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FULL PAPER

Identification of multiple genetic loci in the mouse controlling immobility time in the tail suspension and forced swimming tests

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Abstract

Depression is one of the most famous psychiatric disorders in humans in all over the countries and considered a complex neurobehavioral trait and difficult to identify causal genes. Tail suspension test (TST) and forced swimming test (FST) are widely used for assessing depression-like behavior and antidepressant activity in mice. A variety of antidepressant agents are known to reduce immobility time in both TST and FST. To identify genetic determinants of immobility duration in both tests, we analyzed 101 F_2 mice from an intercross between C57BL/6 and DBA/2 strains. Quantitative trait locus (QTL) mapping using 106 microsatellite markers revealed three loci (two significant and one suggestive) and five suggestive loci controlling immobility time in the TST and FST, respectively. Results of QTL analysis suggest a broad description of the genetic architecture underlying depression, providing underpinnings for identifying novel molecular targets for antidepressants to clear the complex genetic mechanisms of depressive disorders.

Key Words: depression, tail suspension test, forced swimming test, mice, QTL analysis

Introduction

Mood disorders such as depression and its correlated anxiety symptoms are the most prevalent diseases among psychiatric disorders and a leading cause for disability worldwide. Understanding the mood disorders in animals is a major challenge because of the complex nature

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of such disorders. Understanding the molecular basis related to stress and mood depression is extremely useful not only with respect to the identification and improvement of therapeutic substances, but also regarding the validation of neurobiological underpinnings.

Depression, officially termed major depressive disorder (MDD), ranks among the most prevalent diseases worldwide. According to the estimations of the World Health Organization, depression will be the second leading cause of disability in 2020[12]. It is known that symptoms of anxiety are seen in many individuals with depression[1]. Anxiety disorders affect 18% of people in the United States each year and are prevalent worldwide and debilitating conditions of the people who suffer from them[12]. Anxiety disorders are variable in behavioral and psychological expression and therefore are likely to be affected by a diverse architecture of genetic factors[4]. Anxiety is well-defined psychological phenomena controlled by homologous brain regions in humans and experimental animal models[8]. Few genes have been consistently identified by forward genetic studies that contribute to our understanding of the etiology of anxiety disorders in either human or animal studies[22].

Two well validated paradigms used to measure depression are tail suspension test (TST) and forced swimming test (FST), both of which were developed as screening tests for assessing depression in rodents[23]. In the FST, mice, when forced to swim in a water-filled glass cylinder from which they cannot escape, will rapidly adopt a characteristic immobile posture, making only those movements necessary to maintain their heads above water. This immobile posture is said to reflect a state of "behavioral despair" on the assumption that the animals have given up hope of escaping[22]. The duration of immobility is reduced by using antidepressant drugs[14]. The TST is another simple behavioral model based on an immobility response to inescapable aversive stimulation. In this test, when mice are suspended by the tail, they become immobile. As in the FST, immobility in the TST is sensitive to a wide variety of antidepressants[19]. The duration of immobility in both tests has been inferred as an index of behavioral despair, where longer durations of immobility imply greater degree of behavioral despair[23].

Several studies have reported inter-strain differences in the response to the TST and FST [21]. Because the reactivity to therapeutics is affected by manifold processes of drug metabolism, we believe that the baseline immobility time can more suitably depict an innate vulnerability to stressors and a predisposition to despair under distress. Therefore, in this study, we aimed to determine constitutive genetic factors in mice that contribute to baseline performance in the TST and FST, by means of quantitative trait locus (QTL) mapping approach.

Materials and Methods

Animals: All experimental procedures involving animals were performed in accordance with the regulation of Hokkaido University and the protocol was approved by the President of Hokkaido University through review by IACUCs of both Veterinary School and University. C57BL/6NCrSlc (B6), DBA/2CrSlc (D2), and F_1(B6 x D2) mice were all purchased from Japan SLC (Shizuoka, Japan). F_2 progenies were produced by mating F_1 mice randomly. Animals were housed under a 12 : 12 h light and dark cycle (LD 12 : 12) and maintained in specific pathogen-free conditions with feeding and drinking *ad libitum*. Behavioral tests were conducted using 8-week-old male mice. In the experimental animal care and handling, the investigators adhered to the Regulation for the Care and Use of Laboratory Animals, Hokkaido University.

Measurement of behavioral phenotype: On day 1, TST was performed. Mice were suspended by their tails using an elastic band (5 cm in

diameter) attached to tail by adhesive tape (approximately 1 cm from the tip of the tail) and the elastic band was hooked on a horizontal rod. The distance between the tip of the nose of the mouse and the floor was approximately 20 cm. Mice were suspended for a period of 7 min with video-track, and the time spent immobile (immobile and not struggling) during the last 6 min of 7 min was scored by an observer blinded to the genotype. On day 2, the pre-session of FST was performed. Each mouse was placed in a plastic cylinder (25 cm high, 15 cm in diameter), containing water at 25°C to a depth of 15 cm, and was forced to swim for 15 min. On day 3, the mice were placed in the same pool, the mice were video-tracked for 7 min, and the duration of immobility during swimming in the last 6 min of 7 min was scored by an observer blinded to the genotype[20].

Genotyping of microsatellite markers: Extraction of genomic DNA from tail clips was performed by the standard methods. A total of 106 microsatellite markers showing polymorphisms between B6 and D2 mice were used for genetic study (Table 1). The average interval of adjacent microsatellite markers was 14 cM. The map positions of microsatellite loci were based on information from the Mouse Genome Informatics (MGI; http://www.informatics.jax.org). PCR was carried out on a Bio-Rad PCR thermal cycler (iCycler, California, USA) with the cycling sequence of 95°C for 1 min (one cycle), followed by 35 cycles consisting of denaturation at 95°C for 30 sec, primer annealing at 58°C for 30 sec, and extension at 72°C for 30 sec. PCR mixture and enzymes (*Ex Taq* DNA Polymerase) were purchased from TaKaRa (Otsu, Japan). The amplified samples were electrophoresed with 10–15% polyacrylamide gel (Wako, Osaka, Japan), stained with ethidium bromide, and then photographed under an ultraviolet lamp.

QTL analysis: QTL analysis was performed with Map Manager QTXb20 software program[11]. In this program, linkage probability was examined by interval mapping. Genome-wide significance thresholds were set, as suggested previously, at the 37th ("suggestive"), 95th ("significant"), and 99.9th ("highly significant") percentiles, which correspond to the chance of finding 1 false positive linkage 0.63, 0.05, and 0.001 times, respectively. For each chromosome, the likelihood ratio statistic (LRS) values were calculated by 5,000 random permutations of the trait values relative to genotypes of the maker loci. For the quantitative trait of immobility time of both tests, suggestive, significant, and highly significant values were 10.9, 19.3, and 51.9 for TST, and 10.9, 19.9, and 54.6 for FST, respectively. Confidence intervals (CI) were estimated by bootstrap analysis instead of the classic 1-LOD (logarithm of odds) support interval, since it has been shown to be more reliable over all QTL strengths[5].

Results

Phenotyping of parental strains and their F_1 and F_2 progenies

We first examined the immobility profiles of two conventionally used mouse strains, B6 and D2, in the FST and TST, to determine strain differences in our measuring system. The choice of these mouse strains is based on reports that these strains have differential response to stress including TST[9,24,25], differential basal anxiety[13], and differ in both, their cognitive abilities[15] and sensitivity to antidepressants[3,17]. When mice were video-tracked for 7 min in both behavioral tests, B6 mice showed much longer immobility time than D2 mice, making these two strains suitable for genetic analysis. Therefore, we obtained F_1 mice intercrossed between B6 females and D2 males. Immobility time for F_1 was assessed and compared to that of parental strains (Fig. 1). B6 mice showed longer immobility time than D2 and F_1 in both tests. The immobility average times for TST were 31.3 ±

Table 1. Microsatellite markers used for genotyping of F_2 mice

Marker	Position (cM)	Marker	Position (cM)	Marker	Position (cM)	Marker	Position (cM)
D1Mit1002	4.94	*D5Mit180*	11.93	*D9Mit18*	71.49	*D15Mit12*	1.8
D1Mit324	29.13	*D5Mit108*	23.91	*D10Mit248*	5.21	*D15Mit5*	16.74
D1Mit415	43.94	*D5Mit258*	33.95	*D10Mit61*	34.8	*D15 Mit156*	32.19
D1Mit191	52.66	*D5Mit208*	48.51	*D10Mit186*	38.56	*D15Mit159*	41.96
D1Mit14	67.71	*D5Mit188*	57.51	*D10 Mit14*	66.75	*D15 Mit161*	52.78
D1Mit291	88.97	*D5Mit370*	65.23	*D10Mit297*	72.31	*D16Mit182*	2.57
D2Mit293	17.24	*D5Mit222*	81.53	*D11Mit226*	5.64	*D16Mit59*	26.86
D2Mit296	21.81	*D6Mit159*	12.36	*D11Mit21*	25.94	*D16 Mit140*	40.3
D2Mit91	39.24	*D6Mit74*	23.7	*D11 Mit4*	41.87	*D16Mit152*	48.23
D2Mit185	55.23	*D6Mit188*	32.53	*D11Mit212*	54.34	*D16Mit106*	57.68
D2Mit62	59.34	*D6Mit104*	51.53	*D11Mit199*	65.48	*D17Mit198*	14.59
D2Mit286	76.74	*D6Mit374*	64.6	*D11 Mit48*	82.96	*D17Mit139*	27.4
D2Mit229	88.99	*D6Mit15*	77.7	*D12Mit219*	9.69	*D17Mit218*	43.76
D2Mit200	102.29	*D7Mit114*	15.42	*D12Mit172*	21.09	*D17Mit221*	59.77
D3Mit164	21.73	*D7Mit82*	32.76	*D12Mit5*	37.16	*D18Mit132*	11.92
D3Mit182	21.73	*D7Mit318*	42.27	*D12Mit101*	51.55	*D18Mit17*	21.09
D3Mit28	39.27	*D7Mit66*	64.3	*D13Mit17*	7.73	*D18Mit124*	32.15
D3Mit14	61.32	*D7Mit333*	82.25	*D13Mit63*	21	*D18Mit184*	39.7
D3Mit129	80.49	*D8Mit4*	18.89	*D13Mit9*	42.19	*D18Mit7*	51.92
D4Mit235	3.57	*D8Mit100*	29.7	*D13Mit148*	56.69	*D19Mit69*	8.93
D4Mit237a	22.38	*D8Mit234*	39.33	*D13Mit262*	63.93	*D19Mit80*	18.24
D4Mit139	29.65	*D8Mit242*	50.07	*D14Mit10*	6.41	*D19Mit33*	51.76
D4Mit152	39.36	*D8Mit200*	61.37	*D14Mit120*	20.88	*DXMit166*	28.26
D4Mit303	45.55	*D9Mit90*	17.8	*D14Mit102*	34.36	*DXMit130*	55.45
D4Mit308	57.66	*D9Mit302*	36.36	*D14Mit225*	39.46	*DXMit186*	76.75
D4Mit54	70.02	*D9Mit133*	45.8	*D14Mit165*	56.16		
D4Mit42	82.64	*D9Mit355*	51.41	*D14Mit266*	64.86		

7.4 sec, 208.6 $\pm$ 21.3 sec, and 86.5 $\pm$ 15.4 sec in D2, B6, and F_1 mice, respectively. On the other hand, the immobility average times for FST were 111.3 $\pm$ 11.2 sec, 219 $\pm$ 11.8 sec, and 175.2 $\pm$ 11.9 sec in D2, B6, and F_1 mice, respectively. There was significant difference for TST and FST among B6, D2, and F_1 mice ($F_{2,28}$ values were 22.338, $P < 0.001$ and 12.088, $P < 0.001$ for TST and FST, respectively) .

Next, we measured phenotype of F_2 progenies. We used a total of 101 F_2 animals for phenotypic and genetic analyses. The distributions of immobility periods in F_2 mice between the TST and FST showed a weak correlation with the correlation coefficient value of 0.379 (Fig. 2).

Genome-wide scan for mapping loci controlling immobility time in F_2 progenies

To map the genes responsible for the immobility time observed in the TST and FST, we genotyped 101 F_2 mice using 106 microsatellite markers covering the whole genome and showing polymorphisms between B6 and D2 mice. QTL analysis was performed with Map Manager QTXb20 software program. In this QTL analysis, bootstrap analysis was performed to detect CI of the QTL. Only peak located in the CI was recognized as a substantial QTL. Two significant QTLs regulating immobility time for TST were detected on chromosomes (Chrs) 4 and 5 and one suggestive QTL was detected on Chr 8 (Fig. 3).

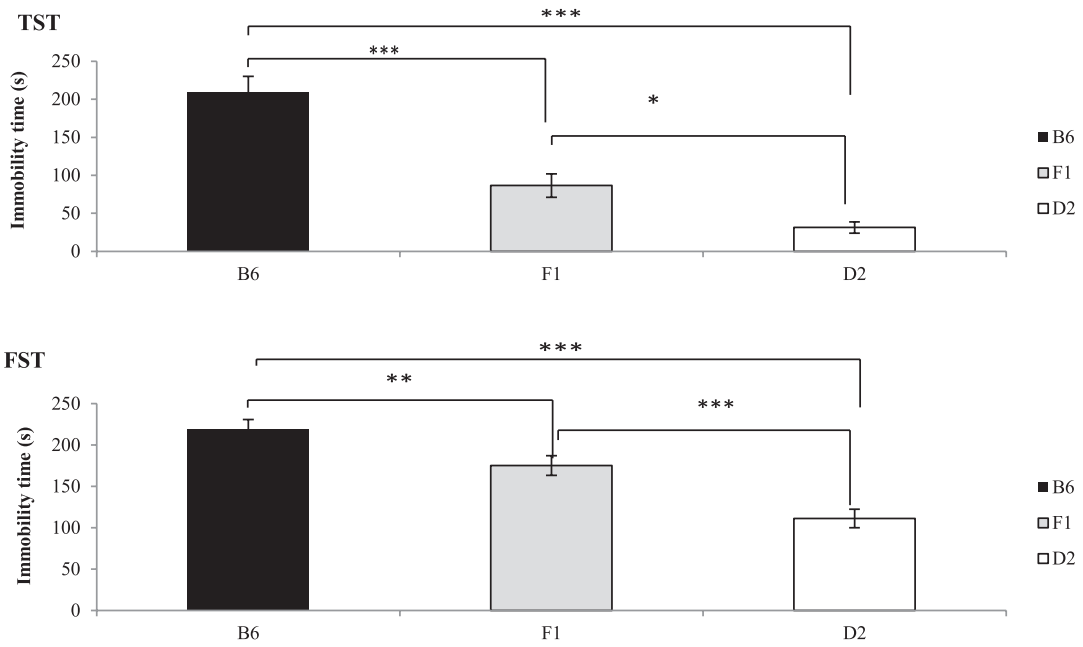

Fig. 1. Immobility time in TST and FST for parental and F_1 mice. The D2 and F_1 mice showed a significantly shorter immobility time than B6 mice in both TST and FST. Statistic analysis was performed with Bonferroni test after one-way ANOVA. *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, at the comparison of each group. $F_{2,28}$ values were 22.338, $P < 0.001$ and 12.088, $P < 0.001$ for TST and FST, respectively, when three groups were simultaneously compared in each behavioral test.

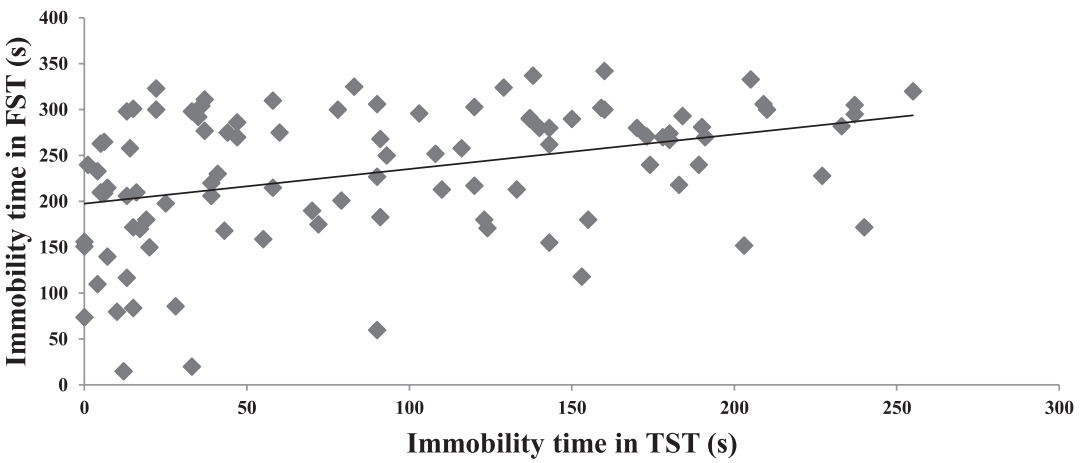

Fig. 2. Correlation of immobility time between TST and FST in F_2 mice. Correlation coefficient, $r = 0.379$, $P = 0.002$.

Table 2 summarizes microsatellite markers linked to the phenotype, showing LRS values, genetic effects, CIs, and phenotypic values in each genotype. For FST, 5 suggestive QTLs showing exceeding suggestive threshold LRS values were detected on Chrs 6, 14, and 17 (Fig. 4). Table 3 summarizes microsatellite markers linked to the phenotype, showing LRS values, genetic effects, CIs, and phenotypic values in each genotype.

Discussion

Our preliminary phenotypic survey showed significant longer immobility duration in B6 mice for both TST and FST, when compared to D2

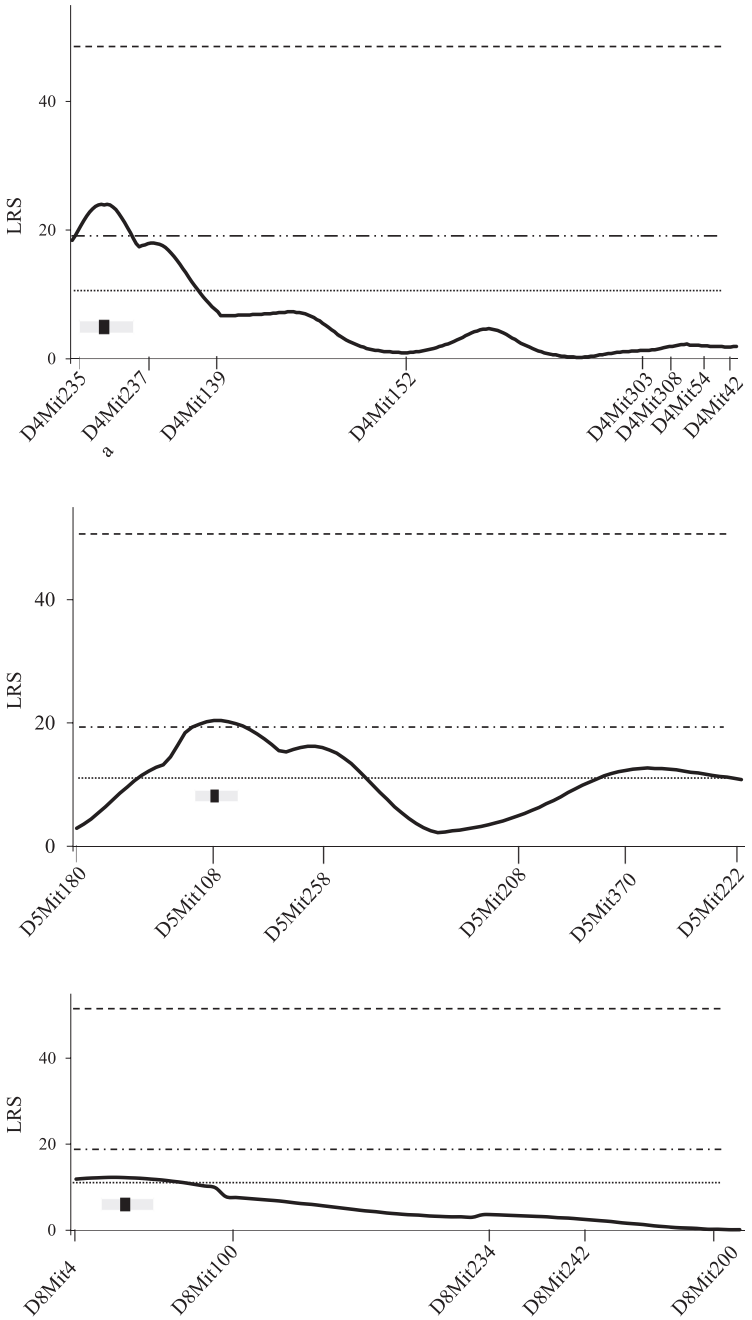

Fig. 3. QTLs detected to influence the immobility time in TST in F_2 mice. The dotted, dashed-dotted, and dashed lines indicate suggestive, significant, and highly significant thresholds, respectively. Shaded bars represent CI calculated by bootstrap analysis with the black bars at the peak position of each QTL.

mice. F_1 mice from B6 and D2 mice showed the intermediate phenotype between the both strains (Fig. 1). However, phenotypes of F_2 progenies did not follow the Mendelian law. The values of immobility time were not segregated into 3 : 1 ratio, but varied with consecutive values. This suggests that the phenotype of immobility time receives multigenic control, which led us to perform QTL analysis. After genotyping of 106 microsatellite markers, QTL analysis was performed using data for 101 F_2 mice. For TST, QTL analysis revealed two main significant

Table 2. Characteristics of QTLs detected with Map Manager QTX for immobility time in TST

Marker	Position		Peak LRS	%[c]	CI[d]	B6/B6[e]	B6/D2[e]	D2/D2[e]
	Mbp[a]	cM[b]						
D4Mit235	8.32	3.57	24.0	17	32	80.2 ± 15.4	71.6 ± 10.4	142.2 ± 15.1
D5Mit108	43.9	23.91	18.1	17	31	83.9 ± 16.4	117.8 ± 17.1	47.6 ± 9.5
D8Mit4	32	18.89	12.3	12	48	67.1 ± 12.6	85.7 ± 12.9	135.6 ± 29.5

a), b) Mbp and cM were expressed based on MGI.
c) Percentage of total variance attributable to locus.
d) 95% confidence interval of QTL location as calculated by Map Manager QTX software.
e) Mean phenotypic value ± SEM for mice homozygous for the B6 allele (B6/B6), heterozygous (B6/D2), and homozygous for the D2 allele (D2/D2).

QTLs located closely to *D4Mit235* and *D5Mit108* and one suggestive locus located closely to *D8Mit4*. Also for FST, we detected five suggestive QTLs located closely to *D6Mit159*, *D14Mit102, D14Mit225, D14Mit165, and D17Mit218*, all of which possibly affect the immobility time cooperatively, although the contribution of each QTL is limited. QTLs detected in TST and FST were not overlapped in this study. This may be reflected from the result that only a weak correlation of immobility time was observed between TST and FST with the correlation coefficient value of 0.379 (Fig. 2). This may suggest that different genetic factors control the immobility time between TST and FST, although these two tests are widely considered to measure depression-like behavior.

Among these QTLs, the *D4Mit235 and D5Mit108* loci are of great interest, because the previous study by Tomida *et al.* revealed QTLs on the same Chrs 4 and 5 by using the same behavioral tests, TST and FST, but using different combination of mouse strains, B6 and mutant strain CS[20]. Tomida *et al*. detected QTLs near the microsatellite markers *D5Mit134* (position at 72 Mbp) on TST and *D4Mit232 and D5Mit113* (positions at 145 Mbp and 77 Mbp, respectively) on FST, whereas we detected QTLs near the microsatellite markers *D4Mit235* (position at 83.2 Mbp) *and D5Mit108* (position at 43.9 Mbp) on TST. Although two studies detected QTLs on the same Chrs, QTLs detected in this study may be different from QTLs detected previously, because the positions of QTLs are different.

Other different QTLs have also been reported to control the immobility time in TST and FST by using a different combination of mouse strains. Yoshikawa *et al*. analyzed QTLs controlling immobility time in TST and FST using intercross between B6 and C3H mice and revealed four loci in the TST (*D4Mit203, D8Mit242, D11Mit271, and D14Mit257* at positions of 129 Mbp, 101 Mbp, 45 Mbp, and 51 Mbp, respectively), and five loci in the FST (*D6Mit289, D8Mit242, D8Mit93, D11Mit271, and D17Mit185* at positions of 128 Mbp, 101 Mbp, 127 Mbp, 45 Mbp, and 68 Mbp, respectively)[23]. Liu *et al.* also performed QTL analysis with respect to the immobility time of TST using other combination of mouse strains, NMRI and 129S6, and obtained QTLs on Chrs 5 (61.0 cM), 12 (43.0 cM), and 18 (51.0 cM)[10]. These QTLs may be different from QTLs detected in this study with respect to their positions, although some QTLs locate on the same Chrs.

Antidepressant drugs are used clinically to combat both depression and anxiety disorders, because the two behavioral disorders often occur together[16]. Therefore, it appears helpful to mention other QTLs reported for anxiety-related paradigms. Flint *et al.* mapped QTLs controlling emotional traits in mice defined by open field test, Y maze performance, and elevated plus maze performance to Chrs. 1, 4, 12, 15, 17, and 18[6]. Furthermore, QTLs for contextual fear conditioning were correlated to Chrs. 1, 2, 3, 10, and 16[2,7].

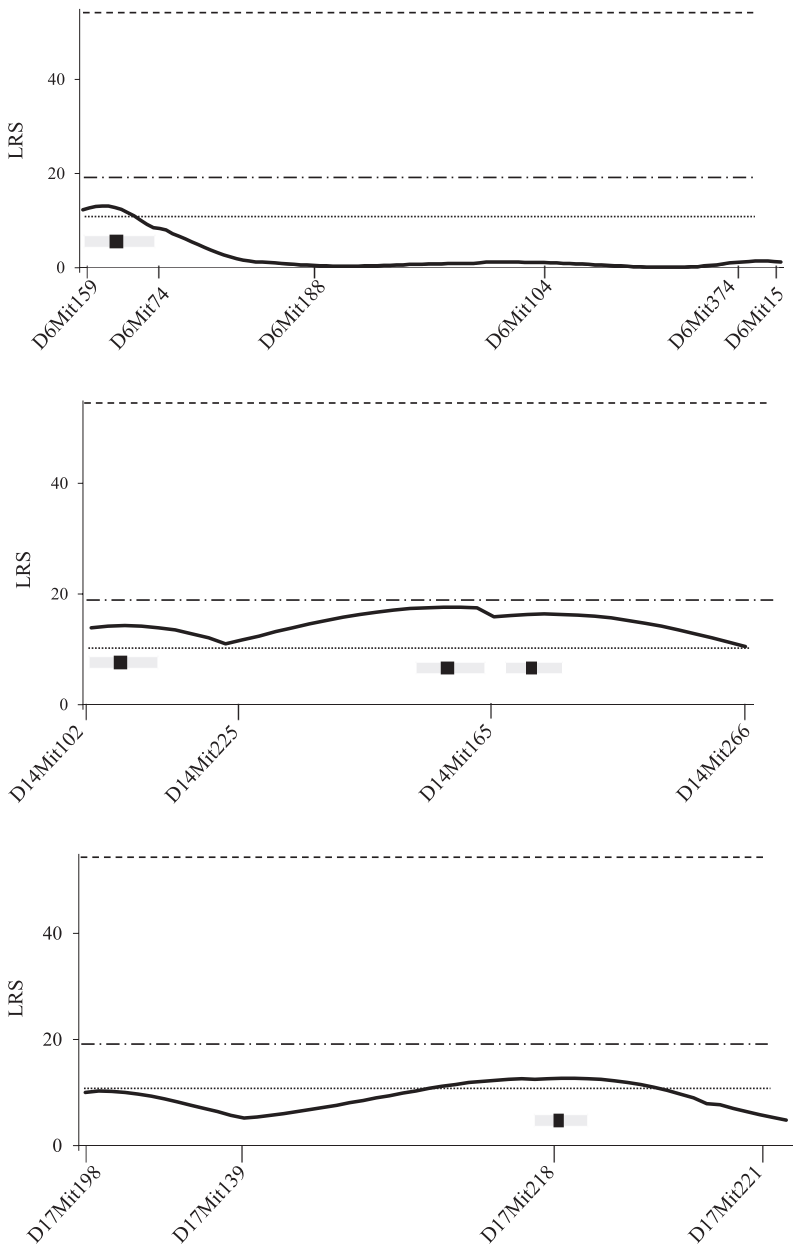

Fig. 4. QTLs detected to influence the immobility time in FST in F_2 mice. The dotted, dashed-dotted, and dashed lines indicate suggestive, significant, and highly significant thresholds, respectively. Shaded bars represent CI calculated by bootstrap analysis with the black bars at the peak position of each QTL.

All genes responsible for immobility phenotype indicating depression and despair are not yet identified and further studies are necessary. The present multipronged QTL analyses and future work may be of benefit in identifying human genes that predispose to general depression and despair, a risk factor for psychiatric disorders, and will provide a rationale for the design of new drugs and similarity between the mouse and human genomes, likely making the mouse an excellent model for elucidating complex traits. We hope that the molecular dissection of the psychological variations in mice could help to identify genetic factors associated with the vulnerability to clinical depression and anxiety in humans,

Table 3. Characteristics of QTLs detected with Map Manager QTX for immobility time in FST

Marker	Position		Peak LRS	%[c]	CI[d]	B6/B6[e]	B6/D2[e]	D2/D2[e]
	Mbp[a]	cM[b]						
D6Mit159	29.7	12.36	12.3	11	46	93.0 ± 18.2	104.9 ± 14.5	56.3 ± 12
D14Mit102	65.8	34.36	14.3	13	41	73.4 ± 15.3	87.6 ± 11.7	117.2 ± 25
D14Mit225	74.9	39.46	17.6	16	51	91.5 ± 17.5	102.1 ± 20.8	85.2 ± 12
D14Mit165	–	56.16	16.4	16	33	73.1 ± 13.3	86.9 ± 12.4	122.4 ± 26.7
D17Mit218	71.8	43.76	12.7	12	32	85.4 ± 18.2	104.3 ± 14.4	64.3 ± 13.4

a), b) Mbp and cM were expressed based on MGI.
c) Percentage of total variance attributable to locus.
d) 95% confidence interval of QTL location as calculated by Map Manager QTX software.
e) Mean phenotypic value ± SEM for mice homozygous for the B6 allele (B6/B6), heterozygous (B6/D2), and homozygous for the D2 allele (D2/D2).

leading to develop novel antidepressants and therapeutics targeting new genes.

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