking	28.2	25.0	24.1	26.9	47.6	60.0	71.0	41.9	14.3	11.5	15.5	3.6	2.9	11.1	13.6	56.8
EPM	Ambulation	1003	1156	1020	1000	1040	991	1081	937	1038	1105	926	1033	978	884	1314	910
	Close-entry	7.6	8.3	4.9	7.6	11.6	9.4	6.4	7.7	9.3	8.4	6.8	8.8	6.9	7.7	12.1	4.8
	Open-entry	7.5	9.1	7.8	4.9	5.8	6.2	7.5	5.9	7.7	8.1	5.7	7.7	6.6	5.8	10.3	8.0
	Open %	25.1	28.7	38.8	25.8	18.1	18.2	31.6	25.3	22.3	25.6	19.9	22.0	20.9	20.1	24.8	29.8
SI	ContDur	95.6	85.8	53.1	99.7		50.6	101.0	76.0	54.1	59.7	66.1	62.3	70.8	78.8	81.2	167.2
	ContNumb	39.7	43.8	39.0	43.3		36.7	42.9	43.6	38.1	41.4	40.6	37.4	36.9	35.9	45.5	43.3

Numbers in each cell shows the mean value of the behavior in each congenic strains.
Colored cell indicated significantly higher : p<.01 : p<.05 : p<.10
or lower : p<.01 : p<.05 : p<.10 than C57BL/6J.

ST2-10min: stretching behavior in two or later minutes, Squeaking: % of animals squeaked when they picked up by its tail with tweezers, ConDur: duration of social contact, ContNumb: number of social contact.

Table 5.3 Genetic and phenotypic correlations between measurements of open-field, elevated-plus maze (EPM), and social interaction test (SI) in the congenic strains of B6-17MSM

			Phenotypic correlation																
			Open-field													Elevated plus-maze			
			AMB	CEN	Cen%	CenT	Def	LO	ST	LE	RE	GR	FW	JP	PA	cheep	Amb	C-ent	O-ent	O-%
Genetic correlation	Open-field	Ambulation		0.73	0.25	0.32	-0.30	0.79	-0.19	0.43	0.34	-0.18	-0.02	0.25	-0.45	0.05	0.36	0.24	0.23	0.00
		Center amb	0.88		0.82	0.72	-0.28	0.57	0.04	0.10	0.35	-0.16	-0.12	0.07	-0.46	0.11	0.20	0.03	0.21	0.17
		Center amb %	0.69	0.94		0.79	-0.17	0.19	0.23	-0.19	0.23	-0.11	-0.13	-0.07	-0.31	0.10	0.01	-0.14	0.13	0.27
		Center time	0.69	0.88	0.91		-0.17	0.26	0.17	-0.17	0.33	-0.14	-0.15	-0.04	-0.36	0.09	0.04	-0.10	0.12	0.23
		Defecation	-0.63	-0.46	-0.29	-0.39		-0.37	0.02	-0.11	-0.23	0.12	0.06	-0.06	0.32	-0.09	-0.05	0.00	-0.03	-0.07
		Locomotion	0.87	0.73	0.55	0.62	-0.71		-0.33	0.51	0.40	-0.19	-0.08	0.24	-0.52	0.05	0.35	0.21	0.22	0.01
		Stretching	-0.04	0.08	0.16	0.00	0.40	-0.26		-0.42	-0.18	-0.18	-0.09	-0.14	0.11	-0.07	-0.22	-0.19	-0.09	0.14
		Leaning	0.21	-0.03	-0.17	-0.14	-0.36	0.45	-0.34		0.17	0.02	0.11	0.36	-0.15	-0.02	0.33	0.32	0.13	-0.23
		Rearing	0.48	0.45	0.38	0.56	-0.61	0.61	-0.52	0.19		-0.08	0.00	0.14	-0.40	0.16	0.20	0.13	0.15	0.06
		Grooming	-0.39	-0.51	-0.54	-0.48	0.22	-0.45	-0.29	-0.13	-0.15		0.05	-0.06	0.08	0.11	-0.09	-0.03	-0.08	-0.03
		Face-wash	-0.44	-0.55	-0.57	-0.57	0.39	-0.56	0.02	0.01	-0.41	0.46		0.06	0.01	0.00	0.00	0.08	-0.07	-0.13
		Jumping	0.14	-0.10	-0.22	-0.14	-0.38	0.36	-0.35	0.73	0.07	-0.03	-0.17		-0.16	-0.02	0.12	0.08	0.15	-0.01
		Pausing	-0.60	-0.66	-0.64	-0.77	0.55	-0.69	0.32	-0.15	-0.59	0.34	0.59	-0.14		-0.09	-0.18	-0.05	-0.11	-0.13
		Squeaking	0.05	0.11	0.14	0.21	-0.20	0.20	-0.64	0.09	0.51	0.30	-0.14	0.20	-0.26		-0.01	-0.02	-0.09	0.03
	EPM	Ambulation	0.38	0.29	0.22	0.24	-0.49	0.47	-0.19	0.41	0.45	-0.26	-0.34	0.36	-0.39	-0.07		0.75	0.47	-0.34
		Close-arm enfry	0.03	-0.18	-0.30	-0.19	-0.33	0.24	-0.37	0.58	0.27	0.03	0.24	0.44	-0.01	0.00	0.52		0.11	-0.66
		Open-arm entry	0.16	0.17	0.19	0.15	-0.22	0.12	0.12	0.06	0.21	-0.29	-0.23	0.00	-0.19	-0.20	0.70	0.23		0.22
		Open-arm %	0.40	0.56	0.64	0.59	-0.15	0.27	0.20	-0.32	0.17	-0.31	-0.64	-0.18	-0.38	0.12	0.10	-0.67	0.31	
	SI	Contact duration	0.30	0.26	0.22	0.21	-0.25	0.37	-0.10	0.34	0.33	-0.01	-0.20	0.20	-0.29	0.21	0.12	-0.10	-0.10	0.13
		Contact number	0.45	0.38	0.28	0.33	-0.45	0.48	-0.45	0.46	0.46	-0.16	-0.18	0.27	-0.54	0.26	0.31	0.03	-0.09	0.07

Correlations exceed 0.70 were represented as red font, and below 0.20 were gray font.

Table 5.4 Factor analysis of the open-field measurements in the congenic strains of B6-17MSM

items	Factor	
	1	2
Ambulation	**0.80**	0.34
Central amb	**0.95**	
Central amb %	**0.94**	
Center time	**0.92**	
Defecation	-0.43	**-0.72**
Locomotion	**0.71**	**0.59**
Stretching		**-0.82**
Leaning		**0.67**
Rearing	0.45	**0.66**
Grooming	**-0.65**	
Face-washing	**-0.69**	
Pausing	**-0.72**	-0.44
Variance explained %	44.041	22.692

Factor loadings over 0.5 are boldfaced and below 0.2 are omitted.