Mamm Genome (2011) 22:329–340
DOI 10.1007/s00335-011-9327-5

The influence of sex and estrous cycle on QTL for emotionality and ethanol consumption

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Received: 8 November 2010 / Accepted: 5 April 2011 / Published online: 24 April 2011

Abstract The inbred rat strains Lewis (LEW) and Spontaneously Hypertensive Rats (SHR) differ with respect to several emotionality- and ethanol intake-related behaviors, one of which (inner locomotion in the open field; OF) is strongly influenced by a locus (*Anxrr16*) on chromosome (Chr) 4. We aimed to further investigate the influence of Chr 4 on these behaviors and to evaluate the role of the estrous cycle in QTL expression. LEW females and SHR males were intercrossed to produce F1 and F2 rats (96–97/sex), which were then tested in the OF, light–dark box (LDB), forced swimming test (FST), and an ethanol consumption procedure (ECP). In addition, another group of 96 F2 females were tested in the OF and LDB according to their estrous cycle phase. All animals were genotyped for microsatellite markers located on Chr 4 and two QTL analyses were performed. A factor analysis of the F2 population produced five factors reflecting different behavioral dimensions. QTL analysis revealed five significant loci in males, some of which with pleiotropic effects on behaviors measured in the OF, LDB, and ECP. The second QTL analysis revealed two significant loci in females in diestrous–proestrous and one in females in estrous–metestrous that influence behaviors in the OF and LDB. Results revealed that *Anxrr16* and four other new QTL influence emotionality- and ethanol-related behaviors in male rats, whereas *Anxrr16* attained suggestive levels only in females in diestrous–proestrous, which raises the need for taking into account factors related to the sex and estrous cycle in behavioral QTL analysis.

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Introduction

The inbred rat strains Lewis (LEW) and Spontaneously Hypertensive Rats (SHR) constitute a valuable genetic model for the study of emotionality. They show contrasting responses to different stressful stimuli, including the open field (OF), light–dark box (LDB), and forced swimming tests (FST), with LEW rats displaying more "anxious" and "depressive-like" behaviors than their SHR counterparts (Hinojosa et al. 2006; Ramos et al. 2002). These strains have also been compared in different ethanol consumption procedures (ECP), where inconsistent results were found, particularly in males, thus pointing to the crucial role of the protocol and of sex in the outcome of genetic studies on alcohol drinking (Chiavegatto et al. 2009; Da Silva et al. 2005; Vendruscolo et al. 2006a). The need for studying sex differences to avoid bias in the conclusions drawn from animal experimentation has been recently emphasized by Wald and Wu (2010) and Zucker and Beery (2010).

Many studies have shown that quantitative trait locus (QTL) analysis is a useful strategy to map genomic regions contributing to complex behavioral traits. Basically, this methodology identifies genomic regions that are potentially correlated with continuous phenotypic variation (Flint et al. 2005). Several different QTL for emotionality and ethanol consumption have already been identified in rodents (Flint

2003; Flint et al. 1995; Moisan et al. 1996; Ramos et al. 1999). Although the number of identified genes is still incomparably smaller than the number of identified QTL, this strategy can ultimately lead us to the identification of specific genes and gene products influencing behavior (Flint et al. 2005; Pravenec et al. 2008; Smoller et al. 2008; Tomida et al. 2009; Yalcin et al. 2004).

Ramos et al. (1999), using LEW and SHR rats, have mapped for the first time a genomic region affecting central locomotion in the open field (OF), a behavior that is thought to be anxiety-related (Angrini et al. 1998; Prut and Belzung 2003). This QTL, which corresponds to a large genomic region (nearly 75 Mb) located on rat chromosome (Chr) 4, was initially found to affect only females, was named *Ofil1* (open field inner locomotion 1) by Ramos et al. (1999) and *Anxrr16* (anxiety-related response 16) by the Rat Genome Database (http://rgd.mcw.edu/). Interestingly, LEW alleles promoted greater (instead of less, as would be expected) central OF locomotion, a phenomenon that is relatively common in QTL studies and is known as the "transgressive effect" (Caldarone et al. 1997; Llamas et al. 2005; Moisan et al. 2003; Silva et al. 2007; Wehner et al. 1997). Further experiments suggested that this genomic region could also modulate ethanol intake, cocaine sensitization, and stress response in LEW and SHR rats (Vendruscolo et al. 2006a, b, 2009). In addition, Hameister et al. (2008), using a different pair of selectively bred rat lines, showed that variations of the *D4Rat59* marker (near the *Anxrr16* peak) were significantly correlated with variations in central locomotion in the OF test. Moreover, three other important QTL have been mapped near this genomic region.

The first QTL, which influences ethanol consumption, was found using the selected rat lines P (alcohol-preferring) and NP (alcohol-nonpreferring) (Carr et al. 1998). Interestingly, these two strains also differ in their approach to the open arms of the EPM (Pandey et al. 2005), another anxiety-related measure. The second QTL also influences ethanol consumption and was reported in a study that used an intercross between high-ethanol-preferring (HEP) and Wistar-Kyoto (WKY) rats (Terenina-Rigaldie et al. 2003a). A subsequent study showed that this QTL had a pleiotropic effect, influencing not only emotional behaviors but also ethanol consumption (Terenina-Rigaldie et al. 2003b). Finally, the third QTL influences the control of corticosterone levels in the LEW and Fischer 344 (F344) inbred rat strains (Potenza et al. 2004). The data revealed by these four distinct genetic models (LEW/SHR, P/NP, HEP/WKY, LEW/F344) suggest that one or several different genes located on rat Chr 4 may affect both emotionality and ethanol consumption in a pleiotropic manner. However, there are doubts about the relative position of the aforementioned QTL, because each was identified using different rat strains and different molecular markers (Ramos and Mormède 2006).

Although the LEW/SHR genetic model is a very promising tool in the search for the neurobiological basis of emotional and consummatory behaviors, no QTL analysis for traits measured in the LDB, FST, and ECP tests (of anxiety-, depression-, and alcohol-related behaviors, respectively) had ever been performed using these two strains. Therefore, through the intercross of LEW and SHR inbred strains, the present study generated F2 rats of both sexes that were tested in the OF, LDB, FST, and ECP before being genotyped with markers covering the entire Chr 4, which, as mentioned above, is likely to harbor genes with a reasonably high impact on emotionality and alcohol drinking. Behavioral data were factor analyzed and, together with the genotypic data, were used in a QTL analysis (experiment 1). In addition, because experiment 1 showed that sex had a major influence on the expression of several QTL, an additional cohort of F2 females was produced, divided in two groups according to their estrous cycle phase, and used in a second QTL analysis for OF and LDB behaviors (experiment 2).

Materials and methods

Animals

The origin of the inbred LEW and SHR rats used herein has been described elsewhere (Chiavegatto et al. 2009). Both strains had been maintained in the Behavior Genetics Laboratory for more than 20 generations under a system of brother-sister mating. F2 rats were produced from intercrosses between LEW females and SHR males. F1 rats were brother–sister mated producing 193 F2 rats (97 males and 96 females) that were used in the first QTL analysis (experiment 1) and 96 females that were used in the second QTL analysis (experiment 2). The animals were weaned and separated by sex at 4 weeks of age and, thereafter, kept in collective plastic cages (5 rats/cage) with food and water available ad libitum under a 12-light:12-dark cycle (lights on at 07:00 h) at 22 ± 2°C. All animals (193 F2 plus LEW, SHR, and F1 with 10/group/sex) from experiment 1 were characterized in the OF (8 weeks of age), LDB (9 weeks), FST (11 weeks), and ECP (13 weeks), with males and females being tested on different days. The 96 F2 females from experiment 2 had their estrous pharmacologically induced and were divided in two groups, diestrous–proestrus (DP) ($n = 43$) and estrous–metestrous (EM) ($n = 53$), and were then characterized in the OF and LDB (10 weeks of age). All behavioral tests were carried out between 13:30 and 18:00 h. Just before tests the rats were transported from the housing room to an adjacent

testing room inside a container that allowed free movement.

Adequate measures were taken to minimize pain or discomfort to the animals. The present experiments were in accordance with the local regulations for the ethical use of animals in research (CEUA/UFSC) which follows the Principles of Laboratory Animal Care from NIH and were covered by valid permission (Protocol PP00019 and 23080.002853/2006-32/UFSC).

Behavioral tests

Open field (OF)

The OF apparatus was described previously (Izídio et al. 2005). The illumination in the test room provided 7 lx in the center of the apparatus. Each rat was placed in the center of the OF, which was novel to the animal, and the following variables were scored for 5 min: number of peripheral (adjacent to the walls) and central (away from the walls) squares crossed with all four paws and total number of fecal boluses. The behavior of each animal was recorded by a video camera positioned above the apparatus, which allowed monitoring in another room via a closed TV circuit. The floor of the apparatus was cleaned with a sponge wetted with ethanol 10% and a dry paper towel between rats. Recording and cleaning methods were unaltered for the LDB test.

Light–dark box (LDB)

The LDB apparatus was described previously (Izídio et al. 2005). Both white and red bulbs were located 30 cm above the floor of apparatus, thus providing 750 lx inside the white compartment and 20 lx inside the black compartment. Each rat was placed in the center of the light compartment and the following variables were registered for 5 min: number of squares crossed and time spent with all four paws in each compartment and number of transitions between compartments (one entry in the light plus one return to the dark). The time spent in the light compartment did not include the initial latency to the first entry into the dark compartment.

Forced swimming test (FST)

The FST consisted of placing the rat twice inside a cylindrical glass tank (40-cm height, 18-cm diameter) that contained clean water up to 20 cm above the bottom and was 25–27°C. The illumination in the test room was about 7 lx. On the first day, after 15 min of forced exposure to the tank (pretest session) with no behavioral observation, the animal was dried with a towel and a heat dryer and returned to its home cage. On the second day, 24 h after the pretest session, the rat was placed once again in the water tank (test session) in the same conditions described above and defecation and total immobility time were recorded for 5 min by an observer sitting beside the water tank. The animal was considered immobile when it only floated or made slight movements to keep the head above the water. The water was changed before the introduction of each animal.

Ethanol consumption procedure (ECP)

Animals were individually housed in plastic cages (21 × 28 × 19 cm) with the floor covered with sawdust and had ad libitum access to food. Briefly, the procedure consisted of two bottles of either ethanol solution or water that were available continuously as a free choice to animals. The protocol consisted of (1) 2 days of habituation to the individual cage and only tap water was available, (2) forced ethanol (10%), and (3) free choice between ethanol (2.5, 5, 10, and 20%) and tap water (2 days for each concentration). The total consumption of each solution and the body weight were measured every 2 days at 18:00 h by weighing the bottles. The positions of the bottles were switched every day to prevent position bias. The data were transformed to grams of ethanol per day per kilograms of body weight. Due to logistic limitations, this procedure was performed only with F2 animals.

Genotyping

Following behavioral tests, all rats were euthanized and their livers and spleens were removed for DNA extraction using a DNAzol commercial kit (GibcoBRL, Carlsbad, CA, USA). All animals were genotyped for 14 polymorphic microsatellite markers (Invitrogen, São Paulo, Brazil) distributed throughout Chr 4. Genotypes were determined by polymerase chain reaction (PCR) in a microtiter plate in a P×2 thermal cycler apparatus. In a 20-ml reaction volume, 50 ng of genomic DNA was mixed with 5 pmol of each primer and 0.4 U of Taq polymerase (Promega, Madison, WI, USA) in Promega type A buffer. The PCR program was (1) one cycle at 96°C for 4 min; (2) 38 cycles at 92°C for 30 s, 58–62°C (depending on the marker) for 1 min, and 72°C for 31 s; (3) one cycle at 72°C for 4 min. Alleles were visualized either on ethidium bromide-stained 3% agarose gels or on 8% polyacrylamide gels.

Estrous cycle

Ninety-six F2 females were first injected in the dorsal region of the neck with estradiol benzoate (10 μg/0.1 ml sc) (Aventis pharma, Suzano, Brazil) and then 48 h later

with progesterone (5 mg/kg sc) (Merck, Darmstadt, Germany), with an injection volume of 1 ml/kg in order to induce estrous. Both drugs were dissolved in sesame oil. Females were then allocated to two different groups according to the stage of the estrous cycle on the experimental day: 43 DP and 53 EM. At 9 weeks of age, females from both groups were tested on the same day in the OF and then immediately in the LDB. After the behavioral tests, vaginal smears were taken to confirm the estrous cycle stage. Briefly, each female was immobilized and a cotton tip containing 0.2 ml of saline solution (0.9%) was introduced into the vaginal cavity. After washing, vaginal fluid was collected and dropped on slides for observation in an optical microscope (10× and 40×).

QTL analysis

Genotype data were first analyzed by QTX software for complex trait analysis (www.mapmanager.org) in order to construct a complete linkage map containing all 14 microsatellite markers used herein with their respective map positions in cM. Following that, phenotypic data were entered into QTX and interval mapping was performed. This program looks for QTL every 1.0 cM throughout the chromosome. A LRS (likelihood ratio statistic) score, which is 4.6 times larger than the LOD score, was used to report the magnitude of the QTL. Significance thresholds were estimated by analysis of 1000 permutations every 1.0 cM. Since behavioral data showed significant sex differences and considering the importance of gender in human psychopathologies, the QTL analysis was performed separately for each sex.

Statistics

Two-way ANOVA (strain and sex) was used to compare behavioral variables in the OF, LDB, FST, and ECP between LEW and SHR rats. All phenotypic variables from experiment 1 were submitted to a principal component analysis (PCA) with a Varimax raw rotation, separately for each sex. Only factors with eigenvalues greater than one were kept. Two-tailed Student's *t*-tests were used to compare female rats in DP and EM. Values of $P < 0.05$ were considered significant. All analyses were performed using the Statistica 6.0 software package (Statsoft, Tulsa, OK, USA).

Results

Experiment 1

Significant differences were found between LEW and SHR rats in all behavioral measures except latency and locomotion in the dark compartment in the LDB (Fig. 1). In all cases, SHR rats approached the aversive compartments of the behavioral tests more and spent less time immobile in the FST than their LEW counterparts, with F1 and F2 rats most of the time somewhere in between the two parental strains (Fig. 1).

The PCA revealed five orthogonal factors with eigenvalues greater than 1 in both males and females. In males, they corresponded to 72% of the total variability. Factor 1, the most significant one, correlated strongly with all putative measures of anxiety/emotionality in the LDB. Factor 2 correlated only with measures from the ECP; factor 3 only with measures from the OF; and factor 4 with ethanol free-choice consumption, forced ethanol, and time of immobility in the FST. Finally, factor 5 was represented only by defecation in the FST (Table 1). In females, the five factors corresponded to 67% of the total variability. Factor 1 correlated strongly with all putative measures of anxiety/emotionality from the LDB and with central locomotion in the OF. Factor 2 correlated with measures of ethanol consumption at high concentrations (10 and 20%); factor 3 with measures of ethanol consumption at low concentrations (2.5 and 5%), forced ethanol, and defecation in the FST; factor 4 with measures from the OF (central and peripheral locomotion) and time of immobility in the FST; and factor 5 was represented only by defecation in the OF (Table 2).

The significant results from the first QTL analysis are given in Table 3 and shown Fig. 2. Five QTL were identified above the significance level (one of which, near *D4Mgh11*, presented pleiotropic effects) and six were found above the suggestive level (data not shown) in male rats. The five significant QTL were found for behaviors related to emotionality/anxiety (OF and LDB) and ethanol consumption (Table 3). The QTL for central locomotion in the OF (Fig. 2), which is one of the most consistent and significant QTL ever identified for anxiety-related behaviors (Mormède et al. 2002; Ramos et al. 1999; Vendruscolo et al. 2006a) (http://rgd.mcw.edu/) and was one of the main focuses of this work, was found for the first time to be significant in males and to have two peaks, with the higher one near its expected map position. Also, as expected, the LEW alleles had a transgressive effect on the phenotype (Table 3) (Ramos et al. 1999).

In females, surprisingly, no significant QTL was identified, but ten QTL above the suggestive level were found to influence behaviors exhibited in the OF, LDB, FST, and ECP (data not shown). An extra QTL analysis performed on the data resulting from the PCA revealed no significant QTL for any of the factors, except for one significant effect for factor 4 in males (LOD score = 3.2; $P = 0.013$; at *D4Rat40*).

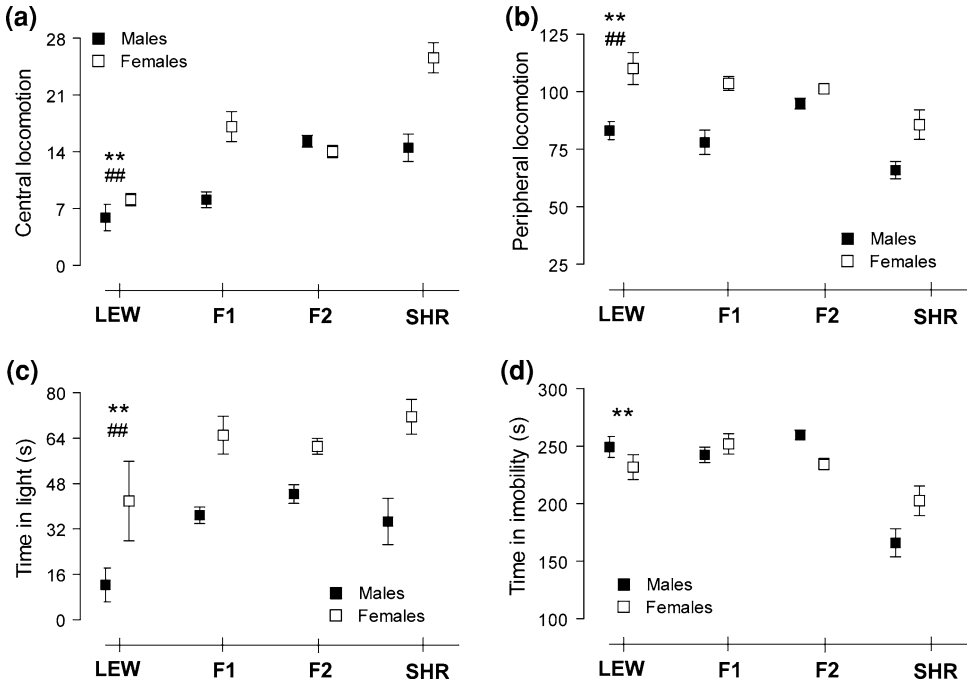

Fig. 1 Means and SEM of the behaviors from LEW, SHR, F1 (LEW × SHR) and F2 (F1 × F1) male and female rats. **a** Central locomotion in the OF. **b** Peripheral locomotion in the OF. **c** Time spent in the light compartment of the LDB. **d** Time of immobility in the FST. ANOVA was performed only with LEW and SHR rats. ** $P < 0.01$; * $P < 0.05$ and $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ represent overall strain and sex effects, respectively

Table 1 Factor analysis of 13 behavioral variables from 97 male F2 LEW/SHR rats

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Central locomotion (OF)			−0.72		
Peripheral locomotion (OF)			−0.71		
Defecation (OF)			0.52		
Time in light (LDB)	0.95				
Time in dark (LDB)	−0.96				
Locomotion in light (LDB)	0.92				
Time of immobility (FST)				0.81	
Defecation (FST)					0.85
Forced ethanol		0.40		0.42	
Ethanol (2.5%)				0.64	
Ethanol (5%)		0.74			
Ethanol (10%)		0.87			
Ethanol (20%)		0.78			
Total variance (%)	23	17	11	12	9

Factor loadings higher than 0.4, produced by a Varimax raw rotation, are shown for each factor

OF open field; *LDB* light–dark box; *FST* forced swimming test

Experiment 2

Females that were grouped according to their estrous cycle phase showed significant behavioral differences in the central (DP > EM; $P < 0.05$), peripheral (DP > EM; $P < 0.01$), and total locomotion (DP > EM; $P < 0.01$) in the OF (Fig. 3). No significant differences were found in the LDB test (data not shown). Two QTL were identified above the significance level (Table 4) and five were found above the suggestive level (Table 5) in DP female rats. The significant QTL influenced defecation in the OF and locomotion in the dark area of the LDB. In EM female rats, one significant (Table 4) and four suggestive (Table 5) QTL were found. The significant QTL and two of the suggestive QTL influenced four different behaviors from the LDB: time spent and locomotion in the light compartment (significant) and time spent in the dark compartment and number of transitions (suggestive).

In this second QTL analysis, two suggestive loci were found for central locomotion in the OF (Fig. 4 and

Table 2 Factor analysis of 13 behavioral variables from 96 female F2 LEW/SHR rats

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Central locomotion (OF)	0.42			−0.52	
Peripheral locomotion (OF)				−0.43	
Defecation (OF)					−0.94
Time in light (LDB)	0.94				
Time in dark (LDB)	−0.94				
Locomotion in light (LDB)	0.89				
Time of immobility (FST)				0.78	
Defecation (FST)			0.44		
Forced ethanol			0.62		
Ethanol (2.5%)			0.71		
Ethanol (5%)			0.76		
Ethanol (10%)		0.83			
Ethanol (20%)		0.78			
Total variance (%)	24	12	13	10	8

Factor loadings higher than 0.4, produced by a Varimax raw rotation, are shown for each factor

OF open field; *LDB* light–dark box; *FST* forced swimming test

Table 3 Significant QTL identified in the first analysis with male F2 LEW/SHR rats

Phenotype	Marker	Localization (cM)	LRS	% variance	*F*	*P*	Model	L/L	L/S	S/S
Central locomotion OF	*D4Mgh27*	73	14.4	14	7.53	0.00092	Free	19.24 ± 1.71	15.19 ± 0.91	11.75 ± 1.12
Defecation OF	*D4Rat76*	48	14.5	14	7.59	0.00087	Free	2.73 ± 0.46	0.91 ± 0.25	2.29 ± 0.44
Peripheral locomotion OF	*D4Rat131*	64	8.8	9	4.84	0.00998	Addictive	102.38 ± 6.67	97.02 ± 2.84	83.58 ± 3.94
Locomotion in light LDB	*D4Mgh11*	104	14.3	14	7.46	0.00098	Free	23.38 ± 1.18	16.90 ± 1.00	18.56 ± 1.61
Time in light LDB	*D4Mgh11*	97	10.4	10	4.96	0.00896	Dominant	55.90 ± 4.12	37.0 ± 3.73	43.19 ± 5.52
Time in dark LDB	*D4Mgh11*	103	17.4	16	9.15	0.00023	Free	203.10 ± 4.66	233.15 ± 4.52	221.96 ± 6.29
Forced ethanol	*D4Rat206*	110	10.8	11	5.59	0.00509	Free	20.92 ± 1.78	15.40 ± 0.80	18.18 ± 1.08

In cases where a QTL was found in the interval between two molecular markers, data from the most significant one is given

OF open field; *LDB* light–dark box; *cM* centimorgan; *LRS* likelihood ratio statistic; *L/L, L/S, S/S* genotype of F2 animals, *L/S* are heterozygous; *L/L* and *S/S* are homozygous (*L* = Lewis and *S* = SHR)

Table 5). Interestingly, the QTL found in DP females (near *D4Mgh27*) was mapped in the same region as the significant QTL found in males in the first QTL analysis. The second suggestive QTL found in EM females was located in a very different region of Chr 4 (near *D4Rat151*) (Fig. 4 and Table 5), thus suggesting that the expression of *Anxrr16* is influenced by the estrous cycle.

Discussion

The behavioral profiles of LEW and SHR rats reported in the present work are totally consistent with previously published data (Hinojosa et al. 2006; Izídio et al. 2005; Ramos and Mormède 1998). The multivariate analysis of all behaviors revealed five main factors in both males and females, although some differences were found in factor structure between sexes. For instance, the different behavioral tests shared more common aspects in females than in males. In males, factor 1 was associated only with emotional variables from the LDB, whereas in females factor 1 was linked with emotional variables from both the OF and the LDB. Recent data suggest that the OF and LDB tests assess very distinct aspects of the emotional behavior, sharing only 5.3 and 11.6% of their total variance in rats and mice, respectively (Archer 1973; Fraser et al. 2010;

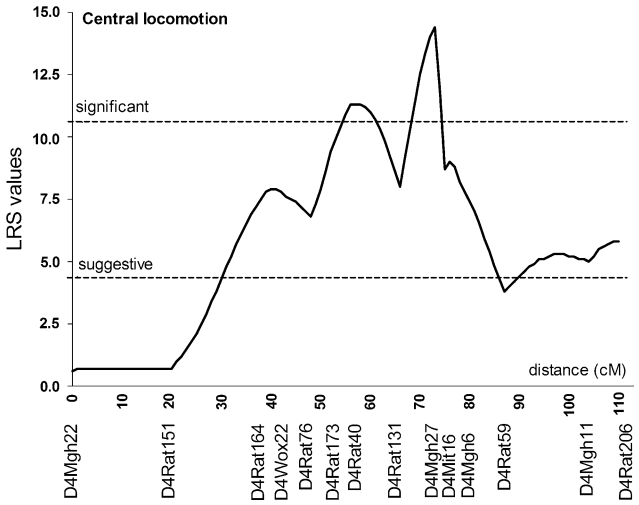

Fig. 2 A significant QTL found in males for central locomotion in the OF (free model). The 14 molecular markers used in the genotyping are shown at the *X* axis, which represents the map distances in cM

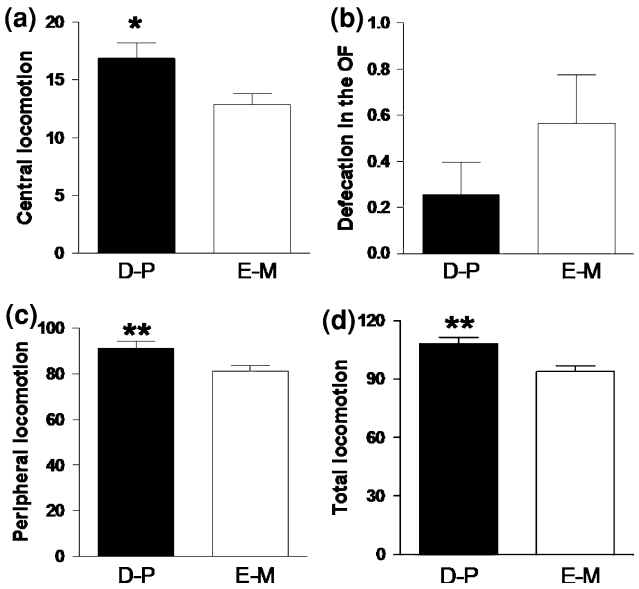

Fig. 3 Behavior in the OF of F2 females grouped according to their estrous cycle phases. * and ** represent an estrous cycle effect ($P < 0.01$ and $P < 0.05$ respectively; *t*-test). *D–P* diestrous and proestrous combined, $n = 43$; E–M estrous and metestrous combined, $n = 53$

Ramos 2008). Moreover, the complex set of emotional behaviors measured herein was shown to be related to two or three independent dimensions (Archer 1973; Cruz et al. 1994; File 1991). Therefore, identifying QTL that are specific for a particular anxiety- or depression-related test is expected to be the rule rather than the exception. Nevertheless, females showed a factor (factor 4) indicating that animals that avoided the central region of the OF also showed higher immobility in the FST. This correlation reinforces the notion that anxiety- and depression-related behaviors may share common underlying mechanisms (Berton et al. 1998; Flint 2004; Gorwood 2004; Hinojosa et al. 2006). Additionally, in females, variables from the ECP were allocated to two different factors: one associated with the consumption of high ethanol concentrations and three with the consumption of low ethanol concentrations, which reinforces the importance of including females in the search for genes influencing ethanol drinking (Cailhol and Mormède 2002; Marinelli et al. 2003; Vendruscolo et al. 2006a).

Five different QTL linked to emotional and ethanol-drinking behaviors were found in males. Except for the QTL for central locomotion in the OF (*Anxrr16*), all the others were original and thus had not been identified in previous studies. In agreement with Ramos et al. (1999), the previously known QTL *Anxrr16* also had a transgressive effect herein, which is a relatively common phenomenon in QTL studies (Caldarone et al. 1997; Kovács et al. 1997; Llamas et al. 2005; Mogil et al. 1997; Moisan et al. 2003; Silva et al. 2007; Wehner et al. 1997), meaning that F2 animals carrying two LEW alleles showed an increased rather than a decreased central locomotion in the OF, as initially expected. Ramos et al. (1999) also found that *Anxrr16*'s effects were exclusive of females, a result that is not supported by the present study. However, Mormède et al. (2002), through the use of a selection-based strategy using molecular markers for *Anxrr16*, have suggested that the effect of this QTL could be found in both sexes. Herein, twice as many animals were used than were used in the QTL analysis performed by Ramos et al. (1999), where the QTL was found to be effective in a group of only 48 females, a factor that gives a higher statistical power to the data from the present study (Crusio 2004; Flint et al. 2005). Hence, it can be suggested that in the previous work by Ramos et al. (1999), the absence of effect of *Anxrr16* in males could be regarded as a false-negative result. Recently, we found a novel single nucleotide polymorphism between LEW and SHR strains in the 3′-UTR region of the α-synuclein gene (localized in *Anxrr16* region) associated with central locomotion in the OF, suggesting that this gene could be a good candidate for *Anxrr16* (Chiavegatto et al. 2009).

Four other significant QTL were currently found in males. The first one influenced defecation in the OF, which was originally proposed as a test of emotionality by Calvin Hall (1934), who suggested that a highly "emotional" animal would ambulate little and defecate a lot in this test. Much more recently, several QTL analyses have identified QTL for defecation in the OF in mouse Chrs 1, 6, 7, 12, 14, 17, and X (Flint et al. 1995; Henderson et al. 2004; Singer et al. 2005; Turri et al. 2001a, b) and in the rat Chrs 3, 6, 19, and X (Fernandez-Teruel et al. 2002). Interestingly, as

Table 4 Significant QTL identified in the second analysis with diestrous-proestrous and estrous-metestrous F2 females

Phenotype	Marker	Localization (cM)	LRS	*F*	*P*	Model	L/L	L/S	S/S
DP									
Defecation OF	*D4Rat151*	12-14	14.3	6.21	0.00449	Free	0.08 ± 0.08	0.08 ± 0.08	1.33 ± 0.84
Locomotion in dark LDB	*D4Rat206*	110	9.9	5.10	0.01061	Dominant	36.62 ± 2.54	28.74 ± 1.44	28.36 ± 2.29
EM									
Time in light LDB	*D4Rat206*	110	15.2	8.02	0.00095	Free	55.00 ± 5.89	25.33 ± 3.31	36.60 ± 5.08
Locomotion in light LDB	*D4Rat206*	110	13.3	7.35	0.00159	Free	23.44 ± 1.31	14.38 ± 1.27	16.15 ± 1.48

OF open field; *LDB* light–dark box; *cM* centimorgan; *LRS* likelihood ratio statistic; *L/L, L/S, S/S* genotype of F2 animals, *L/S* are heterozygous; *L/L* and *S/S* are homozygous (*L* = Lewis and *S* = SHR)

Table 5 Suggestive QTL identified in the second analysis with diestrous-proestrous and estrous-metestrous F2 females

Phenotype	Marker	Localization (cM)	LRS	*F*	*P*	Model	L/L	L/S	S/S
DP									
Central locomotion OF	*D4Mgh27*	71	6.0	2.76	0.07528	Free	20.83 ± 3.21	13.95 ± 1.56	18.20 ± 2.12
Defecation OF	*D4Rat76*	48	14.5	0.19	0.83076	Free	0.18 ± 0.13	0.25 ± 0.25	0.40 ± 0.40
Peripheral locomotion OF	*D4Rat131*	64	8.8	3.23	0.04995	Free	97.75 ± 2.53	83.53 ± 5.19	96.75 ± 5.88
Time in center OF	*D4Mgh11*	104	14.3	1.31	0.28008	Free	32.13 ± 3.50	39.59 ± 4.48	29.30 ± 650
Total locomotion OF	*D4Mgh11*	97	10.4	2.06	0.14035	Free	115.75 ± 3.36	100.29 ± 6.19	109.30 ± 8.26
Time in dark LDB	*D4Rat206*	110	9.5	5.07	0.01087	Free	248.77 ± 5.63	229.11 ± 5.44	250.73 ± 4.95
Time in light LDB	*D4Mgh6*	78	5.7	2.89	0.06717	Dominant	47.62 ± 6.63	33.38 ± 3.36	34.22 ± 5.37
Locomotion in light LDB	*D4Mgh6*	77	5.8	2.78	0.07428	Free	19.92 ± 1.81	15.81 ± 1.04	18.89 ± 1.34
EM									
Central locomotion OF	*D4Rat151*	24	5.3	2.45	0.09637	Free	17.56 ± 2.69	11.97 ± 1.31	11.79 ± 1.52
Locomotion in dark LDB	*D4Rat151*	87	9.7	3.97	0.02514	Free	24.44 ± 2.72	31.20 ± 1.40	26.14 ± 1.73
Defecation OF	*D4Rat76*	48	14.5	1.94	0.15488	Free	1.17 ± 0.75	0.18 ± 0.18	0.95 ± 0.47
Peripheral locomotion OF	*D4Rat131*	64	8.8	0.42	0.66021	Free	80.14 ± 8.47	83.56 ± 2.74	78.91 ± 4.33
Total locomotion OF	*D4Rat59*	97	10.4	1.41	0.25384	Free	86.71 ± 10.70	99.04 ± 3.83	91.14 ± 4.08
Time in dark LDB	*D4Rat206*	109	8.9	4.18	0.02091	Free	223.67 ± 7.81	252.96 ± 5.18	238.40 ± 6.80
Transitions in LDB	*D4Rat206*	110	8.7	4.66	0.01397	Free	4.22 ± 0.32	2.50 ± 0.29	3.10 ± 0.37

OF open field; *LDB* light–dark box; *cM* centimorgan; *LRS* likelihood ratio statistic; *L/L, L/S, S/S* genotype of F2 animals, *L/S* are heterozygous; *L/L* and *S/S* are homozygous (*L* = Lewis and *S* = SHR)

seen in comparative genomic studies, most of mouse Chr 6 is syntenic to Chr 4 in rats (Rat Genome Sequencing Project Consortium 2004), suggesting that common genes for OF defecation could be present in the two different species.

The second new QTL influenced the peripheral locomotion in the OF. Traditionally, peripheral locomotion is viewed as a locomotion index, but the meaning of this measure may be more complex than it appears (Paulus and Geyer 1993; Ramos et al. 2003). For example, in the present study, it correlated positively with central OF locomotion, a typical index of anxiety/emotionality. Several QTL have already been described for locomotor activity in the OF on mouse Chrs 1, 4, 6, 7, 9, 12, 13, and 15 (Eisener-Dorman et al. 2010; Takahashi et al. 2008; Turri et al. 2001a) and on rat Chrs 1, 5, and 8 (for a review see Moisan and Ramos 2010).

The third QTL showed pleiotropic effects on three different behavioral variables from the LDB test. Some QTL for activity and time spent in both compartments of the LDB on mouse Chrs 1, 4, 11, 12, 14, 15, 16, 18, and X have already been described (Flint et al. 1995; Henderson et al. 2004; Turri et al. 2001a, b). However, to our knowledge, this is the first study to report a QTL for anxiety-related behaviors in the LDB using rats. This suggests that the distal portion of Chr 4 is important in the regulation of the emotional behaviors exhibited in the LDB.

Finally, the fourth new QTL affected forced ethanol intake (10%). Some QTL have been described as influencing both intake and preference for ethanol on mouse

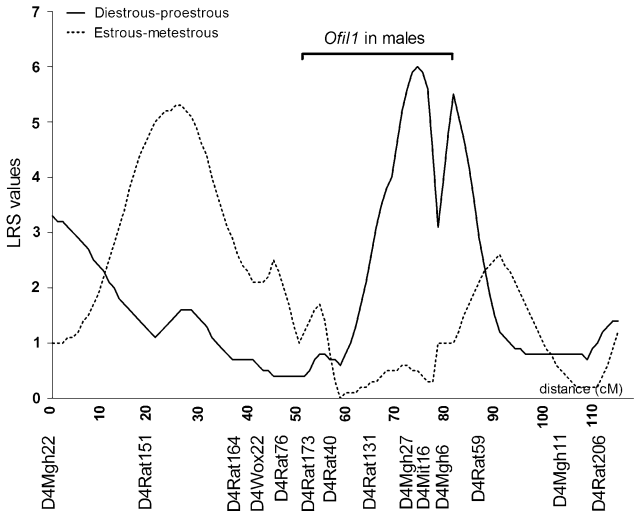

Fig. 4 Two suggestive QTL found for central locomotion in the OF in diestrous–proestrous females (*black line*) or in estrous–metestrous females (*dotted line*), both in a free model. The 14 molecular markers used in the genotyping are shown at the *X* axis, which represents the map distances in cM. We showed the estimated 96.8% confidence interval of *Anxrr16* for the QTL found in males

Chr 6 (Hitzemann et al. 2009; Tabakoff et al. 2008; Vadasz et al. 2007), which, as mentioned before, is in most part syntenic to rat Chr 4 (Rat Genome Sequencing Project Consortium 2004). In rats, a QTL (named *Alc14*, *Alc16*, or *CoEt5*) acting in both sexes has been reported for free-choice ethanol consumption on Chr 4, in a region that overlaps with *Anxrr16* (Moisan and Ramos 2010; Terenina-Rigaldie et al. 2003b). Indeed, the α-synuclein gene that lies near *Anxrr16* and has been associated with ethanol consumption in P/NP rats also cosegregates with increased central locomotion in the OF (Liang et al. 2003; Ramos et al. 1999). Herein, in the ECP, we also found one suggestive QTL in males near *D4Rat164*, a molecular marker localized close to the QTL found in P/NP rats (Bice et al. 1998; Carr et al. 1998), thus suggesting that the same genes could also be regulating ethanol consumption in the LEW/SHR genetic model. However, none of the five significant QTL identified in males influenced behaviors from more than one test, suggesting that different genes are involved in the regulation of these different behaviors. Moreover, an additional QTL analysis using the scores emerging from the PCA revealed a significant QTL for only one (factor 4 in males) of the ten resulting factors (5 per sex). The lack of QTL affecting most of the representative factors, which are meant to synthesize the overall phenotypic information obtained from all animals, is compatible with previous findings that show that the majority of the QTL are related to very specific behaviors rather than to coherent groups of behaviors (Ramos and Mormède 2006; Turri et al. 2001a).

If, on the one hand, the effect of *Anxrr16* in males confirms and extends the data previously reported about the effects of this promising genomic region on the regulation of emotional behaviors, on the other hand, the lack of effect in females is intriguing and does not confirm the original study (Ramos et al. 1999). Some hypotheses were raised to explain this point. First, one can suggest that idiosyncratic factors related to the laboratories and/or the experimental conditions could explain the differences in the expression of the QTL in females (Bell et al. 2006a, b; Chesler et al. 2002; Van Der Staay and Steckler 2002; Wahlsten et al. 2003; Würbel 2002). For example, Crabbe et al. (1999) have shown that even with enormous efforts to standardize the test environment, different results for strain comparisons can be obtained in different laboratories. In humans, some genes are known to increase the prevalence of affective disorders only when combined with specific environmental contexts (Caspi et al. 2003; Gordon and Hen 2004). Thus, the fact that the original study performed by Ramos et al. (1999) and the present one were conducted in different laboratories and in different countries could potentially explain any interstudy inconsistency. Alternatively, there could be genetic differences between the LEW and SHR substrains used in the two studies. Recent phylogenetic studies point to LEW and SHR rats as the strains with the largest genetic diversity among different populations widespread around the world (STAR Consortium 2008). Accordingly, there are more than 15 substrains of LEW and more than 25 substrains of SHR rats (Rat Genome Sequencing Project Consortium 2004), probably due to the distribution of these animals to different laboratories before they were completely inbred and to the events of new mutations.

Finally, one can hypothesize that hormonal factors could explain the inconsistent effect of *Anxrr16* in females. In order to address this hypothesis, we performed a second QTL analysis taking into account the estrous cycle, which was artificially induced at the moment of the behavioral tests. The results showed that females from the DP group (i.e., the sum of diestrous and proestrous females) exhibited lower levels of anxiety-related behaviors in the OF test than those from the EM (i.e. estrous plus metestrous) group. It is well known that estrous cycle and fluctuations in ovarian hormones may lead to behavioral changes related to anxiety/emotionality (Díaz-Véliz et al. 1997; Maguire et al. 2005; Meziane et al. 2007; Mora et al. 1996; Palanza et al. 2001), with estradiol, progesterone, and their metabolites exerting an anxiolytic effect in different paradigms (Chikahisa et al. 2007; Frye et al. 2004; Mora et al. 1996; Rodgers and Johnson 1998). In spite of some controversy found in the literature, the present results agreed with previous data suggesting that females in DP exhibit

reduced anxiety/emotionality (Contreras et al. 1998; Frye et al. 2000; Maguire et al. 2005).

The second QTL analysis revealed two QTL above the significance level and five QTL above the suggestive level in DP females and one significant QTL and four suggestive QTL in EM females. It is interesting to note that at least two of these QTL totally corroborated the results from males in the first QTL analysis and pointed to the extremity of Chr 4 as an important region regulating anxiety-related measures in the LDB. Most importantly, DP females, differently from EM females, showed a suggestive QTL for central locomotion in the OF in the same region as the original QTL *Anxrr16* found in females (Ramos et al. 1999) and in the same region as the QTL for this same variable found in the males of the present study. Interestingly, in all cases, the QTL was transgressive, thus suggesting that the same emotionality-related QTL was in fact revealed in all three analyses and that its expression depends on physiological and molecular factors related to the females' estrous cycle. The estrous cycle lasts around 4–5 days, with the longest phase the diestrous, which lasts approximately 57 h (Hebel and Stromberg 1986). Therefore, in a randomized experiment without any type of cycle control and at any given time, there is a higher probability that the experimenter will phenotype females that happen to be in DP rather than in EM, which could explain, at least in part, the contradictory results between the present and the former study by Ramos et al. (1999). According to this hypothesis and considering that females housed together tend to synchronize reproductive cycles (Olsson et al. 2003), it would be unlikely that two randomized, uncontrolled QTL studies would lead to the same results by representing, each of them, a balanced average of all estrous phases. Thus, we reinforced in the present study the idea that major sex-based differences may exist in molecular or gene expression studies, and for this reason more attention must be paid to the interpretation of studies performed only with male rats and mice (Grove et al. 2010; Wald and Wu 2010; Zucker and Beery 2010). Nevertheless, further studies are necessary to confirm this hypothesis and, in this case, to better elucidate the mechanisms through which each phase of the estrous cycle influences the phenotypic expression of *Anxrr16*.

The effects of *Anxrr16* reported herein, at least in males, reinforce the importance of this genomic region in the regulation of anxiety-related behaviors. We have also mapped new QTL on Chr 4 for anxiety- and ethanol-drinking-related behaviors. Finally, to our knowledge, we found for the first time that behavioral QTL, including *Anxrr16*, have differential expression in females depending on their estrous phases, suggesting an important role of sex hormones in the genetic control of emotional reactions.

Acknowledgments This study was supported by grants from Pronex 427/2003 and from Fapesc/CNPq. A. Ramos had a fellowship from CNPq; G. S. Izídio, E. Pereira, L. C. Oliveira, and L. F. G. Oliveira had scholarships from CNPq; T. D. Wehrmeister had a scholarship from Capes. The authors also thank A. P. Costa, F. B. Oliveira, and N. B. Mello for their technical assistance.

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