

Evidence for a female-specific effect of a chromosome 4 locus on anxiety-related behaviors and ethanol drinking in rats

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Previous studies using the inbred rat strains Lewis (LEW) and spontaneously hypertensive rats (SHR) led to the mapping of two quantitative trait loci, named *Ofil1* (on chromosome 4 of the rat) and *Ofil2* (on chromosome 7), for open-field inner locomotion, a behavioral index of anxiety. Studies using other strains showed that the region next to *Ofil1* influences measures of not only anxiety but also ethanol consumption. In view of the high prevalence of psychiatric disorders such as anxiety and alcoholism, as well as the comorbidity between them, the present study was designed to better characterize the contribution of these two loci to complex emotional and consummatory responses. Rats deriving from an F2 intercross between the LEW and the SHR strains were selected according to their genotype at markers flanking the loci *Ofil1* and *Ofil2* and bred to obtain lines of rats homozygous LEW/LEW or SHR/SHR for each of the two loci, thus generating four genotypic combinations. These selected animals as well as purebred LEW and SHR rats of both sexes were submitted to a battery of tests including measures of locomotor activity, anxiety, sweet and bitter taste reinforcement and ethanol intake. Lewis rats displayed more anxiety-like behavior and less ethanol intake than SHR rats. *Ofil1* (on chromosome 4) affected both the activity in the center of the open field and ethanol drinking in females only. These results suggest that *Ofil1* contains either linked genes with independent influences on anxiety-related responses and ethanol drinking or a pleiotropic gene with simultaneous effects on both traits.

Keywords: Alcoholism, anxiety disorder, behavior genetics, elevated plus maze, marker-assisted selection, open field, oral self-administration paradigm, quantitative trait locus

Anxiety disorders are the most frequent psychopathologies, with approximately 25% of the population being affected sometime in their lives (Anagnostaras *et al*. 1999) and with a clear preponderance of women being found among anxious patients (Blanchard *et al*. 1995). Alcohol (ethanol) abuse, another frequent psychiatric disorder, is also considered as a major problem of public health (Pearson 2004). Besides the fact that it increases the risk of several life-threatening diseases, such as cancer and liver cirrhosis, ethanol-dependent individuals have high rates of co-occurring psychiatric conditions such as anxiety, depression and schizophrenia (Le Fauve *et al*. 2004). The influence of genetic factors on such psychopathologies has been widely demonstrated (Cloninger 1987; van de Wetering *et al*. 1999), but this effect is still poorly understood at the molecular level. Therefore, the identification and characterization of predisposing genes would certainly be a major advance for preventing and treating psychological disorders such as alcoholism and anxiety.

Quantitative trait locus (QTL) analysis has been employed to study the underlying molecular bases of complex (and probably polygenic) phenotypes. Several anxiety- and ethanol-related QTLs have already been identified, mostly in mice but also in rats (Flint 2003). Although it has been proven difficult to identify genes controlling behavioral phenotypes through QTL analysis, this approach has provided abundant information on the genetic architecture of behaviors, and specific genes are beginning to be identified (Flint 2003; Korstanje & Paigen 2002; Liang *et al*. 2003; Yalcin *et al*. 2004).

One frequently used method for QTL detection consists in the production of a segregating population (F2, FX or backcross) by crossing two inbred strains that are contrasting for a trait of interest. The inbred rat strains Lewis (LEW) and spontaneously hypertensive rats (SHR) provide a useful genetic model for the study of anxiety. Lewis rats show higher indices of anxiety-related behaviors when submitted to a variety of behavioral tests such as the elevated plus maze, open field, black/white box and elevated T maze compared with SHR rats, without differing in relation to several measures of general locomotion in either novel or familiar

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environment (Ramos *et al*. 1997; Ramos *et al*. 1998; Ramos *et al*. 1999; Ramos *et al*. 2002). Consistent responses of SHR and LEW rats injected with anxiolytic and anxiogenic drugs (Ramos *et al*. 1997; Vendruscolo *et al*. 2003) corroborate the idea that these behavioral differences may be associated with different baseline levels of anxiety. In an effort to investigate the molecular bases of these differences, a genome-wide QTL search using a LEW × SHR intercross was performed (Ramos *et al*. 1999). This study revealed a complex genetic structure of several anxiety-related behaviors. Two female-specific QTLs were found for the inner locomotion in the open field (being therefore named *Ofil1* and *Ofil2*). Although one of these QTLs increased the trait value when its alleles were inherited from the SHR strain (*Ofil2* on chromosome 7), the other one acted on the opposite direction, with SHR alleles reducing rather than increasing the trait (*Ofil1* on chromosome 4) (Ramos *et al*. 1999). In a subsequent study, Mormède *et al*. (2002) confirmed the role of these QTLs on anxiety-like behaviors, by producing two rat lines with extreme genotypes and by showing that the high line (LEW/LEW at *Ofil1* and SHR/SHR at *Ofil2*) displayed more locomotion in the aversive areas of the open field and elevated plus maze than the low line (SHR/SHR at *Ofil1* and LEW/LEW at *Ofil2*). However, this study did not allow sorting apart the respective influences of *Ofil1*, *Ofil2* and their interaction.

Terenina-Rigaldie *et al*. (2003a) reported a QTL near *Ofil1* influencing anxiety-related behaviors in a high-ethanol preferring vs. Wistar-Kyoto rats intercross. Curiously, the anxiety-like profile observed for this locus was also inverted in comparison with the parental lines, i.e. with high-ethanol-preferring ('less anxious strain') alleles increasing avoidance of the aversive areas of the open field and elevated plus maze. Moreover, other QTLs in the same region have been found to influence ethanol drinking and saccharine reinforcement (Carr *et al*. 1998; Terenina-Rigaldie *et al*. 2003a, 2003b). Furthermore, one recent study reported a QTL on chromosome 4 controlling corticosterone levels (Potenza *et al*. 2004), which have been implicated in several psychiatric disorders such as anxiety, depression and drug addiction. The aforementioned evidence suggests that this region of chromosome 4 may contain genes controlling not only anxiety-related behavior but also stress reactivity and ethanol drinking in rats.

In order to better characterize the contribution of each of these two emotionality-related loci (*Ofil1* and *Ofil2*) as well as their interaction on behavioral responses, rats deriving from an F2 intercross between LEW and SHR strains were selected for breeding based on their genotype for polymorphic markers flanking the two QTLs. Animals whose genotypes were homozygous LEW/LEW or SHR/SHR at each one of the two loci were selected to produce an F3 generation with a known genotype at these loci only, the rest of the genome being a random assortment of linked blocks of alleles from one or the other founder strain. The phenotypic differences among these groups would therefore be the result of genetic variations within these chromosomal loci. A similar approach has been used previously with success (Bennett *et al*. 1997; Mormède *et al*. 2002; Terenina-Rigaldie *et al*. 2003a). The four new rat lines as well as the parental strains were subsequently submitted to a battery of behavioral tests of activity, anxiety, sweet and bitter taste preferences and ethanol intake.

Materials and methods

Animals

Male and female LEW/CRLIFO (LEW) and SHR/CRL (SHR) rats were received from Charles River/IFFA CREDO. To obtain the F1 population, we bred three LEW and six SHR and three SHR and six LEW, males and females, respectively. F1 rats were bred to produce the F2 generation. A total of 453 rats (F2) were selected based on polymorphic markers at *Ofil1* (D4Wox22, 37.38 cM, and D4Mgh6, 58.99 cM) and *Ofil2* (D7Rat35, 6.83 cM, and D7Mgh11, 2.3 cM). Animals which had inherited the genotypes homozygous LEW or SHR at each locus were used as founders. Three to five breeder pairs for each genotypic line were used in the study, and the litters were culled at eight pups. To better delineate *Ofil1* the animals belonging to the F3 generation were further genotyped with D4Rat61 (73.62 cM), and the rats were required to be homozygous at all three markers. Adult F3 rats (8 weeks old) of the four new lines were used in the behavioral tests ($n = 11$ and 14 from L4/L7 line; 5 and 5 from L4/S7 line; 8 and 5 from S4/L7 line; 16 and 12 from S4/S7 line; males and females, respectively). Additional groups of LEW (10 males and 10 females) and SHR (6 males and 10 females) rats at the same age were concurrently tested. All the animals were kept in collective plastic cages (2–4 rats/cage) having food and water available *ad libitum* and maintained in a room with controlled temperature ($21 \pm 2\ ^{\circ}C$), under a 12:12 L : D cycle (lights on at 0700 h). The blood pressure of all animals was measured as previously described (Ramos *et al*. 1998; Ramos *et al*. 1999) and, as expected, the SHR rats were hypertensive in relation to their LEW counterparts. However, no differences were found between the new genotypic rat lines (data not shown). All procedures used in the present study complied with the 'Principles of laboratory animal care' from NIH.

Genotyping

Primers for microsatellite markers were purchased from Eurogentec (Seraing, Belgium) or from Research Genetics (Huntsville, AL). Genomic DNA was extracted from tail tissue using a commercial kit (Promega, Charbonnières, France). Genotype determinations were performed by polymerase chain reaction (PCR). In a 20-μl reaction volume, 50 ng of genomic DNA was mixed with 5 pmol of each primer and

0.4 U of Taq DNA polymerase (Promega) in Promega type A buffer. Amplification was performed in microtitre plates on a Hybaid OmniGene thermocycler (Hybaid Limited, Teddington, UK). The PCR conditions were initial denaturation at 96 °C for 5 min followed by 35 cycles of 92 °C for 40 seconds, 55 °C for 1 min and 72 °C for 30 seconds and one cycle at 72 °C for 2 min. Alleles were separated on 3% agarose gels and visualized with ethidium bromide staining under ultraviolet light.

Phenotyping

All tests were carried out between 0800 and 1230 h. The animals were submitted successively to a battery of behavioral tests with at least 3 days of interval between tests. The test battery is described (in the order of testing) below.

Activity cages

Animals were placed individually for 60 min in wire-mesh cages with transparent plastic sides (38.5 × 23.5 × 23 cm) located inside a rack equipped with infra-red photo beams connected to a computer which recorded the front-to-back traverses (Imetronic, Pessac, France). This test was conducted under low-light conditions (<25 lux). In this test, two variables were analyzed: locomotion during the first 10 min, which represent locomotor reactivity in a novel environment, and total locomotion (60 min), which is a better measure of general locomotion.

Open field

The apparatus was made out of laminated wood with a white floor of 100 × 100 cm (divided by black lines into 25 squares of 20 × 20 cm) and 40-cm high white walls. Illumination inside the open field was 530 lux. Each rat was placed in the center of the open field and the number of peripheral squares (adjacent to the walls) crossed (peripheral locomotion), central squares (away from the walls) crossed (central locomotion) and fecal bolus dropped were registered for 5 min.

Elevated plus maze

The apparatus was made of opaque Perspex, with black floor and grey walls, and had four elevated arms (66 cm above 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 but with a 1-cm lip, having at their intersection a central platform (10 × 10 cm) which gave access to any of the four arms. The central platform was under 530 lux of illumination. Each rat was placed on the central platform facing an open arm, and the number of entries and the time spent (with all four paws) inside each type of arm were registered for 5 min. In both open field and elevated plus maze, the behavior of each animal was recorded via a video camera positioned above the apparatus and monitored in another room via a closed circuit TV camera.

Two-choice saccharine

A standardized testing procedure for saccharine, quinine and ethanol consumption was used (Terenina-Rigaldie *et al.* 2003a, 2003b). After a 5-day habituation period in individual plastic cages, the animals were given free choice between two bottles containing either saccharine solution (7.5 mM as the sodium salt; Sigma, Saint Quentin Fallavier, France) or water on two consecutive days. The bottles were weighed, refilled and their position was changed each day within the same 2-h interval. The data were expressed as an index that measures the increase in fluid ingestion when saccharine was available according to the following formula: volume of saccharine plus the volume of water consumed in the same 24-h period divided by the normal volume of water consumed in 24 h (measured during the two preceding days), expressed as a percentage.

Two-choice quinine

This test followed the same protocol as for saccharine, with a 2-μM quinine solution being used.

Forced ethanol

The animals were given 10% (v/v) ethanol (Prolabo, Saint Quentin Fallavier, France) as their sole source of drinking fluid for 2 days. Volumes consumed were measured, and the positions of the bottles were changed each day. Data are expressed as the mean ethanol intake during the 2 days in g/kg of body weight. Between saccharine, quinine and forced ethanol conditions, water was given as the sole source of drinking fluid for 2 days.

Two-choice ethanol

Immediately following forced ethanol, the animals were given a choice between water and 10% (v/v) ethanol. The bottles were weighed (refilled if necessary) every 2 days for seven 2-day periods (i.e. over 14 days). Data are expressed in g/kg/day (ethanol intake).

Ethanol consumption vs. concentration

In order to test the hypothesis that some animals may adjust their ethanol intake based on its concentration, we varied ethanol concentrations at 2.5, 5, 10 and 20% (v/v), given as a free choice vs. water. This protocol began after 4 days of ethanol deprivation with water as the sole source of drinking fluid. The animals were divided into four groups, and the order of presentation of the different concentrations was based on a replicated Latin-square design. In the consumption tests, the body weight of the rats was recorded at least once a week.

Statistical analysis

Initially, the parental strains (LEW and SHR) were compared with each other as well as with the two recombinant lines (L4/L7 and S4/S7) that are equal to either one of the purebred strains at the loci *Ofil1* and *Ofil2* but not at the rest of the genome. Therefore, a two-way ANOVA (rat line and sex) was followed by an LSD test to compare LEW vs. SHR; LEW vs. L4/L7 and SHR vs. S4/S7. These comparisons should reveal the effect of overall differences between LEW and SHR genotypes as well as differences derived specifically from genes not located in either of the two QTLs. To specifically analyze the influence of L or S alleles at each QTL as well as their interaction, a three-way ANOVA (*Ofil1*, *Ofil2* and sex factors) was performed exclusively with the data of the four recombinant rat lines. When an interaction with sex was observed, an additional two-way ANOVA (*Ofil1* and *Ofil2*) was performed separately for each sex. The accepted level of significance for all tests was $P < 0.05$. Data are presented in the text and figures as means and SEM.

Results

Activity cages

Figure 1 illustrates the results of locomotor activity measured for 1 h in a novel environment in (a) male and (b) female rats. The two-way ANOVA revealed significant line ($F_{5,92} = 2.59$; $P < 0.03$) and line vs. sex interaction ($F_{5,92} = 2.86$; $P < 0.02$) effects on the locomotor activity in the first 10 min of test. The LSD *post hoc* tests indicated that LEW males were more active than L4/L7 males ($P < 0.001$) and that SHR females were more active than LEW females ($P < 0.02$). For total locomotion (60 min), females were more active than males ($F_{1,92} = 6.86$; $P < 0.01$). Three-way ANOVA (sex, *Ofil1* and *Ofil2* factors) revealed only a sex effect (females > males) in the total locomotion ($F_{1,60} = 8.26$; $P < 0.006$).

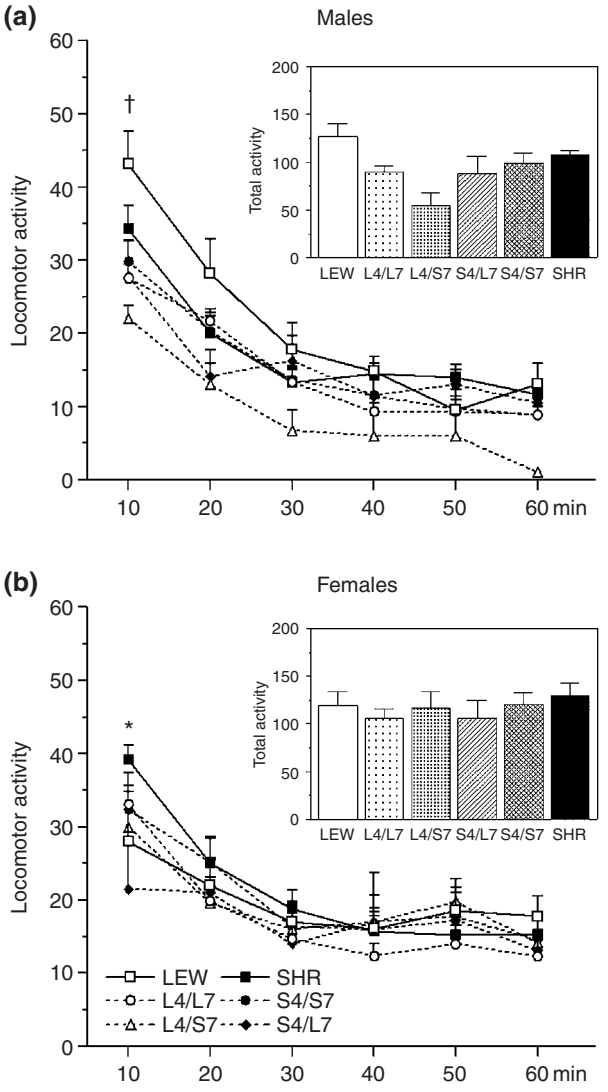

Figure 1: Number of front-to-back traverses in activity cages made by male (a) and female (b) Lewis (LEW), spontaneously hypertensive rats (SHR) and F3 rats (according to line). Results are given by 10-min bins (total activity over the hour is shown in the inset). *Significantly different from LEW rats. †Significantly different from L4/L7 rats (LSD test, $P < 0.05$).

Open field

The results for (a) central and (b) peripheral locomotion in the open field are illustrated in Fig. 2. The two-way ANOVA revealed an overall effect of line ($F_{5,100} = 8.55$; $P < 0.0001$), of sex ($F_{1,100} = 26.52$; $P < 0.0001$) and a line vs. sex interaction ($F_{5,100} = 3.11$; $P < 0.01$) on the central locomotion in the open field. The *post hoc* tests indicated that SHR rats of both sexes crossed more central squares in the aversive area of the open field than LEW rats ($P < 0.05$). Moreover, SHR females showed more central locomotion than S4/S7 females, and LEW females showed less central locomotion than L4/L7 females ($P < 0.0001$). For peripheral locomotion, the two-way ANOVA revealed an overall effect of line ($F_{5,100} = 2.39$; $P < 0.05$) and of sex ($F_{1,100} = 27.84$; $P < 0.0001$). Lewis females crossed less peripheral squares than SHR and L4/L7 females ($P < 0.02$). The three-way ANOVA (sex, *Ofil1* and *Ofil2* factors) revealed a significant effect of sex ($F_{1,68} = 15.19$; $P < 0.0002$) and a sex vs. *Ofil1* interaction ($F_{1,68} = 5.36$; $P < 0.02$) on the central locomotion in the open field. An additional two-way ANOVA (*Ofil1* and *Ofil2* factors) performed separately for each sex revealed that females with LEW alleles at *Ofil1* (i.e. L4) crossed more central squares ($F_{1,32} = 4.81$; $P < 0.04$) than females with SHR alleles (i.e. S4). For peripheral locomotion, a sex effect was observed ($F_{1,68} = 16.34$; $P < 0.0001$), with female rats showing higher scores than male rats. Defecation was almost absent, so that no clear effect could be observed.

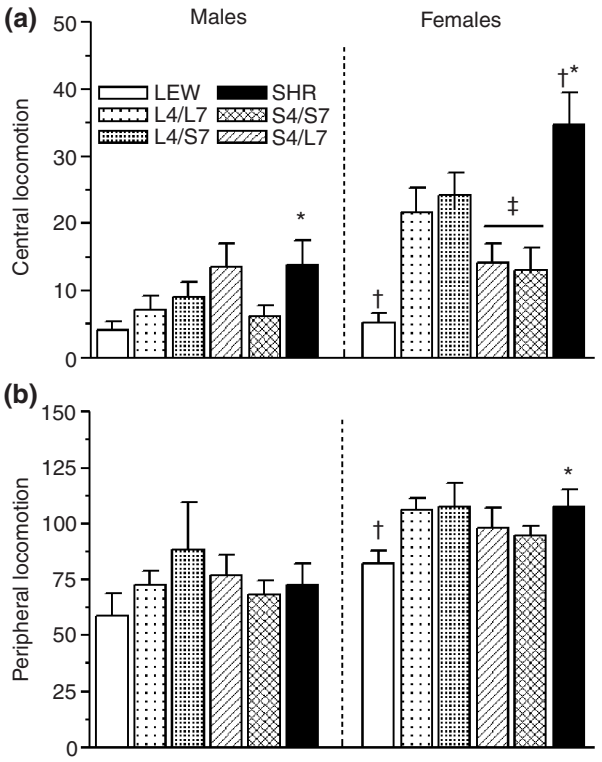

Figure 2: Locomotion in the central (a) and peripheral (b) areas of the open field (530 lux) for 5 min of Lewis (LEW), spontaneously hypertensive rats (SHR) and F3 rats (according to line) of both sexes. *Significantly different from LEW rats (LSD test, $P < 0.05$). †Significant difference between LTW and L4/L7 or between SHK and S4/S7 (LSD tests, $P < 0.05$). ‡Indicates significant *Ofil1* (on chromosome 4) effect (ANOVAS, $P < 0.05$).

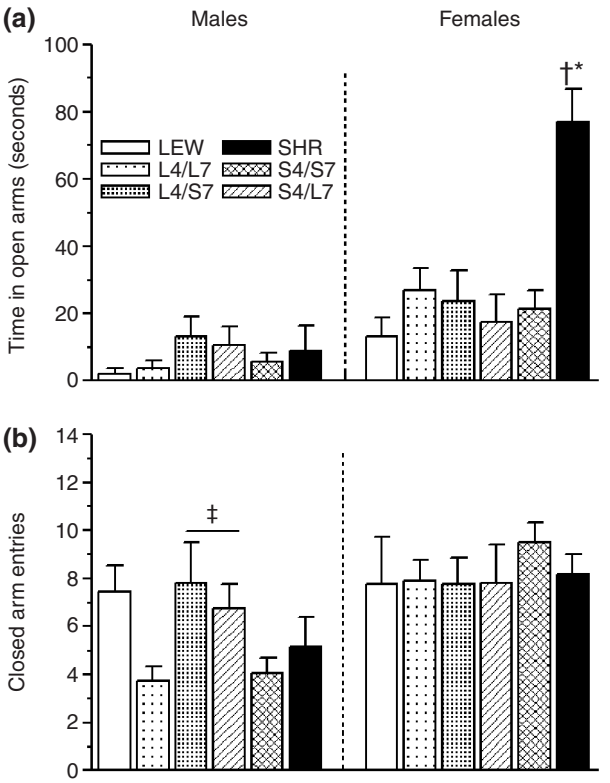

Figure 3: Time spent in the open arms (a) and number of entries in the closed arms (b) of the elevated plus maze for 5 min of Lewis (LEW), spontaneously hypertensive rats (SHR) and F3 rats (according to line) of both sexes. *Significantly different from LEW (LSD test, $P < 0.05$). †Significantly different from S4/S7 rats (LSD test, $P < 0.05$). ‡Indicates significant *Ofil1–Ofil2* interaction (ANOVAS, $P < 0.05$).

Elevated plus maze

Figure 3 illustrates the results for (a) the time spent in the open arms and (b) the number of closed arm entries in the elevated plus maze. The two-way ANOVA revealed overall effect of line ($F_{5,94} = 7.93$; $P < 0.0001$), of sex ($F_{1,94} = 37.89$; $P < 0.0001$) and a line vs. sex interaction ($F_{5,94} = 6.33$; $P < 0.0001$) on the time spent in the open arms. The *post hoc* tests indicated that SHR females spent more time in the open arms than LEW and S4/S7 females ($P < 0.0001$). For closed arm entries, the ANOVAS revealed only a sex effect ($F_{1,94} = 12.20$; $P < 0.0007$), with females showing more crossings than males. The three-way ANOVA (sex, *Ofil1* and *Ofil2* factors) revealed that females spent more time in the open arms than males ($F_{1,64} = 11.55$; $P < 0.001$). For closed arm entries, there was a significant effect of sex ($F_{1,64} = 13.44$; $P < 0.0005$; females > males) and a sex vs. *Ofil1* vs. *Ofil2* interaction ($F_{1,64} = 8.85$; $P < 0.0041$). An additional two-way ANOVA for each sex revealed a significant *Ofil1* vs. *Ofil2* interaction for male rats ($F_{1,35} = 13.83$; $P < 0.0007$), with L4/L7 and S4/S7 rats displaying less closed-arm entries than L4/S7 and S4/L7 ats.

Two-choice saccharine

Figure 4 illustrates the increase of fluid intake induced by the availability of a saccharine solution (7.5 mmol) in free choice with water for two consecutive days. The two-way ANOVA revealed an effect of line ($F_{5,100} = 5.27$; $P < 0.0002$) and of sex ($F_{1,100} = 14.81$; $P < 0.0002$). The *post hoc* test indicated that LEW rats of both sexes showed lower saccharine consumption than SHR and L4/L7 rats ($P < 0.02$). The three-way ANOVA revealed a significant effect of sex ($F_{1,68} = 10.92$; $P < 0.001$; female > males), of *Ofil2* ($F_{1,68} = 7.74$; $P < 0.007$), with the animals with LEW alleles at *Ofil2* showing higher saccharine reinforcement than the animals with SHR alleles and an *Ofil1* vs. *Ofil2* interaction ($F_{1,68} = 4.65$; $P < 0.04$), with L4/L7 rats displaying more saccharine consumption than L4/S7 rats.

Two-choice quinine

The two-way ANOVA revealed a significant line effect ($F_{5,100} = 3.18$; $P < 0.01$) on the quinine consumption. The *post hoc* test indicated that female LEW rats showed lower

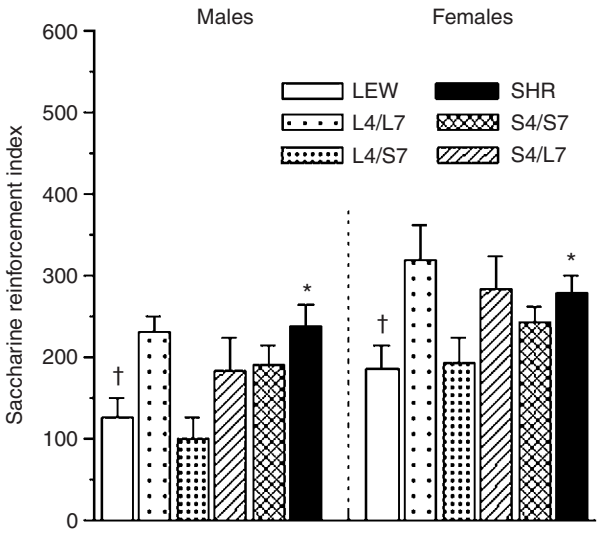

Figure 4: Increase of fluid consumption induced by the availability of a saccharine solution (7.5 mmol) in free choice with water (mean of two consecutive days) of Lewis (LEW), spontaneously hypertensive rats (SHR) and F3 rats (according to line) of both sexes. Data are expressed as an index that consists of the volume of fluid ingested when saccharine is available to the animal as compared to fluid ingested when water only is available. *Significantly different from LEW (LSD test, $P < 0.05$). †Significantly different from L4/L7 rats (LSD test, $P < 0.05$).

increase of fluid intake after quinine presentation than female SHR and S4/S7 rats ($P < 0.009$). The three-way ANOVA (sex, *Ofil1* and *Ofil2* factors) revealed only a significant effect of sex ($F_{1,68} = 5.92$; $P < 0.02$), with a higher quinine reinforcement being found in females. The means ± SEM of the index of quinine consumption for males were: LEW, 8.1 ± 2.5; SHR, 22.6 ± 8.3; L4/L7, 11.9 ± 2.6; L4/S7, 15.2 ± 7.1; S4/L7, 18.4 ± 7.2 and S4/S7, 12.9 ± 3.5. Means ± SEM of the index of quinine consumption for females were: LEW, 3.3 ± 3.6; SHR, 22.8 ± 5.9; L4/L7, 32.7 ± 6.4; L4/S7, 29.5 ± 5.3; S4/L7, 22.3 ± 8.3 and S4/S7, 16.6 ± 5.1.

Forced ethanol (ET 10%)

The results for forced ethanol consumption for two consecutive days are depicted in Fig. 5(a) (left). The two-way ANOVA revealed a significant effect of line ($F_{5,100} = 11.63$; $P < 0.0001$) and of sex ($F_{1,100} = 79.93$; $P < 0.0001$) on the forced ethanol intake. The *post hoc* comparisons indicated that male and female LEW rats consumed less ethanol than SHR and L4/L7 rats ($P < 0.03$). In addition, SHR females consumed more ethanol than S4/S7 females ($P < 0.0001$). The three-way ANOVA revealed that females consumed more ethanol than males ($F_{1,68} = 49.90$; $P < 0.0001$), but no difference was found for either *Ofil1* or *Ofil2*.

Two-choice ethanol (ET 10% vs. water)

The results of two-choice ethanol drinking are presented in Fig. 5(a) (right). The two-way ANOVA revealed a significant effect of line ($F_{5,100} = 5.97$; $P < 0.0001$), of sex ($F_{1,100} = 36.12$; $P < 0.0001$) and a line vs. sex interaction ($F_{5,100} = 3.28$; $P < 0.009$) on the ethanol intake in free choice with water. The *post hoc* comparisons indicated that SHR females drank more ethanol than LEW and S4/S7 females ($P < 0.0001$). The three-way ANOVA revealed a significant effect of sex ($F_{1,68} = 30.51$; $P < 0.0001$) and an interaction between sex and *Ofil1* ($F_{1,68} = 3.99$; $P < 0.05$) on the ethanol intake. An additional two-way ANOVA for each sex revealed that female rats with SHR alleles at *Ofil1* (i.e. S4) showed higher ethanol intake ($F_{1,32} = 4.51$; $P < 0.04$) than female rats with LEW alleles (i.e. L4).

Ethanol consumption vs. concentration

The results of two-choice ethanol drinking behavior (vs. water) at different ethanol concentrations (ET 2.5, 5, 10 and 20%) are presented in Fig. 5(b). The two-way ANOVA revealed, at all ethanol concentrations, an overall effect of line (ET 2.5%, $F_{5,100} = 6.68$; $P < 0.0001$, ET 5%, $F_{5,100} = 8.58$; $P < 0.0001$, ET 10%, $F_{5,100} = 4.48$; $P < 0.001$, ET 20%, $F_{5,100} = 5.44$; $P < 0.0002$) and of sex (ET 2.5% $F_{1,100} = 32.35$; $P < 0.0001$, ET 5%, $F_{1,100} = 31.85$; $P < 0.0001$, ET 10%, $F_{1,100} = 22.92$; $P < 0.0001$, ET 20%, $F_{1,100} = 26.99$; $P < 0.0001$). For ethanol intake at 10%, a significant line vs. sex interaction was also found ($F_{5,100} = 2.76$; $P < 0.02$). The *post hoc* comparisons indicated that SHR males drank more ethanol at 2.5 and 5% than LEW males ($P < 0.009$), and SHR females drank more ethanol than LEW and S4/S7 females at all ethanol concentrations ($P < 0.002$). The three-way ANOVA revealed a significant sex effect for ethanol intake at all concentrations (ET 2.5%, $F_{1,68} = 27.48$; $P < 0.0001$, ET 5%, $F_{1,68} = 15.65$; $P < 0.0002$, ET 10%, $F_{1,68} = 26.36$; $P < 0.0001$, ET 20%, $F_{1,68} = 29.12$; $P < 0.0001$), with females showing higher ethanol consumption than males. Yet, the analysis revealed a significant overall *Ofil1* effect for ethanol intake ($F_{1,68} = 4.63$; $P < 0.04$) at 10%, with the animals with SHR alleles (i.e. S4) showing higher ethanol intake than the animals with LEW alleles (i.e. L4).

Discussion

The present results are in agreement with previous studies demonstrating that LEW rats display higher levels of anxiety-like behaviors than SHR rats. Moreover, in the oral self-administration procedure, SHR rats consumed significantly more saccharine, quinine and ethanol than LEW rats, thus confirming previous data (Da Silva *et al.* 2004, Da Silva *et al.* 2005). The most important finding of the present study, however, was that the locus *Ofil1* (Ramos *et al.* 1999) on chromosome 4 affected significantly the locomotion in the

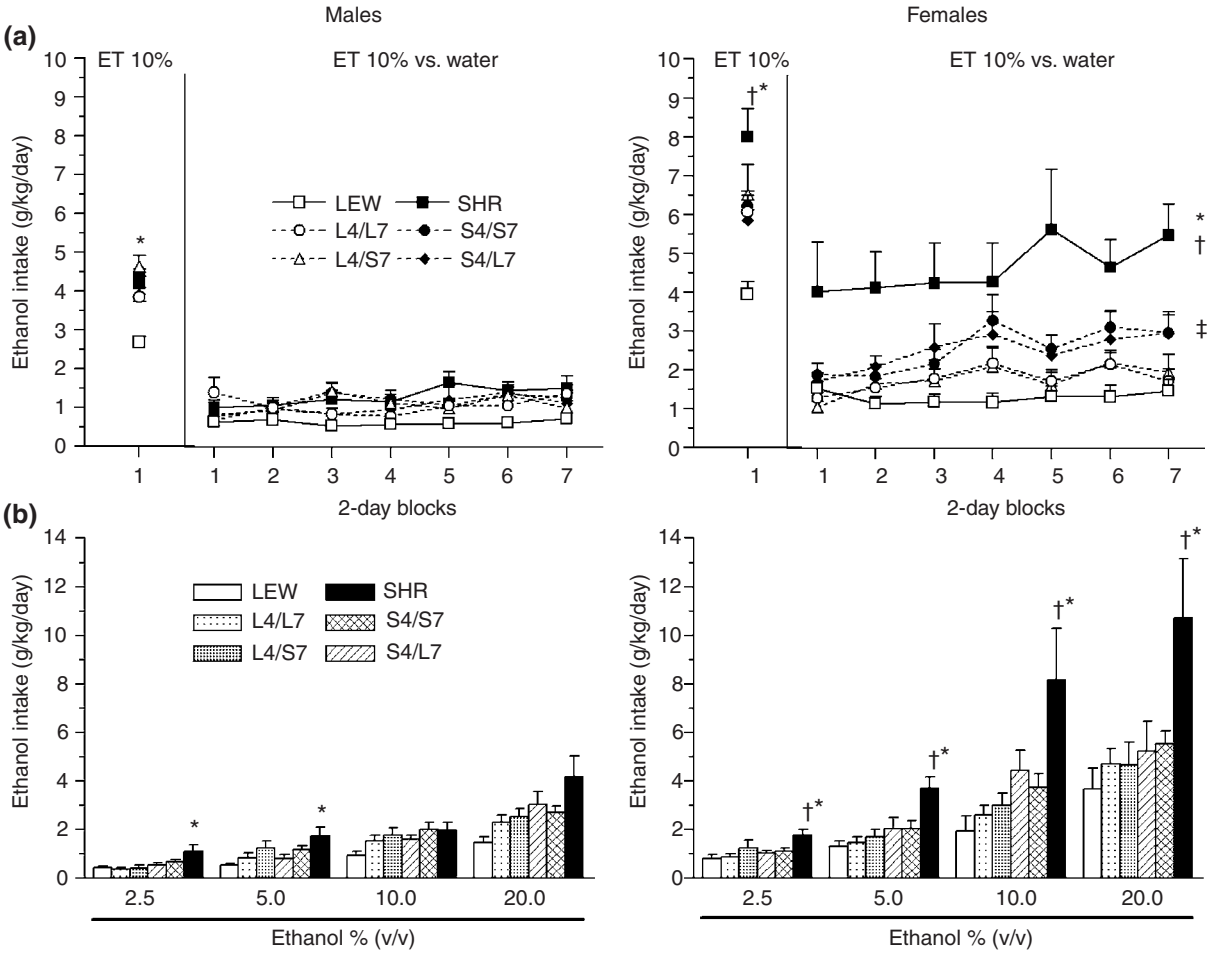

Figure 5: (a) Forced ethanol consumption (ET 10%; at left). The animals were given ethanol at 10% (v/v) as their sole source of liquid for two consecutive days. Two-choice ethanol vs. water (ET 10% vs. water; at right) across 14 days (seven 2-day blocks). Animals were given a choice between ethanol 10% (v/v) and water. (b) Ethanol drinking as a function of concentration. All animals were given a choice between ethanol at 2.5, 5, 10 and 20% (v/v) and water for 2 days each in a balanced Latin-square design. Data are expressed as the amount of ethanol in gram per kilogram body weight per day (g/kg/day). *Significantly different from Lewis (LEW) rats (LSD, $P < 0.05$). †Significantly different from S4/S7 rats (LSD test, $P < 0.05$). ‡Indicates significant *Ofil1* (on chromosome 4) effect.

aversive central area of the open field (a putative measure of anxiety) as well as ethanol drinking (an experimental index of alcoholism) in female F3 rats derived from the intercrossing between the LEW and the SHR strains. Their locomotion in a novel minimally challenging environment was not affected by this locus. Together, the present results suggest that this genomic region may contain one or more genes with simultaneous effects on anxiety and ethanol drinking in female rats.

Besides the expected behavioral contrasts between the purebred strains LEW and SHR, the present study also revealed significant differences between LEW and L4/L7 rats and/or between SHR and S4/S7 rats for measures of locomotion in the activity cages, central and peripheral locomotion in the open field, time spent in the open arms of the plus maze, saccharine reinforcement, forced ethanol intake and free-choice ethanol intake at different concentrations. For the anxiety-related measures from the open field and plus maze and for the indices of alcohol consumption, these differences were female specific. When compared with each other, LEW vs. L4/L7 rats and SHR vs. S4/S7 rats are theoretically equal for the loci *Ofil1* and *Ofil2* on chromosomes 4 and 7, but they differ for the rest of the genome. Therefore, the aforementioned differences within line pairs indicate that besides the two QTLs which are the focus of the present study, other genomic regions are acting on these emotional and consummatory behaviors thus explaining part of the differences between the parental LEW and SHR strains. Regarding central locomotion in the open field, because such additional regions were not detected in our previous whole-genome QTL analysis (Ramos *et al.* 1999), it can be suggested that they are

numerous, highly significant when taken as a whole but with small individual effects when analyzed separately. Such a genetic profile is consistent with the polygenic nature of quantitative behavioral traits (Flint 2003).

Regarding the influence of the QTLs *Ofil1* and *Ofil2*, Ramos *et al*. (1999) found that they were female specific, one of them was highly significant (*Ofil1*, LOD score = 7.22) and the other one only suggestive (*Ofil2*, LOD score = 3.66), and they had a specific effect on central locomotion in the open field. The effect of *Ofil1* was inverted in relation to the parental strains (i.e. LEW alleles promoted more instead of less central locomotion), whereas *Ofil2* acted in the expected direction (i.e. LEW alleles reducing the trait). The results of the present study clearly demonstrated that the animals carrying two LEW alleles at the locus *Ofil1* showed higher central locomotion in the open field than the animals carrying two SHR alleles. This locus, however, did not affect peripheral locomotion in the open field or locomotion in a novel but less challenging environment, which is consistent with its anxiety-specific profile. The effect was female- and test-specific, because the open-arm time in the plus maze (considered as a good index of anxiety; Cruz *et al*. 1994) was not affected. These findings are interesting for two main reasons: (a) anxiety-related disorders are more prevalent in females; and (b) anxiety disorders are not simple phenomena, because various subtypes of anxiety-related pathologies are known in spite of the molecular bases remaining largely obscure.

One of the most puzzling aspects of anxiety disorders is the occurrence of dramatic sex differences in prevalence rates and course. In spite of the fact that anxiety disorders are more prevalent in women, the vast majority of the animal studies involving anxiety-related behaviors are carried out with male subjects (Blanchard *et al*. 1995). Numerous hypotheses have been proposed to explain these gender differences. Both organizational and activational influences of gonadal hormones could be involved. It is known, for example, that the incidence of psychiatric disorders is greater during women's reproductive lives (Bebbington *et al*. 1998). In rodents, it is also known that sexual hormones play an important role in anxiety-like behaviors, mainly in females (Palanza 2001). The biological mechanisms underlying hormonal influences on behavior are complex and not fully understood. Moreover, genetic mechanisms, independent of hormonal action, may trigger sexual differentiation of brain and behavior (Arnold 1996). As mentioned previously, the salient finding of the present study was observed among females. Further studies are needed to clarify the specific role of *Ofil1* on females' behavior.

There is increasing evidence for the fact that different tests may assess distinct types of anxiety in rodents (Ramos & Mormède 1998). Therefore, the test-specific genetic effects observed herein are likely related to the different types of emotional stress that the open field and the plus maze tests may evoke on the animals. The elevated plus maze is a model of anxiety that is based on the natural tendency of rodents to avoid open alleys (Montgomery 1955; Pellow *et al*. 1985). The approach toward its open arms can be increased by benzodiazepines and decreased by anxiogenic substances whereas serotonin-related compounds produce variable results (Handley & McBlane 1993; Hogg 1996; Pellow *et al*. 1985; Treit *et al*. 1993). On the other hand, the open field was originally developed as a test of emotionality (Hall 1934; Hall 1936) and is generally considered to be a stressful, fear-arousing environment; more anxious animals tend to stay away from the central part of the arena where they cannot perform thigmotaxis. The effects of anxiolytic drugs have not been universally verified in this test (Angrini *et al*. 1998; Fisher & Hughes 1996), but as suggested by Prut and Belzung (2003), the open field seems to be a good model to test classical benzodiazepines and 5-HT_{1A} agonists which are effective in the treatment of generalized anxiety disorder. Conversely, the open field is not sensitive to compounds (alprazolam and chronic selective serotonin reuptake inhibitors) that are effective in anxiety disorders such as panic, obsessive-compulsive disorder, phobias and post-traumatic stress disorder (Prut & Belzung 2003). Therefore, the open-field test seems to model some aspects of generalized anxiety disorder as well as some aspects of non-pathological anxiety. In this context, it is important to consider that clinical features of anxiety disorders share similarities with normal personality traits related to anxiety (Finn *et al*. 2003).

Concerning the inverted action of the *Ofil1* locus, hypotheses have been proposed elsewhere (Ramos *et al*. 1999). Opposite effect of other QTLs or epistatic interactions with other genomic regions should be the cause of its unexpected action. Similar opposite-acting QTLs have been reported in various other studies on physiological and behavioral variables (Caldarone *et al*. 1997; Jiang *et al*. 1997), including central locomotion in the open field (Terenina-Rigaldie *et al*. 2003a). The hypothesis that epistatic interactions of *Ofil1* would involve the locus *Ofil2* on chromosome 7, as far as central open-field locomotion is concerned, is not supported by the present results, because the effect of *Ofil1* did not depend on this second locus. Therefore, it can be suggested that other locus(ci) located somewhere else in the genome, in spite of not having been detected in our previous whole-genome analysis, interact with *Ofil1* and thus are responsible for its inverted effect.

The locus *Ofil1* also influenced the amount of 10% ethanol consumed by females at pharmacologically relevant doses (Ferraro *et al*. 1991; Weiss *et al*. 1993) when offered as a free choice with water. The possibility that a single gene in that region affects, through common neurobiological mechanisms, anxiety-related behaviors as well as ethanol drinking is interesting and deserves further investigation. Nevertheless, the fact that the same locus influences different traits does not necessarily mean that these traits are under the control of the same gene(s), because closely

located genes can independently modulate disparate traits. Studies aiming to breakdown this large region are currently underway. Interestingly, a QTL linked to free-choice 5% ethanol consumption in a high ethanol preferring × Wistar-Kyoto intercross (Terenina-Rigaldie *et al*. 2003a; 2003b) and another one influencing free-choice 10% ethanol consumption (Carr *et al*. 1998) in an intercross between the alcohol-preferring and alcohol-non-preferring rat lines were described and localized approximately in the same region as *Ofil1* on chromosome 4. Recently, the α-synuclein gene, which regulates dopamine synthesis, was mapped on rat chromosome 4 at the peak of a QTL for ethanol consumption (Carr *et al*. 1998; Liang *et al*. 2003) and is thus considered as a candidate gene for ethanol preference (Liang *et al*. 2003). Therefore, we confirm that the locus *Ofil1* on chromosome 4 should contain gene(s) affecting ethanol intake.

For males, no clear effect of either *Ofil1* or *Ofil2* was found. However, we found an interaction between these two loci. This epistatic effect was observed for closed-arm entries of the elevated plus maze and saccharine reinforcement. The L4/L7 and S4/S7 rats made less closed-arm entries in the elevated plus maze than L4/S7 and S4/L7 rats. A priori, one could attribute this finding to anxiety-related differences, because the rat lines did not differ for locomotion in the less-threatening activity cage. However, attempts to distinguish between activity- and anxiety-related behaviors in the elevated plus maze for males can be misleading, and the results should be interpreted with caution. Furthermore, L4/L7 and S4/S7 rats appear to be slightly more sensitive to saccharine reinforcement than the two other recombinant lines. These findings demonstrate the complexity of genetic influences on behavioral traits, probably involving gene–gene, gene–gender and gene–environment interactions.

In conclusion, the present results demonstrate that the locus *Ofil1*, located on chromosome 4, influences a specific anxiety-related behavior and modulates ethanol drinking in female rats. These results point to two possibilities. The presence of two (or more) linked genes independently controlling anxiety-like responses and ethanol drinking or one single gene affecting simultaneously both traits. Further dissection of this locus should give information about sex and genetic mechanisms influencing anxiety and alcoholism as well as their comorbidity, thereby opening up new perspectives for psychiatric therapies in humans. An interaction between *Ofil1* and *Ofil2* was also observed for some traits but not for others, such as central open-field locomotion, demonstrating the complexity of genetic influences on behavior and suggesting that other loci, yet to be identified, interact with *Ofil1*.

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