QTL analysis identifies multiple behavioral dimensions in ethological tests of anxiety in laboratory mice

Maria Grazia Turri,* Susmita R. Datta,* John DeFries,† Norman D. Henderson‡ and Jonathan Flint*

Background: Ethological tests of anxiety-related behaviors, such as the open field arena and elevated plus maze, are often carried out on transgenic animals in the attempt to correlate gene function with a behavioral phenotype. However, the interpretation of such tests is problematic, as it is probable that different tests measure different aspects of behavior; indeed, anxiety may not be a unitary phenomenon. Here, we address these questions by asking whether behaviors in five ethological tests of anxiety are under the influence of a common set of genes.

Results: Using over 1600 F2 intercross animals, we demonstrate that separate, but overlapping, genetic effects can be detected that influence different behavioral dimensions in the open field, elevated plus maze, square maze, light-dark box, and mirror chamber. We find quantitative trait loci (QTLs) on chromosomes 1, 4, and 15 that operate in four tests of anxiety but can be differentiated by their action on behavior in threatening and nonthreatening environments and by whether habituation of the animals to an aversive environment alters their influence. QTLs on chromosomes 7, 12, 14, 18, and X influenced a subset of behavioral measures.

Conclusions: The chromosome 15 QTL acts primarily on avoidance behavior, the chromosome 1 QTL influences exploration, and the QTL on chromosome 4 influences activity. However, the effects of loci on other chromosomes are not so readily reconciled with our current understanding of the psychology of anxiety. Genetic effects on behaviors in these tests are more complex than expected and may not reflect an influence on anxiety.

Addresses: *Wellcome Trust Centre for Human Genetics, Oxford, OX3 7BN, UK. †Institute for Behavioral Genetics, University of Colorado, Boulder, Colorado, 80309, USA. ‡Oberlin College, Oberlin, Ohio, 44074, USA.

Correspondence: Jonathan Flint
E-mail: jf@molbiol.ox.ac.uk

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Background

The open field test is one of the most widely used instruments in animal psychology, its popularity primarily due to the simplicity of the apparatus and the commonly held view that it represents a test of anxiety in animals, attested by its use in the development of anxiolytic agents [1, 2]. More recently it has been included, along with other ethological tests, in phenotypic assessments of genetically modified animals and in surveys of mutagenized mice as a method of determining genetic effects on emotional behavior [3, 4]. The apparatus consists of an open, brightly lit space of which an animal has no prior experience and from which there is no escape. Typically, open field ambulation (OFA) is recorded, under the assumption that the novel, potentially threatening environment will lead to a decrease in activity.

Despite its widespread use and the commonly held view that the open field arena is a test of anxiety, for a number of reasons the interpretation of open field measures has been the subject of debate. First, the underlying constructs that the test purports to measure (fearfulness, emotionality, and susceptibility to anxiety) are themselves not well defined, leading to circular operational definitions (such as emotionality is that which is measured in the open field arena), anthropomorphic validation (it looks like human fearfulness), or the use of debatable indices of validity (such as intertrial decreases in scores, effects of altering test parameters, and correlated behavioral changes in another apparatus) [5, 6]. Second, the individual measures in the open field may themselves consist of multiple, possibly interacting, factors, many of which are of no interest to the experimenter [7, 8].

One way of determining whether behaviors in the open field arena, and other ethological tests, reflect the action of a single construct, such as anxiety, is to see whether they are under the influence of a common set of genes. Most behaviors in tests of anxiety are measured quantitatively, and the numerous genes that are thought to influence the measures reside at quantitative trait loci (QTL). Consequently, it should be possible to distinguish phenotypes by determining whether they are represented by a unique combination of QTLs, or, when there is complete or substantial overlap for QTLs influencing multiple traits, whether QTLs contribute differentially to each phenotype.

Our experiment was designed to address four questions. First, whether a common set of genes act in five ethological tests of anxiety in which anxiolytic drugs have been shown to alter behavior in a direction that is consistent with their action in human subjects: the open field arena, the elevated plus maze [9], the square maze [10], the light-dark box [11], and the mirror chamber [12].

Second, we asked whether the presumed anxiogenic components of the tests have different genetic influences than the nonanxiogenic. QTLs influencing anxiety will have the most influence on measures taken in anxiogenic regions of the apparatus. In each test, the animal is presented with a choice between threatening and nonthreatening environments. In the elevated plus maze and square maze, mice have a choice between two relatively anxiogenic regions (the open arms) and two relatively safe regions (the closed arms) [9, 13, 14]. The light-dark box and mirror chamber also permit the animal to explore a novel situation or remain in a relatively nonthreatening dark enclosure. The difference lies in the nature of the novel environment: either a well-lit exposed area or a mirrored chamber [15]. Even within the open field, there are thought to be distinctions in the level of threat the exposed area provides: the periphery is safer than the center, and latency to reach the center of the maze is thought to measure the same behavior as latency to emerge from the light-dark box or enter a mirrored chamber [6].

Third, we asked whether the genetic effects are specific to the aversive situation that is common to the ethological tests of anxiety. We did this by including two controls, one for locomotor activity and one for novelty as an aversive stimulus. All the ethological tests use a change in the animal's locomotor activity as an index of its presumed emotional state, so we also measured activity in a nonthreatening environment (the home cage). To determine whether the genetic effects were common to all aversive stimuli, we included a tail suspension test in our battery. The tail suspension induces stress in rodents, as assessed by tail suspension- induced immobility [16].

Finally, we asked whether the same genes influence behavior after habituation to the aversive situations (as measured by retesting the animals). Retesting is expected to reduce the anxiogenic potential of the apparatus, leading to a relative reduction in the effect of the QTLs influencing anxiety [17–19]. Consequently, in two tests, the open field arena and light-dark box, animals were tested twice, on separate days.

Our experiment used two intercrosses of the DeFries strains of mice [20]. These strains are inbred derivatives of lines subjected to 30 generations of bidirectional selection in the open field. Selection was carried out in two separate experiments so that there are four lines, termed H1 and H2 (for high activity) and L1 and L2 (for low activity). We have already shown that the same, relatively small number of QTLs contributes to differences in OFA and OFD (open field defecation) [21]. We have replicated these results and have demonstrated that there are QTLs common to the selected strains for measures in the open field, elevated plus maze, and light-dark box [22]. Here, we report the analysis of a large set of ethological measures of anxiety to explore in detail the relationship between behavioral phenotype and genotype.

Table 1

LOD score significance levels.

	5%	2.5%	1%
Single phenotype	3.22	3.51	3.98
9 phenotypes	4.06	4.35	4.82
22 phenotypes	4.56	4.85	5.32

Results

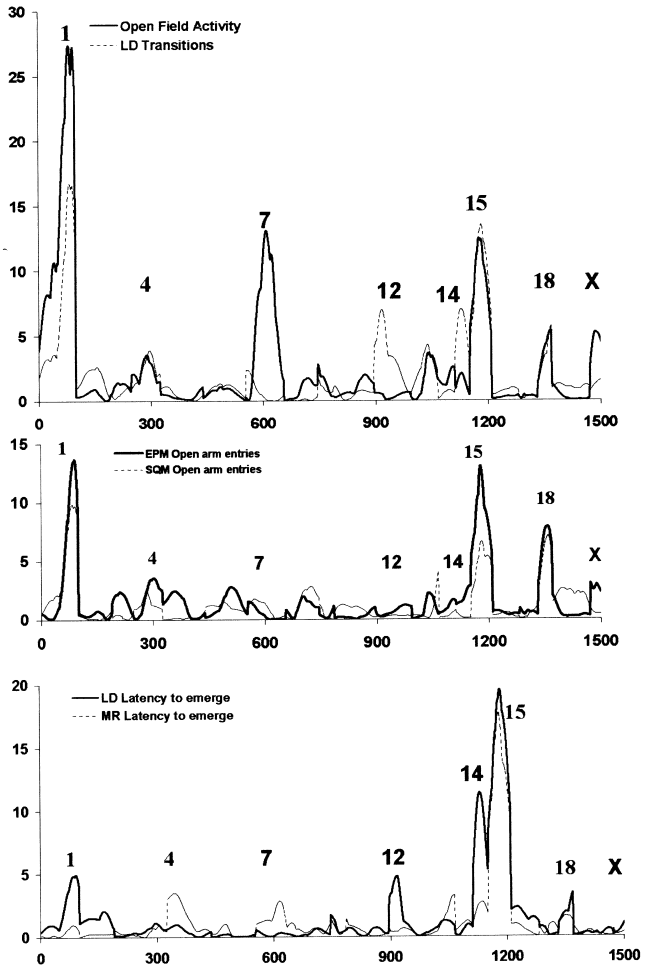
QTLs influencing measures of anxiety in five ethological tests

In a first experiment, we sought to determine whether there are QTLs that influence behavior in all ethological tests of anxiety. We obtained phenotypic and genotypic data from a total of 1636 animals, from the two intercrosses described in a separate paper where we present mapping data for a subset of the phenotypic measures (day 1 measures only for open field activity and defecation; day 1 measures only in the light-dark box: transitions, latency to emerge, and time spent in the light compartment of the light-dark box; time spent, as well as number of entries, into the open and closed arms of the elevated plus maze) [22]. The results from the two crosses were highly consistent and, to maximize power and mapping resolution, we combined data from both groups and determined the likely positions of QTLs using MAPMAKER-QTL and QTL-CARTOGRAPHER. Table 1 gives appropriate significance levels and thresholds for the analyses (see Materials and methods).

Figure 1 shows a genome scan for measures from each test believed to reflect an animal's level of anxiety or emotionality. QTLs on eight chromosomes exceeded the 5% significance threshold. Some are test specific (such as the QTL on chromosome 14 for two measures in the light-dark box), but those on 1, 4, 12, 15, 18, and the X influence more than one test. The QTL on chromosome 15 influences all measures.

Consistent with previous findings, the effect sizes of the QTLs were generally small. The locus on chromosome 1 accounts for 10% of the total phenotypic variance of open field activity (LOD 28). Because the number of

Figure 1



LOD scores (vertical axis) for measures from five ethological tests of anxiety. The top panel shows two measures of activity, transitions in the light-dark box (LD) and total activity in the open field arena; the middle panel shows entries into the open arms of the elevated plus maze (EPM) and square maze (SQM); the lower panel shows two measures of latency to emerge into a novel environment in the light-dark box and the mirror (MR) chamber. The horizontal axis represents the distance in centimorgans (cM) across the whole genome, from chromosome1 on the left to the X chromosome on the right. The chromosomes with LOD scores exceeding the 5% threshold are shown as numbers on the graph.

animals used is large and almost identical for each phenotype, the relationship between the LOD score and the percentage variance explained is constant: by linear regression the slope is 0.361 (standard error 0.004, intercept 0.003). The percentage variance explained is, thus, about a third of the value of the LOD scores.

QTLs influencing spatio-temporal measures in ethological tests of anxiety

We asked whether the QTLs influence all or only a subset of measures taken in the ethological tests of anxiety. In three tests, the elevated plus maze, square maze, and light-dark box, we obtained measures of activity in both threatening (open) and nonthreatening (enclosed) regions. We also mapped the latency to enter the more threatening regions of each apparatus (the center of the open field, light compartment of the light-dark box, mirror chamber, and open arms of the elevated plus maze) and the time spent in these compartments. QTLs with LOD scores exceeding the 5% threshold for a single phenotype are shown in bold typeface in Table 2. For each QTL, we looked at the direction of effect of the allele from the less active strain (L1 and L2, referred to as the low allele), and the direction of the allelic effect is also shown in Table 2 as a plus (+) or minus (−), according to whether the QTL allele increases or decreases the trait.

Considering first the constellation of measures that each QTL influences, the locus on chromosome 15 emerges with a profile most consistent with a role in anxiety. Its effects are almost entirely restricted to activity in anxiogenic compartments (open arms of the elevated plus maze and square maze and light-dark box light compartment); furthermore, the genetic influence on latency measures is derived almost entirely from chromosome 15. Indeed, no other QTL contributes to all latency measures or even to latency and all other tests. The locus on chromosome 1, although, in general, having a larger effect than the locus on chromosome 15, influences a broader range of measures, in both anxiogenic and nonanxiogenic compartments.

The QTL on chromosome 7 has similar effects to that of chromosome 1 but its effects are more test specific (Table 2). The QTL influences visually mediated behavior: it influences activity in the brightly lit center of the open field (LOD 13.5), time spent in the center of the open field (LOD 7.3), and open arm activity in the square maze (LOD 4.4). It also has a significant effect on elevated plus maze closed arm entries and activity and square maze closed arm activity. These results are consistent with the effect coming from the albino c locus on chromosome 7 [23]. The locus on chromosome 4 has an influence on all tests, but not on time spent in any compartment, which is consistent with a role restricted to locomotor activity.

Since each apparatus that was used is designed to contain regions that differ in their potential to induce anxiety, the effect that a QTL allele has on different components of the apparatus can be used for its identification. An allele that increases anxiety will decrease time spent in the apparatus and the decision to enter an anxiogenic region (as measured by the number of entries), while an allele that decreases locomotor activity will have no effect on either of these measures. In addition, if the nonanxiogenic regions are truly nonthreatening, the anxiety allele should have no effect on activity levels here (for instance, in the closed arms of the elevated plus maze).

Table 2

LOD scores and direction of effect of QTLs influencing five ethological tests of anxiety.

Apparatus	Test	Chr 1	Chr 4	Chr 5	Chr 6	Chr 7	Chr 8	Chr 11	Chr 12	Chr 14	Chr 15	Chr 18	Chr X
Open field	Total activity	**27.4** −	**3.5** −	1.0	1.1	**13.1** −	1.7	0.6	**3.6** −	2.0	**12.5** −	**5.4** −	**5.3** −
	Center activity	**14.8** −	**6.0** −	2.2	0.9	**13.5** −	0.9	0.9	3.2	1.7	**5.1** −	**6.2** −	2.2
	Center time	**3.9** −	2.2	0.9	1.2	**7.3** −	0.5	0.9	3.7	1.1	**5.2** −	**6.0** −	0.8
	Latency to approach center	1.8	2.2	0.6	1.3	**4.3** −	0.8	1.9	0.9	0.6	**9.0** +	1.5	1.1
Elevated plus maze	Closed arms entries	**9.4** −	**5.5** −	1.8	1.0	**16.5** −	**5.9** +	0.7	2.0	0.4	1.5	**7.1** −	1.9
	Closed arms activity	**6.7** −	**6.5** −	1.5	1.2	**15.8** −	**6.2** +	0.8	2.0	0.6	1.2	**6.4** −	2.3
	Closed arms time	**4.3** +	0.4	2.1	**4.4** +	1.1	1.3	0.4	0.5	1.8	0.2	2.0	1.5
	Open arms entries	**13.7** −	**3.6** −	2.4	2.7	1.5	1.9	1.1	2.2	2.5	**13.0** −	**7.9** −	2.9
	Open arms activity	**12.1** −	**3.5** −	2.3	0.6	2.5	0.4	0.8	0.8	1.1	**11.8** −	**7.7** −	**4.1** +
	Open arms time	**3.4** −	0.8	**3.6** −	2.3	2.2	0.5	1.3	1.0	2.0	**4.5** −	1.2	**5.3** −
	Latency to enter open arms	0.9	1.0	0.6	0.7	0.6	0.5	2.5	2.4	2.3	**10.5** +	2.4	1.3
Square maze	Closed arms activity	**21.3** −	**3.2** −	0.5	1.9	**9.7** −	**5.1** +	0.7	0.6	1.1	2.9	**10.4** −	**3.5** −
	Open arms activity	**11.3** −	**4.9** −	1.0	1.7	**4.4** −	1.5	2.5	0.9	2.8	**15.4** −	**6.9** −	**5.4** −
	Open arms entries	**9.2** −	**3.6** −	1.8	1.8	2.0	1.9	2.5	1.5	0.8	**9.3** −	**4.6** −	2.2
	Latency to enter open arms	1.8	1.7	1.3	1.0	0.7	0.7	0.6	0.9	2.1	**5.7** +	1.8	0.9
Light-dark box	Light box time	**13.0** −	2.2	0.7	1.0	1.5	0.5	**5.9** −	1.1	**6.2** +	**11.9** −	**3.5** −	0.8
	Light box activity	**24.8** −	2.9	0.6	1.2	2.2	0.4	**6.0** −	2.3	**5.6** +	**11.4** −	**4.1** −	1.4
	Dark box activity	**7.4** −	**6.2** −	2.7	0.6	0.3	2.0	1.1	**5.5** −	2.2	2.1	1.7	**3.7** −
	Transitions	**16.7** −	**3.9** −	1.1	1.3	2.3	0.3	**6.9** −	**4.3** −	**7.0** +	**13.5** −	**5.7** −	1.5
	Latency to emerge	**4.9** +	1.1	0.9	0.2	0.5	0.6	**4.8** +	1.2	**11.4** +	**19.6** +	2.4	1.3
Mirror chamber	Latency to emerge	0.9	0.9	**3.5** +	1.0	2.8	0.9	0.6	3.2 +	2.7	**17.7** +	1.5	1.2

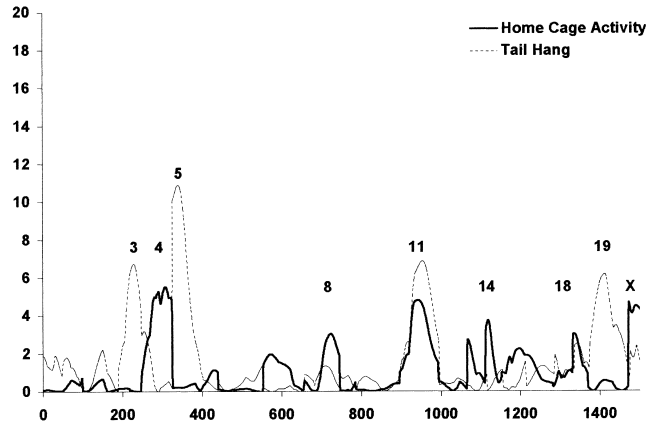
Significant LOD scores are shown in bold. The direction of the allele derived from the low strain is shown next to the LOD score. The locus on chromosome 7 is recessive; all other loci fit an additive model.

Table 3

LOD scores for defecation in the open field arena, elevated plus maze, and light-dark box.

	Chromosome 1			Chromosome 8			Chromosome 12			Chromosome 14			Chromosome X		
Defecation phenotype	LOD	Pos.	Dir.	LOD	Pos.	Dir	LOD	Pos.	Dir.	LOD	Pos.	Dir.	LOD	Pos.	Dir.
Open field day 1	14.50	74	+	1.92	4	–	1.71	38	+	9.43	20	–	5.36	50	–
Open field day 2	13.25	74	+	0.87	20	–	3.21	34	+	7.61	14	–	3.47	0	–
Elevated plus maze	7.41	72	+	3.59	38	–	1.18	18	+	7.10	26	–	8.92	50	–
Light-dark box day 1	5.59	84	+	1.86	52	–	2.87	34	+	4.17	10	–	3.72	0	–
Light-dark box day 2	6.63	84	+	2.44	50	–	2.83	28	+	1.43	36	–	4.05	0	–
Mirror chamber	3.33	74	+	2.23	58	–	1.95	8	+	2.46	28	–	5.64	50	–

Figure 2



Whole genome LOD plot for tail hang (continuous line) and home cage activity (dotted line). The graph uses the same format as that described for Figure 1.

For each QTL, we looked at the direction of effect of the allele from the less active strain (L1 and L2, referred to as the low allele); the results are shown in Table 2. Allelic effects at only one QTL are consistent with a role restricted to locomotor activity: an allele on chromosome 4 decreases activity in all test apparatus, with no effect either on the time spent in a particular test component or the decision to enter an anxiogenic region (as indicated, for example, by entries into the open arms of the elevated plus maze).

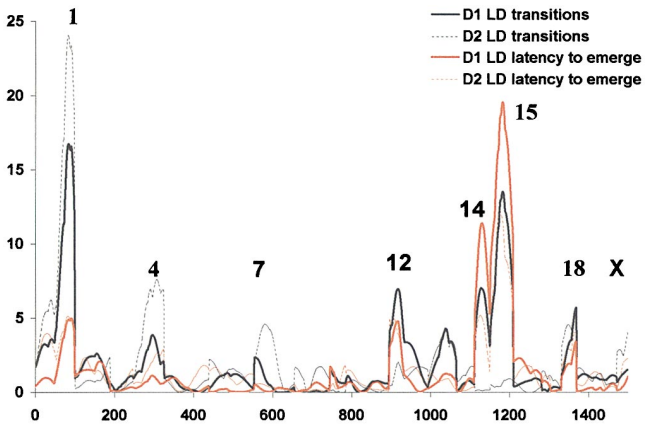
The locus on chromosome 15 has allelic effects indicating an effect on anxiety. The low allele decreases time spent in and the chances of entering into the anxiogenic compartments of the test apparatus as well as increases the latency to enter such compartments. The allele always decreases anxiety in anxiogenic regions and has no effect in the closed arm of the elevated plus maze (a relatively safe, nonthreatening region). QTL allelic effects on chromosome 1, 7, and 18 are almost identical and substantially overlap with the QTL on chromosome 15, differing only in that they influence elevated plus maze measures that are more activity based (closed arm entries and close arm activity).

QTLs influencing home cage activity and tail suspension

We next sought to determine whether the QTLs that influence activity in the home cage during the dark cycle and QTLs that influence freezing behavior during tail suspension are the same as those that influence behavior in the ethological tests of anxiety.

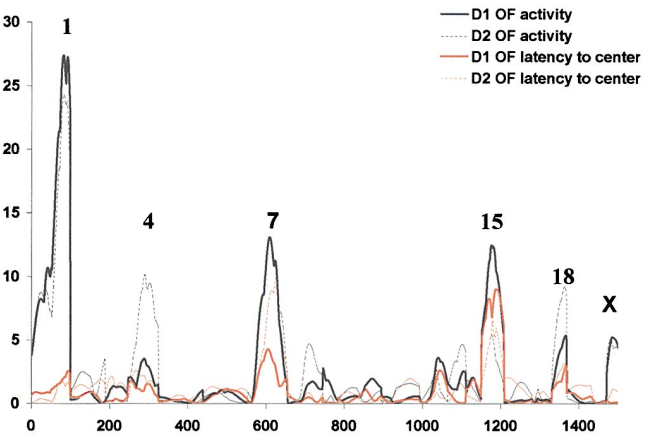
Figure 2 shows the LOD plot across the genome for home cage activity (continuous black line) and tail suspension (dotted black line). The graph shows that only one QTL influencing tail suspension coincides with QTLs already

Figure 3



Whole genome LOD plot for measures taken on two separate days (D1 and D2) for the number of transitions and the latency to emerge into the light compartment of the light-dark box. The graph uses the same format as that described for Figure 1.

Figure 4



Whole genome LOD plot for measures taken on two separate days (D1 and D2) for total activity in the open field latency to reach the center of the apparatus. The graph uses the same format as that described for Figure 1.

identified, such as that on chromosome 11, specific for four measures in the light-dark box. However, loci on 4, 18, and X are common to home cage activity and other measures. The simplest explanation of this finding is that these QTLs represent a locomotor component of measures of anxiety. By contrast, QTLs on chromosomes 1, 12, and 15 are specific to measures from the ethological tests.

Mapping QTLs for defecation

We next asked if the same loci influenced defecation. Table 3 gives the mapping results for defecation in each apparatus. We cannot differentiate defecation scores by components of the test apparatus and thus are unable to see if the scores reflect the presumed differences in anxiogenic potential of open and closed arms, light or dark compartments, or perimeter or center of the open field. Nevertheless, the results are remarkably consistent for each apparatus: QTLs on chromosomes 1 and the X influence defecation in every test, while QTLs on chromosomes 8, 12, and 14 act in at least one test.

The direction of effect of the allele helps to determine how the QTL operates. We find that alleles on chromosome 1 increase defecation, which is consistent with the view from the previous analyses in which we found that this QTL decreases activity. The QTL on chromosome 14 appears to have an anxiolytic action, since it decreases defecation (Table 3), time, and activity in the dark area of the light-dark box (Table 2) and increases time and activity in the light area (Table 2). However, we observe no effects on other measures taken in the open field or elevated plus maze and a negative effect on home cage activity.

Repeated measures

We next mapped repeated measures taken in the light-dark box and open field. In Figure 3, day 1 light-dark box measures are shown as solid lines, and day 2 measures are shown as dotted lines, with latency measures shown in red. In the light-dark box on day 2, there is a dramatic fall in the importance of the chromosome 15 locus: for the latency measures, it drops from a peak LOD score of 20 to 11, while the LOD score for transitions falls from 14 on day 1 to a nonsignificant value of 0.75 on day 2. As shown in Figure 4, a similar but less marked drop in day 2 LOD scores can be seen with respect to open field activity and latency measures on the chromosome 15 locus. In contrast, on chromosome 4, day 2 LOD scores increase for both light-dark box transitions and open field activity. On chromosomes 1 and 7, day 2 LOD scores increase for light-dark box transitions but show little change for open field activity. In general, LOD score differences between first and second testing sessions were smaller for open field measures than for light-dark box measures.

In order to determine whether differences in LOD scores between the replicates could be due to chance, we obtained the variance associated with a replicate QTL mapping experiment by simulation. We simulated QTLs explaining between 2% and 15% of the phenotypic variance on a chromosome of 100 cM in size. We placed six markers on the chromosome, with an intermarker distance of 15 cM and the QTL placed in the center of the chromosome midway between two markers. Five hundred replicates, each of 1600 animals, were generated, and the QTL was mapped using MAPMAKER-QTL. We found that the standard deviation is approximately the square root of the

LOD score, or 4.4 for a LOD score of 20 (as observed in day 1 measures of latency to emerge from the light-dark box). Therefore, there is a significant change in the LOD score for transitions in the light-dark box.

Bootstrap test for pleiotropy versus close linkage

A critical question is whether a single QTL at one chromosomal location accounts for the effects on multiple traits, or whether there are closely linked QTLs specific for each phenotype. In an accompanying paper, we show that a combination of composite interval mapping for multiple traits and haplotype analysis decreases the intervals containing QTLs and goes some way in demonstrating pleiotropic action [22]. In addition, we used a bootstrap method to differentiate pleiotropy from coincident location on the same measures and chromosomes [24]. Five hundred bootstraps were generated that conformed to the QTL model estimated from our original data. The distance between the estimated QTL positions of phenotypes was calculated, and the 95% confidence interval was calculated by taking the lower and upper 2.5% percentiles. In each case, the interval contained the value 0, so we cannot reject the hypothesis that the loci are pleiotropic.

Discussion

It is generally assumed that the various ethological tests of anxiety in mice, such as the open field arena, the elevated plus maze, and the light-dark box, measure the same psychological construct [6, 25]. Consequently, consistent behavior across tests is interpreted as evidence that an animal is anxious. For example, using this approach, mice with engineered mutations in the serotonin 5-HT_{1A} receptor [26, 27], glucocorticoid receptor [28], and elements of the corticotrophin hormone-releasing system [29, 30] have all been shown to exhibit changes in anxious behavior. However, it is not clear that the ethological tests do measure the same phenomenon, or indeed whether anxiety in rodents is a unitary phenomenon [7].

The genetic mapping data we have collected from five ethological tests of anxiety provide a powerful tool to address this question. We have found QTLs on chromosomes 1, 4, 15, and 18 that influence at least one measure obtained in all five tests. Other loci, on chromosomes 7, 8, 12, and the X, influence behaviors in some, but not all, tests, and QTLs on chromosomes 11 and 14 are test specific. We also find a small number of QTLs that influence just one measure taken in a single test (for example, a QTL on chromosome 6 that influences time spent in the closed arms of the elevated plus maze). We have established that the QTLs do not influence behavior in all aversive situations, since we find almost no overlap with QTLs affecting immobility in the tail suspension test.

Finding QTLs with effects on all fives tests substantiates the view that anxiety, as assessed here, consists of correlated traits. However, the QTLs do not influence all behaviors equally. The tests contain measures intended to extract information about different components of behavior; for example, the animal is faced with a choice between spending time in ostensibly anxiogenic environments in the open arms of the elevated plus maze and the light compartment of the light-dark box apparatus. When we examine genetic effects on different components of the apparatus, we are able to discern a more complex pattern.

We can clearly separate out genetic effects on nonspecific locomotor activity from those operating on activity measures in the tests. The QTL on chromosome 4 acts on activity in the home cage (a nonthreatening environment) and on activity in tests of anxiety, regardless of the supposed anxiogenic potential of the region in which activity is measured. It has no effect on the time spent in anxiogenic regions. Furthermore, the effect of the QTL is approximately equal in each component of every test, explaining $\sim$2% of the phenotypic variance of each measure. Thus, the chromosome 4 QTL fits the pattern of a locus that influences locomotor activity.

By contrast, QTLs on chromosomes 1 and 15 do not influence home cage activity and also have effects on time measures in tests of anxiety, so that the allele that decreases activity in an anxiogenic component also decreases the time spent there, as expected of QTLs that influence anxiety.

However, more detailed examination of the phenotypes upon which these loci act shows that their effects can be dissociated in three ways. First, the locus on chromosome 15 has reduced or no influence on behavior in nonthreatening regions (such as the enclosed arms of the elevated plus maze and the dark compartment of the light-dark box), while the locus on chromosome 1 has equal if not greater effects in nonthreatening regions. This is not merely that the chromosome 15 QTL effects slip below the level at which they can be detected: the LOD score for activity in the open arms of the elevated plus maze is over 11, but only 1.2 for activity in the closed arms (Table 2).

Second, the QTL on chromosome 15 is the only locus with consistent and large effects on the time taken to enter aversive regions of the test apparatus. Again, the difference cannot be accounted for by a failure to detect a small effect, since small chromosome 1 effects are readily detected on other measures (Table 2).

Third, we have demonstrated that prior exposure to the apparatus diminishes the chromosome 15 QTL's effect. The QTL has no detectable influence on transitions in the light-dark box in the second day of testing, while the LOD score for the first day is almost 14 (Figure 3). The

difference is less marked in the open field, where the animal is faced with no possibility of escape, but shows the same pattern (Figure 4).

We can summarize these results as follows. Three QTLs influence behavior in all tests; the QTL on chromosome 4 influences the general level of motor activity, the QTL on chromosome 15 acts primarily (and possibly only) to promote avoidance behavior, and the QTL on chromosome 1 influences exploratory behavior. We can interpret the findings of the retest experiments in the light-dark box as a decrease in avoidance behavior following prior exposure to the tests on day 1 with a consequent increase in exploration and locomotor activity, shown by increased genetic effects on chromosomes 4 and 1, as if the chromosome 15 QTL is releasing its effect.

We have also identified loci whose roles are more complex to interpret, possibly representing other constructs involved in the tests. The QTL on chromosome 7 probably reflects the well-known action of the tyrosinase mutation on behavior [23, 31, 32]. Whether this action reflects abnormal sensitivity to light is not clear [33]. Certainly our data do not fit with that simple interpretation, since the QTL has no influence on measures taken in the light-dark box (where the brightness of the light compartment is expected to have an aversive action, as it does in the open field, elevated plus maze, and square maze). While none of these observations rule out an influence on anxiety, they again point to a more complex genetic determination of the psychological trait than we have hypothesized. Similar comments apply to the loci on chromosome 12, 14, and the X chromosome. There is also a complex relationship between defecation and the other measures. The QTL on chromosome 1, but not 15, influences defecation and other ethological measures of anxiety, suggesting that emotional elimination and avoidance behavior are genetically independent [34].

We have used a genetic method to demonstrate a complex basis for anxiety. Similar complexity has already been observed in investigations of drug response and alcohol and drug preference in mice, using both recombinant inbred strains and intercross designs [35–48]. We can expect more examples to emerge as genetic analyses are applied to other psychological constructs.

In the analysis of mouse transgenes, genetic effects on emotional behavior are almost always inferred from the use of one or two measures from the open field, elevated plus maze, and light-dark box [49]. Our data show a much more complex picture of genetic action than is allowed by the measures generally reported in transgene analysis. We have shown that it is possible to interpret function from the pattern of behaviors that are influenced, but we also find that many QTLs have narrow ranges of influence that are not so easily understood. Genetic action, therefore, cannot be reliably interpreted from a limited behavioral repertoire.

Materials and methods

F2 intercrosses

All mice were bred in the specific pathogen-free colony of the Institute for Behavioral Genetics. Animals were weaned at 25 days and housed with like-sex littermates (two or three per cage) until day 40, at which time they were individually housed prior to testing. All testing was done during the first 4 hours of the dark cycle.

Open field

The open field is a 61 cm white plastic-lined square box, 38 cm deep. The apparatus is covered, and a compact florescent bulb in the center of the ceiling provides an illuminance of ~600 lux on the floor below. The field was divided into an 8 × 8 grid of 76 mm squares, using infrared emitters and photo detectors to monitor horizontal movement. In addition, four emitters at each corner were placed 6 cm from the floor to detect rearing on and near the walls of the open field. Animals were placed in a plexiglass cylinder in one corner of the apparatus, the cylinder was removed, and activity was monitored for a 5 min interval. At the end of the session, the number of faecal boli deposited was recorded, and the floor was cleaned.

Elevated plus maze

The elevated plus maze consists of four runways (5 cm × 30 cm) arranged in a cross and elevated 37.5 cm above the ground. Two arms are enclosed by 21 cm clear acrylic plastic walls, and two arms are open, except for a slight raised (0.25 cm) edge that largely eliminates the problem of mice falling from the open runways. A low- output compact florescent bulb is located over the center of the maze and provides ~20 lux illuminance on the floor of the maze. The clear walls allow equal illumination levels on both open and closed runways. Movement was detected by infrared emitter-detector pairs located around the perimeter of the apparatus. Beams were positioned to detect both horizontal activity and vertical rearing in each runway and scanning over the edges and ends of open runways. Animals were placed in a rectangular, bottomless start box in the center of the maze. The start box was lifted, and mice were tested for a 5 min period. Activity and time spent in each arm was recorded as well as transitions into different arms. At the end of the session, the number of faecal boli deposited was recorded, and the floor was cleaned.

Elevated square maze

The square maze differs from the elevated plus maze in providing a continuous circuit for the animal to traverse, with alternating enclosed and open regions. The maze is a 35 cm square, with 5 cm runways, two of which, on opposing sides, are enclosed by 20 cm walls. The open runways contains .25 cm edges, similar to those on the plus maze. Illumination level and infrared activity monitoring methods are the same as those described for the elevated plus maze. Animals were placed in a bottomless start box in a corner of one closed arm. The start box was lifted, and mice were tested for 5 min. Activity and time spent in each arm was recorded as well as transitions into different arms. At the end of the session, the number of faecal boli deposited was recorded, and the floor was cleaned.

Light-dark box

The light-dark box consists of two chambers. The black-walled, enclosed compartment is 27 cm × 15 cm and has an exit that is 8 cm wide and 9 cm high in the middle of the wall adjoining the white-walled light compartment (31 cm × 27 cm). A small, shaded florescent bulb, positioned at the top of the divider wall in the light compartment, provides 20 lux illuminance to the light side of the box. Infrared emitters and detectors record horizontal activity in both light and dark compartments. Animals were placed in the dark chamber, and movement was recorded over a 5 min period. Measures of latency to emerge from the dark

compartment, time spent in both compartments, activity counts in both compartments, and light-dark transitions were recorded. At the end of the session, the number of faecal boli deposited was recorded, and the floor was cleaned

Mirror chamber

The mirror chamber, designed to detect anxiolytic agents, is based on the principle that many species show approach-avoidance conflict behavior when faced with a mirror image [12]. The outer box containing the chamber is 40 cm × 40 cm × 30.5 cm high. Located within this box is a 30.5 cm cube, open on one end. The three inner walls, ceiling, and floor of the cube are mirrored. The illuminance within the mirrored chamber is ~10 lux. The space between the cube and the outer box provides the animal with a 4.6 cm dark-walled dim (1–2 lux) alley surrounding the cube. The mouse was placed in the narrow alley at the farthest point from the opening to the mirrored chamber. Alley activity and rearing were recorded as well as latency to enter the mirrored chamber using infrared emitters and detectors. At the end of the session, the number of faecal boli deposited was recorded, and the floor was cleaned.

Home cage activity

All mice were caged singly several days prior to home cage activity monitoring and subsequent testing. Activity in home cages was measured during the first 2 hours of the dark cycle on two consecutive days. Two different activity monitoring systems were used simultaneously. The first consists of two infrared photo emitter-detector pairs, located outside the clear plastic home cage that divide the cage into three equal areas. This system records activity in a manner similar to that used in the test apparatus, recording gross locomotor activity on the floor of the home cage as the animal breaks the photo beams. The beam-break activity score consists of the square root of the number of beam breaks for each detector summed across the two test days. Spearman-Brown test-retest reliability of the measure is .70. The second home cage activity monitoring system employs an infrared motion detector located above the top of the home cage. This system converts all motion sensed within the home cage into arbitrary units, based on the magnitude of activity detected. For example, rapid gross motor activity, such as climbing on wire cage lids and cage floor locomotion, generates more score units per second than grooming or nest activity. Unlike the beam-break system, which only monitors floor locomotion, mice showing high levels of activity restricted to one area of the home cage can obtain relatively high activity scores with motion detection monitoring. Spearman-Brown test-retest reliability of the motion detector activity measure is .79. The correlation between the beam-break and motion detector activity scores is .52 across the two test sessions.

Tail suspension

Approximately 2 cm of the animal's tail (beginning 1 cm from the tip) was taped to the vertical surface of a narrow horizontal board. The mouse was observed for 3 minutes; with the total number of seconds the mouse remained immobile recorded for each minute. The dependent variable is total seconds of immobility during the final 2 minutes of the test session.

DNA extraction and genotyping

DNA was extracted from spleens as previously described [21]. All PCRs were carried out in 96-well plates using Taq polymerase, the manufacturer's buffer (Boerhinger), with 2 mM $MgCl_2$. PCR conditions were 30 s at 95°C, 30 s at either 50°C, 55°C, or 60°C, and 30 s at 72°C, and the annealing temperature was determined by a test reaction to assay which temperature gave a specific product. We used MIT markers previously used in the initial H1× L1 cross [21]. We screened these markers on the parental inbred strains H2 and L2, looking for strain differences, and when we found that a marker was not polymorphic in this cross, we screened the parental strains with nearby markers. We chose markers with a difference between the two alleles of at least 6 bp, so that all products could be analyzed on 4% agarose gels. Alleles were scored using a semiautomated system, coded in IDL. Alleles were classified at each locus as either homozygous for the L parent allele, homozygous for the H parent allele, or heterozygous. The order of all markers was checked using the MAPMAKER software package, and results were compared with radiation hybrid maps.

Statistical analyses

Using the map distances derived from the MAPMAKER software, we analyzed all data by interval mapping [50] in QTL-MAPMAKER [51] and composite interval mapping [52] in QTL-CARTOGRAPHER [53]. We used permutations to establish appropriate significance thresholds [54, 55]. On the basis that none of the phenotypes are correlated, dividing the P values of a single phenotype by the number of phenotypes analyzed will give the appropriate significance threshold. However, measures taken within a behavioral test apparatus are highly correlated, so this approach is unduly conservative and would result in an increased false negative rate. A more accurate significance threshold is obtained by dividing P values by the number of behavioral tests (9, including the repeated measures) rather than the total number of measures (22). Table 1 gives the uncorrected significance levels and thresholds corrected for both 9 and 22 phenotypes. For multiple trait composite interval mapping, we used Jzmapqtl [53].

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