



## ORIGINAL RESEARCH ARTICLE

# Identification of female-specific QTLs affecting an emotionality-related behavior in rats

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The influence of genetic factors on psychological traits and disorders has been repeatedly demonstrated; however, the molecular mechanisms underlying such an influence remain largely unknown. Anxiety-related disorders constitute the most common class of mental disorder in humans, with women being diagnosed far more frequently than men. A better understanding of the genetic and gender-related mechanisms mediating anxiety traits should enable the development of more rational methods for preventing and treating anxiety disorders. In this study we have aimed to identify, for the first time, quantitative trait loci (QTL) influencing anxiety/emotionality-related traits in rats. To this end, two strains—Lewis (LEW) and Spontaneously Hypertensive Rats (SHR)—that differ for several behavioral measures of anxiety/emotionality were intercrossed. A QTL analysis of the F<sub>2</sub> population revealed suggestive loci for various traits, including behaviors in the elevated plus-maze and blood pressure. In addition, one major QTL explaining 50.4% of the total variance (LOD = 7.22) was identified on chromosome 4 for the locomotion in the central and aversive area of the open field. Two other relevant QTLs have been recently mapped near this chromosomal region in the rat, which also harbors *Tac1r*, the gene encoding for the substance P receptor. Our major QTL affected females but not males and its effect depended on the type of cross (LEW or SHR grandmothers). The present results reveal a complex genetic basis underlying emotional behaviors and they confirm the existence of interactions between genetic factors and sex for this kind of trait. Further investigation of the loci identified herein may give clues to the pathophysiology of psychiatric disorders such as anxiety-related ones.

**Keywords:** QTL; quantitative trait locus; behavior genetics; anxiety; emotionality; elevated plus-maze; open field; blood pressure; rat; sex differences

## Introduction

It has been proposed that emotional reactivity is an important component of some psychiatric disorders. According to the diathesis-stress hypothesis, an individual's predisposition to display certain types of emotional reactions, combined with stressful life events, may lead to the development of psychopathologies. Such a predisposition varies within a population and is influenced by genetic factors. Therefore, investigating the genetic components underlying the interindividual variability of emotional responses may help us to better understand the mechanisms involved in the etiology of several psychiatric disorders.<sup>1–4</sup> Among the different psychopathologies already described, anxiety-related disorders are the most frequent ones in humans, with a clear preponderance of

females being found among different categories of anxious patients.<sup>5–7</sup> Twin and adoption studies on normal human personality indicate that genetic factors explain approximately 50% of the total variability found in some anxiety-related traits.<sup>8,9</sup> Moreover, genetics also influences the development of anxiety-related disorders, despite the frequently predominant role played by non-genetic factors.<sup>7,10–13</sup>

Genetic analyses of animal models help to dissect complex psychological traits into simpler components.<sup>3</sup> In mice, for example, recent quantitative trait locus (QTL) mapping techniques have allowed the identification of discrete genomic regions affecting different behaviors,<sup>14–17</sup> including emotionality-related ones.<sup>18–21</sup> The future identification of the genes involved will represent an important step forward in psychiatric research, since it will allow the molecular investigation of complex psychological traits such as anxiety-related behaviors. Extending these findings from mice to rats will encourage further physiological studies due to the larger size and widespread use of the latter species in biomedical research.

We have recently reported the first behavioral QTL analysis on rats, which revealed one major locus affect-

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ing motor activity.<sup>22</sup> To our knowledge, no QTL study on the emotional reactivity of rats has been published so far. The aim of the present study, thus, was to identify for the first time QTLs influencing anxiety/emotionality-related behaviors in rats.

We have previously shown<sup>23,24</sup> that the inbred rat strains Lewis/Nico (LEW) and Spontaneously Hypertensive Rats/Nico (SHR) display contrasting responses when tested in the elevated plus-maze (EPM), the open field (OF) and the black/white box, three behavioral models of anxiety/emotionality.<sup>25</sup> SHRs displayed higher levels of approach towards the open arms of the EPM, the central area of the OF and the white compartment of the black/white box,<sup>23</sup> thereby suggesting<sup>26–29</sup> that SHRs are less anxious than LEWs. Pharmacological tests involving anxiolytic and anxiogenic drugs supported our hypothesis that LEWs and SHRs differed in an anxiety-related dimension.<sup>23</sup> Moreover, SHRs were shown to have higher systolic blood pressure when compared to LEW rats.<sup>24</sup>

In a genetic study using LEW/SHR intercrosses, heritability estimates for several emotionality-related behaviors and blood pressure varied from 0.10 to 0.59, indicating that genetic factors may play a major role in the modulation of some of these phenotypes.<sup>24</sup> Of all the variables analyzed, the locomotion in the central area of the OF and systolic blood pressure were found to be the most heritable ones, both being influenced by sex-genotype interactions.<sup>24</sup> These findings suggested that genetic mechanisms affected emotional reactions of males and females in distinct ways and they revealed that LEW and SHR strains constitute an interesting model for further studies on the genetic- and sex-dependent mechanisms involved in emotionality/anxiety.

In the present study, using males and females of a LEW/SHR F2 population, we have performed a QTL analysis of several behaviors measured in the EPM and the OF. The EPM is one of the most widely accepted models for the study of anxiety in mice and rats and it has been thoroughly validated through pharmacological, physiological and behavioral approaches.<sup>27,30</sup> The OF is likely to be the most frequently used test in behavioral research, being classically considered as a test of emotionality.<sup>30–32</sup> Measuring an animal's ambulation in the peripheral and central areas of the apparatus allows the estimation of its tendency to avoid open spaces (considered as an index of fear), where it cannot perform thigmotaxic behavior.<sup>28,33</sup> The level of approach of mice and rats towards the center of the OF has been shown to be selectively modulated by pretreatment with different types of anxiolytic and anxiogenic drugs, being thus considered as an index of anxiety by several authors.<sup>28,29,33–35</sup>

Because SHR rats have been genetically selected in the past for spontaneously high blood pressure and considering the potential relationship between this trait and emotionality, measures of systolic blood pressure were also included in the present analysis. We report here the identification of several suggestive QTLs affecting different behavioral measures and

blood pressure and one major QTL on chromosome 4 affecting the central OF locomotion of female but not of male rats.

## Materials and methods

### *Animals and phenotyping*

F2 rats were produced from intercrosses between the strains Lewis/Nico (LEW) and SHR/Nico (SHR) bred in our laboratory but originally purchased from IFFA CREDO (L'Arbresle, France) in 1995. Two simultaneous reciprocal crosses (LEW female  $\times$  SHR male and SHR female  $\times$  LEW male, with F1 animals being brother-sister mated) produced 192 F2 rats that could be divided into four groups ( $n = 48$ ) according to sex and type of cross. The rats were weaned and separated by sex at 4 weeks of age and, thereafter, kept in collective plastic cages (four rats/cage) with food and water available *ad libitum*, under a 12-h light/dark schedule with lights on at 07:00 h.

We have characterized (phenotyped) all F2 animals in two behavioral tests, the elevated plus-maze (EPM) and the open field (OF), as previously described.<sup>24</sup> The rats were tested in the EPM at 8 weeks and in the OF at 9 weeks of age. Briefly, the EPM apparatus was made of Perspex, with four elevated arms (66 cm from the floor) 45 cm long and 10 cm wide. The arms were arranged in a cross-like disposition, with two opposite arms being enclosed (by 50-cm high walls) and two being open, having at their intersection a central square platform (10  $\times$  10 cm) which gave access to any of the four arms. Each rat was placed in the central platform of the EPM facing an open arm and its behaviour was video-recorded for 5 min. The number of entries and the time spent (with all four paws) inside each arm were recorded and the percentage of open arm entries was calculated in relation to the total number of entries in both types of arms. The OF apparatus was made of wood and had a white floor of 100  $\times$  100 cm divided into 25 squares of 20  $\times$  20 cm. The walls, 40 cm high, were also painted white. The test room had a dim illumination, with 7 lux being measured inside the apparatus. One week after being tested in the EPM, each rat was placed in the center of the OF and the following variables were recorded, by the use of a video-camera, for 5 min: number of outer squares (those adjacent to the walls) crossed, number of inner squares (those not touching the walls) crossed and total number of fecal boli.

Between 10 and 11 weeks of age, systolic blood pressure (mm Hg) was also measured at the tail of conscious rats by a noninvasive indirect method using a sphingomanometric system. The behavioral tests and the measures of blood pressure were carried out between 12:30 and 18:00 h (light cycle). Prior to the first test (EPM), the animals were naive to all manipulation, except for regular cage-cleaning. Each rat left its social partners and home cage only during testing periods, when it was transported by the experimenter inside a small plastic cage to a separate testing room. A list of all phenotypic measures considered for the

QTL analysis and a summary of the results from previous phenotypic comparisons between the two parental strains (LEW and SHR) are presented in Table 1. Further details concerning animals, crosses and phenotypic measures have been described elsewhere.<sup>24</sup>

### Genotyping

Following phenotyping, all rats were killed and their livers removed for DNA extraction using classical phenol/chloroform methods. Initially, all animals were genotyped for 77 polymorphic microsatellite markers distributed throughout the whole genome at 22 cM intervals on average. Following a first genome scan, the total number of markers was increased to 100 to better cover some larger map intervals remaining from the first genotyping and to improve the precision of localization of a major QTL on chromosome 4 and a suggestive QTL on chromosome 7. Rat microsatellite markers were purchased either from Research Genetics Inc (Huntsville, AL, USA) or from Genosys (Cambridge, UK). Genotypes were determined by polymerase chain reaction (PCR) in microtitre plate in a Hybaid Omnigene apparatus. In a 20- $\mu$ l reaction volume, 50 ng of genomic DNA was mixed with 5 pmol of each primer and 0.4 U of *Taq* polymerase (Promega, Charbonnières, France) in Promega type A buffer. The PCR program was: (i) one cycle at 96°C for 4 min; (ii) 35 cycles at 92°C for 40 s, 55–63°C (depending on the microsatellite) for 1 min and 72°C for 30 s; (iii) one cycle at 72°C for 2 min. Alleles were visualized either on ethidium bromide-stained 3% agarose gels or on standard denaturing sequencing gels, transferred onto a nylon membrane (Pall, Champs-sur-Marne, France) and hybridized with a [ $\alpha^{32}$ P] dCTP labeled primer using terminal transferase (Boehringer, Meylan, France).

**Table 1** Phenotypic measures considered for the QTL analysis

<i>Open field</i>	<i>Elevated plus-maze</i>	
Outer locomotion <sup>a</sup> (LEW = SHR)	Time open arms <sup>d</sup> (LEW < SHR)	Blood pressure <sup>g</sup> (LEW < SHR)
Inner locomotion <sup>b</sup> (LEW < SHR)	Entries open arms (%) <sup>e</sup> (LEW < SHR)	
Defecation <sup>c</sup> (LEW = SHR)	Entries closed arms <sup>f</sup> (LEW = SHR)	

<sup>a</sup>Number of peripheral squares (adjacent to the walls) crossed.

<sup>b</sup>Number of central squares (away from the walls) crossed.

<sup>c</sup>Number of fecal boli.

<sup>d</sup>Total time spent in the open arms (s).

<sup>e</sup>Percentage of open arm entries in relation to the total number of entries in both types of arms.

<sup>f</sup>Total number of entries in the closed arms.

<sup>g</sup>Systolic blood pressure measured at the tail of conscious rats (mm Hg).

'LEW = SHR' and 'LEW < SHR' refer to previous reports of phenotypic comparisons between the two parental strains.<sup>24</sup>

### QTL analysis

Genotype data were first analyzed by MAPMAKER/Exp (version 3.0b) in order to construct a complete linkage map containing all the microsatellite markers used herein and their respective map positions in cM. Nevertheless, only those markers mapped with highest significance levels (LOD >3.0) were used as framework markers for further QTL analyses. Map and phenotype data were then entered into MAPMAKER/QTL (version 1.1).<sup>36</sup> This program looks for QTLs every 2.0 cM throughout the entire genome. Assuming that a QTL is located at each of these points, MAPMAKER/QTL calculates models for the maximally likely manner in which the putative QTLs affect a trait. A LOD score, also called 'log-likelihood', is the log<sub>10</sub> of the likelihood that a proposed model does explain the observed phenotypic data. For each likelihood peak, the program determines a confidence interval that defines the width of the peak before a drop of 2.0 is observed in the log-likelihood. Single-point analyses of variance were performed for genotype effects of each putative QTL to confirm MAPMAKER/QTL's results and better illustrate the phenotypic changes due to a QTL. Since previous data showed sex differences and sex/genotype interactions regarding some of the phenotypes considered<sup>24</sup> and considering the importance of gender in human anxiety disorders,<sup>6</sup> the QTL analysis was performed both in the overall population and in male/female subpopulations. Following a first whole-genome scan carried out in this way, the identification of a major QTL which was specific for females derived from a LEW grandmother led us to perform a second whole-genome scan on the F2 animals grouped by sex and by type of cross (LEW or SHR grandmother).

### Results

#### *First whole-genome scan*

The results from the first phase of whole-genome scan are shown in Table 2. Several QTLs were identified above the suggestive level (LOD >2.8) and one QTL (located on chromosome 4) was found above the significant level (LOD >4.3).<sup>37</sup> Three suggestive QTLs were found to be associated with three different behavioral measures in the overall population. These were the outer locomotion in the OF (chromosome 5), the percentage of entries in the open arms of the EPM (chromosome 6) and the number of entries in the closed arms of the EPM (chromosome 7). Outer locomotion in the OF and closed-arm entries in the EPM are thought to represent locomotor reactivity to novel environments, whereas the percentage of open-arm entries in the EPM is an index of anxiety widely used in the study of anxiolytic drugs.<sup>23,25,30</sup> No previous differences between LEW and SHR strains had been found for the two aforementioned locomotion variables. For the percentage of open-arm entries, however, SHR rats had higher scores than LEW rats (see Table 1). The phenotypic effects of the QTL located on chromosome 6 were thus in the expected direction, since the SHR

**Table 2** QTLs identified in the first whole-genome scan

Phenotype	Chr	Marker + cM	LOD	% Var	F	P <	Popul	L/L	L/S	S/S
Outer locomotion	5	D5Mit11 + 0	3.7	8.4	8.7	0.0002	overall	93 ± 4	77 ± 3	72 ± 4
Inner locomotion	4	D4Mit15 + 18	<b>10.4</b>	61.3	13.8	0.00001	female	13.1 ± 1.6	6.0 ± 0.9	5.2 ± 0.9
Entries open arms (%)	6	D6Mit1 + 0	2.8	6.5	6.6	0.0017	overall	13.5 ± 1.9	21.4 ± 1.9	25.1 ± 2.4
Entries closed arms	7	D7Rat20 + 25	2.9	18.4	5.4	0.0052	overall	5.1 ± 0.3	5.1 ± 0.3	6.4 ± 0.3
Blood pressure	6	D6Mit4 + 6	4.0	22.3	8.9	0.0003	male	170 ± 2	179 ± 3	191 ± 5
Blood pressure	12	D12Mit3 + 4	4.2	10.7	9.5	0.0001	overall	167 ± 2	174 ± 2	182 ± 3

Only suggestive (LOD > 2.8) and significant (LOD > 4.3, in bold) QTLs are shown.<sup>37</sup>

Chr: chromosome. Marker + cM: closest marker and distance from the QTL peak. LOD: maximum logarithm of the likelihood for the QTL. % Var: percentage of total phenotypic variance explained by the QTL. F and P <: obtained from single-point analysis of variance for genotype effects considering the closest marker as independent variable. Popul: Population considered in the analysis to allow detection of suggestive or significant QTLs. L/L, L/S, S/S: mean phenotypic values and SEMs for rats homozygous for the LEW allele, heterozygous and homozygous for the SHR allele, respectively.

alleles cause a near two-fold increase of the visits to the open arms of the EPM.

Two suggestive QTLs were found to affect systolic blood pressure. The first one, located on chromosome 6, acted only on male rats whereas the second one, on chromosome 12, acted on the whole population. In both cases, as expected, the SHR alleles increased blood pressure (by 21 and 15 mm Hg, respectively).

The major QTL detected on chromosome 4 was found to affect, with a high significance level, the locomotion in the center of the OF, a behavior thought to be related to anxiety.<sup>28,29,34</sup> This QTL, named *Ofil1* (for open field inner locomotion 1), was found to act only in female rats. Moreover, the direction of its phenotypic effects was opposite to what would have been expected, since the LEW alleles highly increased the phenotype instead of decreasing it.

In order to facilitate interpretation of the QTL data, we show in Table 3 means and SEMs of the parental

strains (LEW and SHR) as well as their reciprocal intercrosses (F1 and F2) regarding all phenotypic traits for which suggestive or significant QTLs have been identified.

#### *Investigation of Ofil1, a major female-specific QTL on chromosome 4*

In the second phase of this study, we focused our attention on *Ofil1*. In order to better characterize this locus, the density of markers in the critical region was increased from six to 19 (only 14 of them being used as framework markers), as illustrated in Figure 1a. Since our F2 rats derived from either of two reciprocal crosses (LEW female × SHR male and SHR female × LEW male) and because cross-specific effects had already been detected in other QTL studies,<sup>15,38</sup> a detailed analysis of *Ofil1* was performed on F2 subpopulations grouped by sex and type of cross. Splitting the F2 population in four such subgroups revealed that this

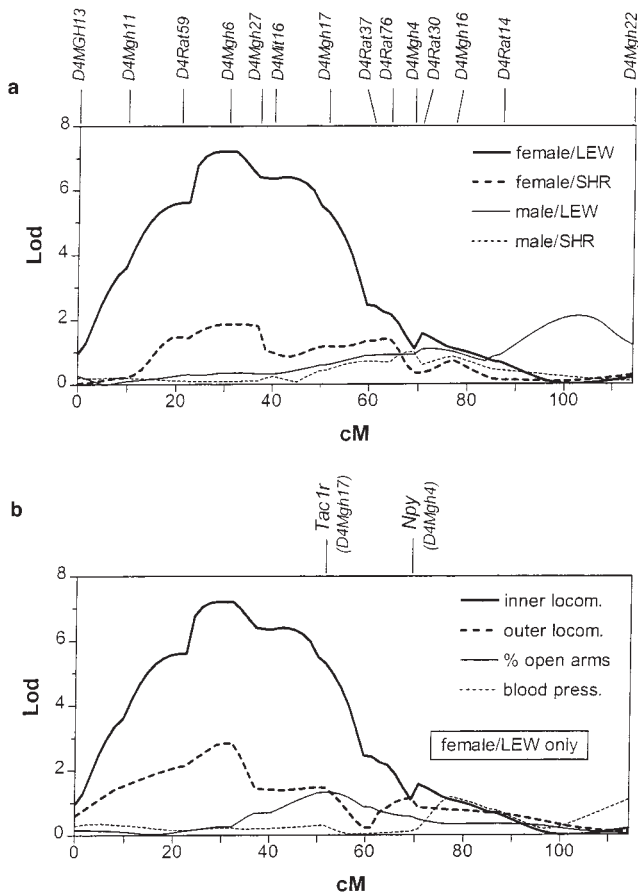
**Table 3** Means ± SEMs of five phenotypic traits for LEW rats, SHR rats and their intercrosses

Genetic type	Outer locomotion	Inner locomotion <sup>a</sup>	Entries open arms (%) <sup>a</sup>	Entries closed arms	Blood pressure <sup>a</sup>
<b>Males</b>					
LEW (n = 5) <sup>a</sup>	47.8 ± 7.5	1.0 ± 0.0	16.3 ± 7.6	3.8 ± 0.7	161.4 ± 3.8
SHR (n = 12) <sup>a</sup>	58.2 ± 4.7	4.7 ± 0.7	28.8 ± 5.0	4.6 ± 0.5	229.2 ± 1.9
F1/LEW (n = 10)	62.1 ± 8.3	5.0 ± 1.1	15.6 ± 4.7	5.2 ± 0.7	180.4 ± 2.0
F1/SHR (n = 10)	52.1 ± 10.9	2.8 ± 0.9	19.2 ± 5.2	5.5 ± 0.6	181.9 ± 2.8
F2/LEW (n = 48)	55.8 ± 4.2	4.3 ± 0.5	17.6 ± 2.6	5.1 ± 0.4	179.9 ± 2.7
F2/SHR (n = 48)	60.5 ± 4.9	4.3 ± 0.9	17.7 ± 2.7	4.5 ± 0.3	176.6 ± 2.8
<b>Females</b>					
LEW (n = 6) <sup>a</sup>	74.3 ± 12.3	1.3 ± 0.2	10.1 ± 6.8	7.0 ± 1.5	157.2 ± 1.6
SHR (n = 12) <sup>a</sup>	79.3 ± 4.4	13.1 ± 2.1	35.3 ± 5.0	5.2 ± 0.4	193.8 ± 1.8
F1/LEW (n = 10)	65.4 ± 7.6	4.3 ± 1.1	23.8 ± 4.7	6.6 ± 0.9	171.9 ± 3.1
F1/SHR (n = 10)	62.6 ± 6.7	3.7 ± 1.2	25.4 ± 5.1	6.9 ± 0.9	170.3 ± 2.3
F2/LEW (n = 48)	78.7 ± 3.1	9.3 ± 1.1	23.6 ± 2.5	5.6 ± 0.3	172.0 ± 2.4
F2/SHR (n = 48)	81.1 ± 4.3	6.7 ± 0.9	21.0 ± 2.0	6.2 ± 0.4	167.7 ± 2.4

LEW: Lewis rats. SHR: Spontaneously Hypertensive Rats. F1/LEW and F1/SHR: F1 rats from either LEW or SHR mothers. F2/LEW and F2/SHR: F2 rats from either LEW or SHR grandmothers.

<sup>a</sup>Data from Ramos *et al.*<sup>24</sup>





**Figure 1** LOD scores for a major QTL (*Ofil1*) on rat chromosome 4. (a) Inner locomotion in the OF. The positions of the 14 framework markers are shown on the top of the figure. This QTL analysis was performed on F2 subpopulations grouped by sex and type of cross (LEW or SHR grandmothers) and 'LEW' and 'SHR' indicate the strain of the grandmother. LOD scores are suggestive when higher than 2.8 and significant when higher than 4.3.<sup>37</sup> LOD scores for the female/LEW group exceeded significance levels (LOD = 7.22), with 50.4% of the total variability being explained by the QTL. LOD scores for the other groups did not reach suggestive levels. (b) LOD scores of four phenotypic traits based on data from female/LEW only. Inner locom and outer locom: inner and outer locomotion in the OF; % open arms: percentage of entries in the open arms of the EPM; blood press: systolic blood pressure. The positions of two candidate genes, *Npy* (*D4Mgh4*) and *Tac1r* (*D4Mgh17*), were determined. Whereas the former falls outside *Ofil1*'s confidence interval, the latter is placed in its right boundary with a LOD of 5.28.

QTL was not only sex-specific, but also dependent on the type of cross (LEW or SHR grandmother) (Figure 1a). Thus, the LOD score for females derived from a LEW grandmother highly exceeded significance levels (LOD = 7.22). For this group, the most likely position of the QTL falls in an interval of 8.6 cM (*D4Rat59*–*D4Mgh6*) at 0.6 cM from *D4Mgh6*, explaining 50.4% of the total phenotypic variance. LOD scores for the other three groups did not even reach suggestive levels (LOD <2.0). Besides inner locomotion in the OF, no other

phenotype was affected by *Ofil1* (Figure 1b) except the outer OF locomotion at a suggestive level (LOD = 2.84).

#### Candidate genes for *Ofil1*

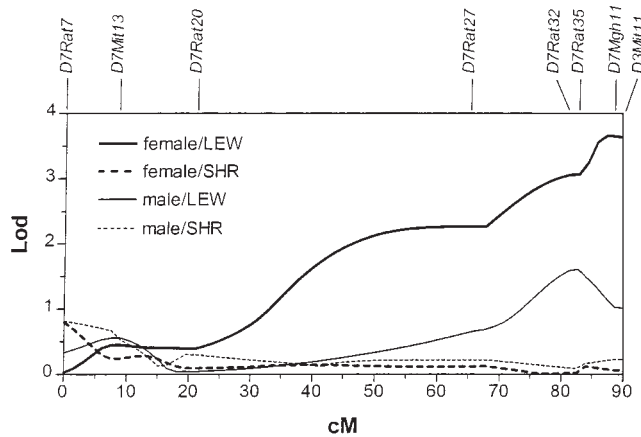
The genes *Tac1r* and *Npy* have been previously mapped on rat chromosome 4 (<http://www.well.ox.ac.uk>) and they encode for the substance P high-affinity receptor NK<sub>1</sub> and for neuropeptide Y (NPY), respectively. Since substance P and NPY are among the neurotransmitters that are able to modulate anxiety in rodents and humans<sup>39–43</sup> we considered *Tac1r* and *Npy* as candidate genes for our QTL. Two microsatellite sequences, *D4Mgh17* and *D4Mgh4*, have been previously identified within *Tac1r* and *Npy*, respectively, being thus considered as markers for these genes (<http://www.genome.wi.mit.edu/rat/public> and <http://www.well.ox.ac.uk>). We have thus localized *Tac1r* and *Npy* in relation to *Ofil1* (Figure 1b) through the genotyping of our F2 rats for *D4Mgh17* and *D4Mgh4*, which have been then added to the map. Whereas *Npy*'s involvement can be excluded, the position of *Tac1r* allows us to continue considering it as a candidate gene. The position of the former falls outside *Ofil1*'s confidence interval, whereas the latter is placed in its right boundary with a LOD score of 5.28.<sup>36</sup>

#### Identification of *Ofil2*, a second female-specific QTL on chromosome 7

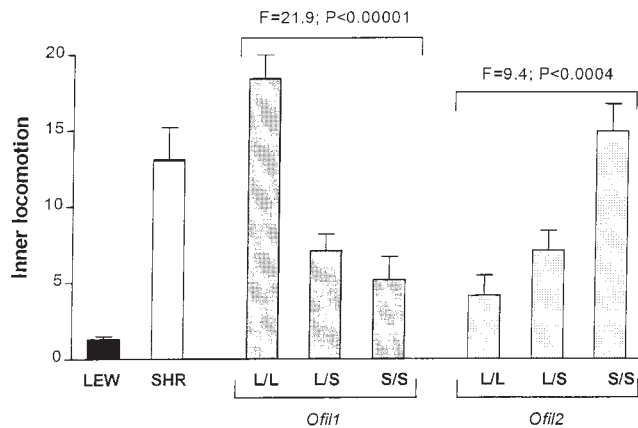
Because *Ofil1* only affected females derived from LEW grandmothers and since its unexpected phenotypic effect suggested the presence of other counteracting QTLs for the same trait, we have performed new genome scans on F2 subpopulations grouped by sex and types of cross. Such analyses revealed a new suggestive QTL for inner OF locomotion on chromosome 7 (Figure 2). This QTL was thus named *Ofil2*. Like *Ofil1*, *Ofil2* acted exclusively in females derived from a LEW grandmother. For this population, the QTL peak was placed in an interval of 6.2 cM (*D7Rat35*–*D7Mgh11*) at 2.2 cM from *D7Mgh11*. No suggestive or significant effects were found for any of the other phenotypes considered in this study (data not shown).

#### The opposite effects of *Ofil1* and *Ofil2*

As shown in Figure 3, *Ofil1* and *Ofil2* had opposite effects on the phenotype. As indicated in the figure, LEW females display a much lower inner OF locomotion than their SHR counterparts. Thus, one could expect the LEW alleles of a QTL for this trait to decrease the magnitude of the phenotype. Nevertheless, *Ofil1* acted in an unexpected direction, with LEW alleles promoting higher (instead of lower) inner locomotion. For *Ofil2*, conversely, the LEW allele decreases inner locomotion as expected. The positive effect of *Ofil1* LEW alleles is seemingly masked in rats from the LEW strain. Therefore, the strong phenotypic impact of *Ofil1* in F2 rats raises the question of whether an epistatic interaction with other gene(s) would be the cause of its unexpected action, where the presence of SHR alleles for one or more further genes would be necessary for *Ofil1*-L/L rats to express their higher pheno-



**Figure 2** LOD scores for a suggestive QTL (*Ofil2*) on rat chromosome 7. Inner locomotion in the OF. The positions of the eight framework markers are shown on the top of the figure. This QTL analysis was performed on F2 subpopulations grouped by sex and type of cross (LEW or SHR grandmothers) and 'LEW' and 'SHR' indicate the strain of the grandmother. LOD scores are suggestive when higher than 2.8.<sup>37</sup> Only LOD scores for the female/LEW group exceeded suggestive levels (LOD = 3.66). No suggestive or significant effects were found for any of the other phenotypes considered in this study (data not shown).



**Figure 3** Inner locomotion in the OF (means and SEMs) for F2 female rats with a LEW grandmother grouped by genotype (*Ofil1* and *Ofil2*). LEW and SHR reference data (females only) are from Ramos *et al.*<sup>24</sup> L/L, L/S, S/S: *Ofil1* or *Ofil2* genotypes of rats homozygous for the LEW allele, heterozygous and homozygous for the SHR allele, respectively. F and P were obtained from single-point analysis of variance for genotype effects.

type. The simultaneous analysis of *Ofil1* and *Ofil2* through MAPMAKER/QTL,<sup>36</sup> however, did not provide enough evidence to demonstrate an epistatic interaction between these two QTLs. MAPMAKER/QTL tests for determining the mode of inheritance controlling the two QTLs indicate that *Ofil1* is best fitted by a dominant model for the SHR allele. Nevertheless, both additive and recessive models for the SHR allele may explain the action of *Ofil2*.

## Discussion

Data accumulated throughout the last two decades on large samples of monozygotic and dizygotic twins (reared either together or apart), provide consistent evidence for the influence of genetic factors on anxiety-related personality traits in humans.<sup>8,9</sup> The important role of genetics in animal models of anxiety/emotionality has also been repeatedly demonstrated.<sup>30</sup> Although recent studies dissecting the individual differences in the predisposition to develop anxiety disorders in humans show that anxiety proneness is etiologically heterogeneous, the contribution of genetic factors has been consistently identified.<sup>7,10,12,13</sup> Heredity can explain as much as 37% of the variability in symptoms of overanxious disorder in children and 40–50% of the individual differences in symptoms of anxiety/depression in adults.<sup>7,13</sup> To date, however, very little is known about the molecular bases underlying the influences of genetic factors on anxiety-related traits and disorders in both humans and animals. The identification of genes and gene products affecting these phenotypes will certainly improve the current understanding of the biological mechanisms controlling anxiety-related psychopathologies.

The present QTL analysis revealed several chromosomal regions putatively associated with emotionality/anxiety-related behaviors, with activity and also with blood pressure. In addition, one major QTL was found to strongly modulate inner OF locomotion.

### The multiple QTLs

The analysis of Table 2 reveals no QTL simultaneously affecting several behavioral indices of anxiety/emotionality, which would be expected if the different measures reflected a unique psychological dimension. Nevertheless, a growing body of evidence shows that emotionality is not a unidimensional construct.<sup>30,31</sup> Regarding anxiety tests, pharmacological and factor analyses have shown a lack of correlation among measures from different types of paradigms,<sup>44–48</sup> which suggests that these models assess different forms of anxiety.

Our finding of a major QTL affecting inner OF locomotion but not EPM anxiety measures confirms indeed the absence of phenotypic correlation that had been previously found between these variables within our LEW/SHR F2 population.<sup>24</sup> Whereas Flint *et al.*<sup>18</sup> identified QTLs in mice affecting measures from different emotionality/anxiety tests, a recent study by Gershensfeld and Paul<sup>20</sup> revealed a lack of overlap among QTLs influencing anxiety-measures from the EPM, the OF and the black/white box. These results highlight the importance of the context in the expression of emotional responses, as pointed out by Lazarus.<sup>49</sup> Phobic disorders, for example, are highly dependent on particular objects or situations.<sup>25</sup> Similarly, the various animal models of anxiety/emotionality differ from each other either in the type of aversive stimulation or in the context of its presentation. Therefore, it is likely that whereas some genes have general behavioral

effects under a variety of emotional situations, others are specific of certain environmental contexts.

Additive genes account for a significant proportion of the individual differences in symptoms of overanxious disorder but not of separation anxiety in children.<sup>7</sup> In adults, genetic factors play a significant role in the development of panic disorder but not of sporadic panic attacks.<sup>12</sup> Thus, the high specificity of some emotionality-related QTLs may in fact help us in the task of breaking down complex phenotypes into simpler components which are genetically independent and individually analyzable, as pointed out by Lander and Kruglyak.<sup>37</sup>

Blood pressure in rats is one of the traits providing the highest number of QTLs in mammals.<sup>50</sup> To our knowledge, however, this is the first report of blood pressure QTLs on chromosomes 6 and 12. That different QTLs were found to influence blood pressure and behavioral variables indicates that the behavioral and blood pressure differences observed between LEW and SHR rats are not genetically linked. These findings are in agreement with our previous report of lack of correlation between behavioral variables and blood pressure in LEW/SHR intercrosses.<sup>24</sup>

#### *The major QTL *Ofil1**

Avoidance of the central part of the OF (where the animal cannot perform thigmotaxis) is considered as an index of anxiety which is sensitive to anxiolytic/anxiogenic drugs.<sup>20,28,29,33–35</sup> Considering the major impact of our QTL located on chromosome 4 (namely *Ofil1*) on OF inner locomotion, we have investigated in more detail the mode of action of this genetic locus.

This QTL was found to affect females only. Sex specificity has been reported in different QTL studies, including behavioral ones.<sup>15,50–52</sup> Despite the well documented sexual dimorphism of emotionality measures in rodents,<sup>6,32,53</sup> the mechanisms responsible for these gender differences are poorly understood. In humans, women are diagnosed more frequently than men for anxiety/depression-related disorders.<sup>5,6</sup> Moreover, experimental evidence on both children and adults suggests that anxiety is more strongly affected by genes in female than in male subjects.<sup>7,13</sup> The present study is, to our knowledge, the first one to report a female-specific QTL for an emotionality-related behavior, which renders *Ofil1* an interesting tool to improve our understanding of sex-genotype interactions in anxiety and of gender-dependent mechanisms of adaptation.

Regarding the cross-specific effect of *Ofil1*, the absence of QTLs on chromosome X led us to rule out the hypothesis of a X-linked gene controlling *Ofil1*. A growing number of traits has been recognized as following non-Mendelian inheritance patterns.<sup>54</sup> Recently, several molecular mechanisms capable of modifying Mendelian rules have been discovered. Genomic imprinting is one such mechanism which plays a predominant role in behavioral traits.<sup>55,56</sup> Indeed, two behavioral mouse QTLs recently identified are possibly

controlled by genomic imprinting.<sup>15,38</sup> In the first study, a QTL for alcohol preference was found to act only in females having received the allele from their mothers. As shown in Figure 1a, *Ofil1* was effective only in females having a LEW grandmother. Skipping generations is not typical of genomic imprinting, which is usually reversed by the progeny with the change of the parental sex.<sup>54</sup> Nevertheless, there are several reports of imprinting and imprint erasure not reversed from one generation to the next<sup>55,57–61</sup>, which could make these effects last longer than one generation. Mitochondrial DNA mutations (always maternally transmitted) are also responsible for several non-Mendelian inherited disorders.<sup>54,62,63</sup> Some of these mutations seem to produce psychiatric manifestations such as depression and alcoholism. Moreover, interactions between nuclear and mitochondrial genes have been described.<sup>62–64</sup> Thus, the hypothesis of mitochondrial genes interacting with *Ofil1* also deserves consideration.

#### *Rat chromosome 4 and its candidate genes*

Several QTL studies suggest that the distal end of mouse chromosome 1 contains emotionality-influencing gene(s).<sup>18,19,21,65</sup> We have thus screened the corresponding region in the rat genome (on chromosome 13) but we found no QTLs for the traits studied herein. Still in mice, Gershenfeld and Paul<sup>20</sup> have recently identified QTLs influencing the time spent in the OF center. One of these loci was mapped on chromosome 6 (which has a high degree of homology with rat chromosome 4), but a detailed analysis of rat/mouse homologies indicates that the chromosome-4 region containing our major QTL for inner OF locomotion does not correspond to that containing the equivalent mouse QTL.

More promising than the search for rat/mouse homologies is the comparison of our data with previous results from QTL studies on rats. Carr *et al*<sup>66</sup> have recently identified a major QTL on rat chromosome 4, in a region not far from the confidence interval of our QTL *Ofil1*, that influences alcohol consumption. Considering the reported genetic relationship between alcoholism and anxiety/depression disorders,<sup>13</sup> further and more powerful studies should be conducted to verify whether or not these two regions overlap and may correspond to a common emotionality-influencing gene. Another recent QTL study using a substrain of LEW rats revealed yet another major QTL on chromosome 4 influencing the susceptibility to induced arthritis.<sup>67</sup> Despite the fact that different markers have been used in the latter study and in the present one, the results point to similar locations for the QTL reported by Lorentzen *et al*<sup>67</sup> and *Ofil1*, reported herein. It is interesting to notice that in a different substrain of LEW rats, that develop induced arthritis, the susceptibility to inflammatory diseases is related to the hyporesponsiveness of their HPA axis to inflammatory agents and to other stressful stimuli such as the OF test.<sup>68,69</sup> Thus, the finding of three major QTLs on rat chromosome 4 potentially controlling stress-related



responses appoints this genomic region as a promising one for future molecular studies.

The tachykinin substance P plays a major role as an excitatory neuromediator in the nervous system. Among the three subtypes of tachykinin receptors, NK<sub>1</sub> is the one with the highest affinity for substance P.<sup>70</sup> In the present study we have mapped the gene for NK<sub>1</sub> (*Tac1r*) on rat chromosome 4, inside the confidence interval of *Ofil1*. In addition to the well known effects of substance P on nociception and cardiovascular functions, recent studies point to the exciting possibility that alterations in the NK<sub>1</sub> receptor may be involved in the pathophysiology of psychiatric disorders such as anxiety and depression. Thus, it has been shown that the stimulation of mouse substance P receptor decreases open-arm entries in the EPM. The opposite effect (anxiolysis) was observed in response to the receptor antagonist FK 888.<sup>39</sup> In rats, administration of another substance P receptor antagonist (CGP 49823) triggered anxiolytic effects in the social interaction test<sup>41</sup> and anxiogenic effects of substance P have also been reported in the EPM.<sup>71</sup> The most compelling results come from a recent study by Kramer *et al*<sup>43</sup> in humans, who showed that an orally available NK<sub>1</sub> antagonist was more efficient than paroxetine in the treatment of patients with major depressive disorder and moderately high anxiety. In what concerns our QTL study, it is noteworthy that SHR rats, used herein, were shown to have a low brain expression of the gene for substance P compared with WKY rats.<sup>72</sup> Considering the localization of our major QTL and the genetic correlations that have been reported between anxiety and depression,<sup>7</sup> further efforts should be concentrated on testing the potential significance of *Tac1r* for the QTL here described.

#### *The opposite effects of Ofil1 and Ofil2*

As shown in Figure 3, *Ofil1* LEW alleles promoted higher (instead of lower, as it could be expected) inner OF locomotion. LEW alleles of *Ofil2*, on the other hand, had the expected phenotypic effect, decreasing OF inner locomotion. Similar opposite-acting QTLs have been reported in studies on blood pressure<sup>73–76</sup> and behavioral variables,<sup>19,21,52</sup> which is genetically sound, since quantitative traits result from the additive action of many genes. Thus, even a strain presenting a high phenotype (ie tending to one extreme) may carry some alleles for a low phenotype (ie tending to the other extreme). Regarding *Ofil1*, however, it is unlikely that its strong phenotypic effect (>50% variability) is simply counteracted by the opposite and additive effects of several other QTLs. A more likely hypothesis is that the phenotypic expression of *Ofil1* depends on epistatic interactions with other gene(s). A recent study in humans has revealed a major locus for anxiety proneness that is modulated by epistatic interactions with three other loci.<sup>77</sup> In the present study, the fact that major positive effects of *Ofil1*'s LEW alleles are detected in a segregating F2 population, being completely masked in pure LEW rats, strongly suggests that such effects would only be possible in the presence of

SHR alleles from other QTL(s). This phenomenon has been tested considering *Ofil2* as the second potentially interacting gene, but due to the low number of female/LEW rats available, such an effect could not be demonstrated herein. Nevertheless, the hypothesis of an epistatic interaction modulating the effects of *Ofil1* remains genetically sound, even though further analyses using larger F2 populations will be necessary before it can be conclusively verified.

The present study points to a complex genetic basis underlying emotional traits in rats. This finding is not surprising if one considers the biological and psychological complexity of these phenotypes,<sup>30</sup> but it reinforces the importance of using adequate and informative crossing designs while genetically analyzing complex traits. Modern genetics is likely to increase even further the list of 'unusual' processes able to modify the rules of Mendelian inheritance<sup>54</sup> and the present work opens an interesting perspective to investigate some of these mechanisms. Most importantly, the present identification of genomic regions strongly influencing an anxiety/emotionality-related response should ultimately contribute to the nosological and etiological understanding of anxiety-related disorders.

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