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# Genetic influences on behavioral and neuroendocrine responses to predator-odor stress in rats

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**Abstract**

The exposure of animals to a variety of stressful events can induce behavioral and physiological responses, which can be modulated by anxiety levels. It is well recognized that genetic factors play a substantial role in both anxiety and stress reactivity. The present study examined the effect of exposure to 2,4,5-trimethylthiazoline (TMT), a component of fox feces, on nociception and corticosterone levels in Lewis (LEW) and Spontaneously Hypertensive (SHR) inbred rat strains (which display genetic differences in anxiety models such as the elevated plus-maze and open-field). The influence of two quantitative trait loci (QTL), named *Ofil1* and *Ofil2*, which are known to affect emotionality in LEW versus SHR intercrosses on the responses to TMT was also investigated. LEW and SHR rats of both sexes displayed similar levels of behavioral and neuroendocrine responses after TMT exposure. As expected, TMT odor stress produced analgesia and enhanced corticosterone levels. *Ofil1* on chromosome 4 affected stress-induced analgesia in males only. *Ofil2* on chromosome 7 had no effect. The results suggest that behaviors measured in classical models of generalized anxiety and reactivity to stress produced by predator odors can be genetically dissociated. Finding a locus with an effect on the behavioral responses to stress represents the starting point in the search for genes responsible for stress-related traits.


*Keywords:* Defensive system; Behavior genetics; Quantitative trait locus (QTL); Genomic regions; Marker-assisted selection; Hypothalamic-pituitary-adrenal (HPA) axis

The exposure of humans and animals to stressful life events can induce a broad range of behavioral and physiological responses. For example, stress can activate endogenous analgesic systems [11], producing a phenomenon named stress-induced analgesia. It has been suggested that this phenomenon is part of the animal defensive system and that it can be modulated by anxiety [9]. There is growing evidence pointing to the fact that genetic factors play a substantial role in shaping both stress-induced analgesia [19] and hypothalamic-pituitary-adrenal (HPA) axis function [13], the primary mediator of neuroendocrine stress responses. However, the genes involved remain largely unknown.

The inbred rat strains Lewis (LEW) and Spontaneously Hypertensive Rats (SHR) show high and low indices of experimental anxiety, respectively, when submitted to a variety of behavioral tests such as the open-field, elevated plus-maze and black–white box [16,21] and are therefore a useful genetic model for the study of anxiety. Moreover, LEW rats submitted to repeated social stress showed more severe and longer-lasting responses, such as body weight loss, hypophagia and anxiogenic-like effects, than SHR rats [2]. LEW rats tend to display lower corticosterone release (i.e. hypoactivity of the HPA axis) under stress compared to SHR rats [5,8]. These findings suggest that LEW and SHR rats display distinct "psychoneuroendocrine" profiles related to stress reactivity and behave differently in various aversive environments. It is unclear, however, whether LEW and SHR differ in relation to other types of emotional cues, such as the odor of predators.

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We have recently reported that a 3-min swim-stress-induced analgesia in LEW and Wistar, but not in SHR male rats. The same stressor, however, induced analgesia in females of all three strains [20]. In Wistar (but not LEW and SHR) females, the stress-induced analgesia seems to involve a nonopioid *N*-methyl-D-aspartate (NMDA) analgesic system. Other studies in mice, however, reported that stress-induced analgesia can be modulated by NMDA receptor antagonists in male and ovariectomized female mice, but not in intact females (see [19] for review). Mogil et al. [12] reported the localization of a quantitative trait locus (QTL), located on mouse chromosome 8, associated with stress-induced analgesia in female mice. This evidence suggests the existence of sex- and sex x genotype influences on stress reactivity.

In an effort to investigate the molecular basis of the anxiety-related differences observed between LEW and SHR rats, a genome-wide QTL search was performed [17]. This study revealed two female-specific QTLs for inner locomotion in the open-field (being thereafter named *Ofil1* and *Ofil2* on chromosomes 4 and 7, respectively). Subsequent studies confirmed the role of these QTLs on anxiety-like behaviors [21]. Potenza et al. [15] reported a QTL on chromosome 4, near *Ofil1*, controlling corticosterone levels, thus suggesting that these genomic regions may contain genes controlling not only anxiety-related behavior but also HPA axis reactivity in rats.

The aim of the present study was to compare LEW and SHR rats in their stress-related responses to predator odor and to evaluate the possible implication of the loci *Ofil1* and *Ofil2* on these responses. To this end, nociceptive sensitivity and plasma corticosterone levels before and after exposure to 2,4,5-trimethylthiazoline (TMT), a component of fox feces, were investigated. To verify the role of *Ofil1* and *Ofil2* on behavioral and neuroendocrine responses induced by TMT, rats deriving from an F2 intercross between LEW and SHR strains were selected for breeding based on their genotype at polymorphic markers flanking the two QTLs. Animals whose genotypes were homozygous LEW/LEW or SHR/SHR at either *Ofil1* or *Ofil2* were selected to produce an F3 generation with a known genotype at these loci only, with the rest of the genome being a random assortment of alleles from one or the other parental strains. The phenotypic differences among these groups would therefore be the result of genetic variations within these chromosomal loci. Similar approaches have been used previously with success [21]. Animals of both sexes were included because there is considerable evidence for quantitative and qualitative sex differences in stress reactivity and because significant gender x genotype interactions have been found in our previous work using this same genetic model [21].

Male and female Lewis/CRLIFO (LEW) and SHR/CRL (SHR) rats were purchased from Charles River/IFFA CREDO. To obtain the F1 population, three LEW males were crossed with six SHR females and three SHR males were crossed with six LEW females. F1 rats were then inbred to produce the F2 generation. A total of 453 F2 rats were selected based on polymorphic markers for *Ofil1* (D4Wox22 at 37.38 cM and D4Mgh6 at 58.99 cM) and *Ofil2* (D7Rat35 at 6.83 cM and D7Mgh11 at 2.3 cM). Animals that inherited the genotypes homozygous LEW/LEW (L) or SHR/SHR (S) at each locus (4 = *Ofil1* and 7 = *Ofil2*) were used as founders of the recombinant lines (L4/L7; L4/S7; S4/L7; S4/S7). To better delineate *Ofil1*, the animals belonging to the F3 generation were further genotyped with D4Rat61 (73.62 cM) and rats were required to be homozygous for all three markers to be included in the study. Adult F3 rats (10-weeks old) of the four new lines and both sexes were tested. The number of rats per group was: 7 and 10 for L4/L7; 5 and 4 for L4/S7; 9 and 10 for S4/L7; 9 and 9 for S4/S7; for males and females, respectively. Purebred LEW (10 males and 9 females) and SHR (10 males and 8 females) rats of the same age were concurrently tested. All animals were kept in collective plastic cages (2–4 rats/cage) with food and water available ad libitum under a 12-h light:12-h dark cycle (lights on at 07:00 h) at 21 ± 2 °C. This study was conducted in conformity with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Primers for microsatellite markers were purchased from Eurogentec (Seraing, Belgium), or from Research Genetics (Huntsville, USA). Genomic DNA was extracted from tail tissue and the genotype determinations were performed by polymerase chain reaction (PCR). Alleles were separated on 3% agarose gel and visualized with ethidium bromide staining under ultraviolet light (see [21] for more details on genotyping).

For measurement of plasma corticosterone, blood samples (150 μl) were collected from a clipped tail vein within 30 s while the rat was gently restrained. Samples were collected in the morning (8:00–9:00) and late afternoon (17:00–18:00) to estimate basal corticosterone levels. Blood was collected in chilled tubes coated with a 10% EDTA solution and centrifuged (4500 g, 15 min, 4 °C). The plasma was then stored at −80 °C for subsequent measurement of corticosterone. As previously described [4], plasma corticosterone concentrations were determined by competitive protein binding following extraction with absolute ethanol and using [$^3$H]-corticosterone (76.5 Ci/mmol; NEN Life Science, Paris, France) as radioligand and transcortin from rhesus monkey plasma as binder.

The hot-plate task was used in accordance with methods previously described [20]. The hot-plate apparatus (Ugo Basile, model-DS37) was maintained at 52.2 ± 0.5 °C. Animals were placed in a glass cylinder of 24 cm diameter on the heated metal surface and the time between placement and shaking or licking the paws or jumping (whichever occurred first) was recorded as an index of latency to nociception. A 45-s cut-off was imposed whereby non-responding animals were removed from the hot-plate to prevent tissue damage.

The TMT odor stress procedure took place 1 week after blood samples collection for basal corticosterone measurements. After evaluation of basal nociceptive behavior on the hot-plate, the animals were placed individually in a plastic cage with a small piece of filter paper placed at the bottom of a cylindrical plastic tube located in one corner of the cage (without sawdust) with 25 μl of 2,5-dihydro-2,4,5-trimethylthiazoline (TMT, Phero Tech Inc., Vancouver, BC, Canada). The amount of TMT and conditions of exposure were based on previous studies [4]. The experimental room was ventilated under negative air pressure relative to the hallway to avoid odor dissemination to other areas. After 10 min of TMT exposure, the animals were removed from the

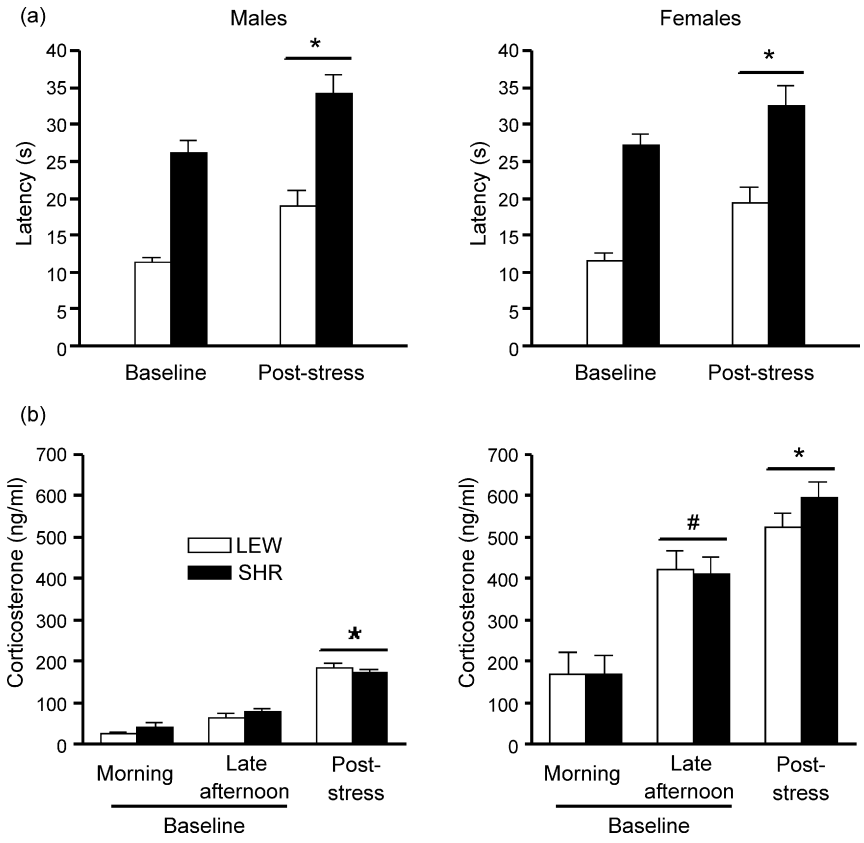


Fig. 1. (a) Latencies in seconds (means and S.E.M.) for thermal nociceptive sensitivity in the hot-plate test (52.2 ± 0.5 °C) of LEW and SHR rats of both sexes tested before (Baseline) and immediately after a 10-min period of TMT odor exposure (Post-stress). (b) Plasma corticosterone values (means and S.E.M.) in the morning and late afternoon (Baseline), and 20 min after the end of a 10-min TMT odor exposure (Post-stress). *Significantly different from baseline levels ($p < 0.05$). #Significantly different from corticosterone levels in the morning ($p < 0.05$).

room and immediately retested on the hot-plate (in an adjacent room). Blood samples were collected 20 min after the end of the TMT exposure to estimate the post-stress corticosterone levels. Male and female rats were tested in alternate days between 8:00 and 12:00 h. Adequate measures were taken to minimize pain or discomfort to the animals.

For comparisons between LEW and SHR, data were analyzed by a three-way ANOVA (strain, gender and time factors) with repeated measures on the time factor (before versus after TMT exposure). To analyze the influence of L or S alleles at each locus and their interaction, four-way ANOVA with repeated measures (*Ofil1*, *Ofil2*, gender and time factors) was performed exclusively with the data of the four new rat lines. Newman–Keuls test was used for post hoc comparisons when appropriate. The accepted level of significance for all tests was $p < 0.05$.

Fig. 1(a) illustrates the results of basal (baseline) and post-stress (exposure to TMT odor) nociception latencies obtained in LEW and SHR rats of both sexes. The three-way ANOVA revealed an overall effect of strain ($F_{(1,33)} = 90.33$; $p < 0.0001$) and of time ($F_{(1,33)} = 49.51$; $p < 0.0001$). As expected, SHR rats of both sexes showed higher nociception latencies than LEW rats. TMT odor stress induced significant analgesia (i.e. increase of nociception latencies) for both strains. No differences were found for the gender factor ($F_{(1,33)} = 0.01$; $p = 0.93$), and for the interactions between strain versus gender ($F_{(1,33)} = 0.02$; $p = 0.88$), strain versus time ($F_{(1,33)} = 0.23$; $p = 0.64$), gender versus time ($F_{(1,33)} = 0.27$; $p = 0.60$) and strain versus gender versus time ($F_{(1,33)} = 0.31$; $p = 0.58$).

The results of basal (morning and late afternoon) and post-stress corticosterone levels obtained in LEW and SHR rats of both sexes are illustrated in Fig. 1(b). Three-way ANOVA revealed a significant effect of gender ($F_{(1,33)} = 333.96$; $p < 0.0001$), of time ($F_{(2,66)} = 76.46$; $p < 0.0001$), and of gender versus time interaction ($F_{(2,66)} = 18.98$; $p < 0.0001$). The post hoc comparisons indicated that the corticosterone levels in males were higher after stress exposure as compared to the baseline levels, i.e. in the morning ($p < 0.0002$) and late afternoon ($p < 0.002$). In females, it was observed the following rank order for corticosterone levels: morning < late afternoon < TMT odor ($p < 0.0002$). Moreover, the corticosterone levels, in baseline and post-stress conditions, were higher in females than in males ($p < 0.0002$). No differences were found for the factor strain ($F_{(1,33)} = 0.64$; $p = 0.43$) and for the interactions between strain versus gender ($F_{(1,33)} = 0.22$; $p = 0.65$), strain versus time ($F_{(2,66)} = 0.22$; $p = 0.80$) and strain versus gender versus time ($F_{(2,66)} = 0.93$; $p = 0.40$).

The results on the influence of the loci *Ofil1* and *Ofil2* on TMT stress-induced analgesia are illustrated in Fig. 2(a). Four-way ANOVA revealed a significant effect of time ($F_{(1,55)} = 15.99$; $p < 0.0002$) and an interaction between *Ofil1* versus gender ($F_{(1,55)} = 4.14$; $p < 0.0467$), gender versus time ($F_{(1,55)} = 6.33$; $p < 0.0148$) and *Ofil1* versus gender versus time

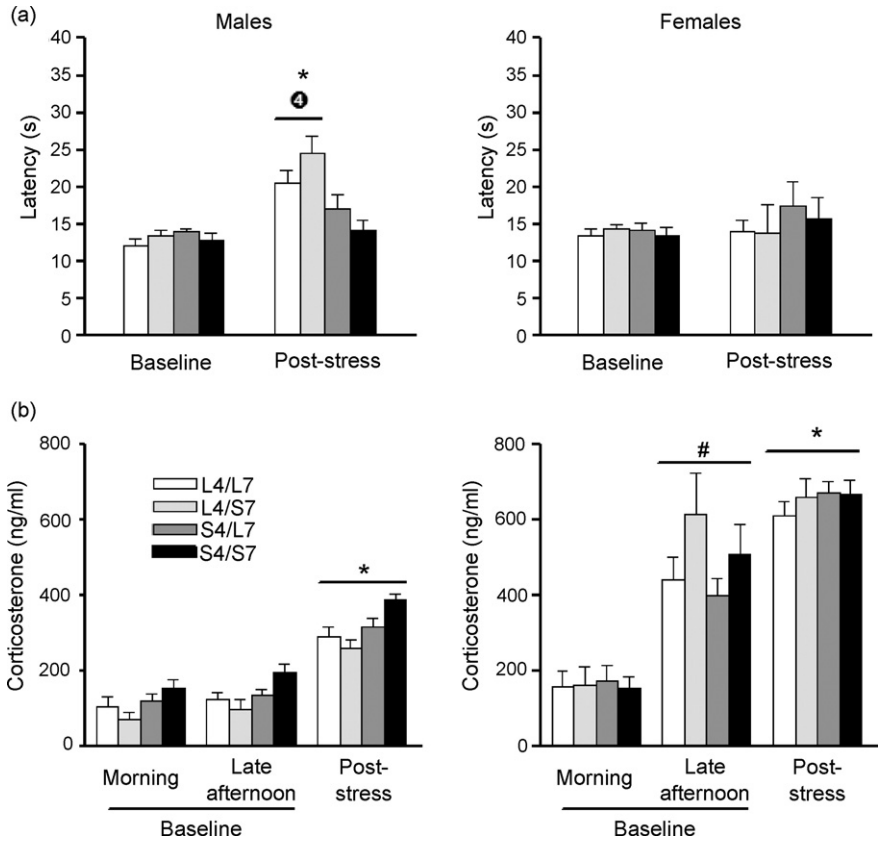


Fig. 2. (a) Latencies in seconds (means and S.E.M.) for thermal nociceptive sensitivity in the hot-plate test (52.2 ± 0.5 °C) of F3 rats (according to line) of both sexes tested before (Baseline) and immediately after a 10-min of TMT odor exposure (Post-stress). (b) Plasma corticosterone values (means and S.E.M.) in the morning and late afternoon (Baseline), and 20 min after the end of a 10-min TMT odor exposure (Post-stress). *Significantly different from respective baseline latencies ($p < 0.05$). ❹ Indicates significant *Ofil1* (on chromosome 4) effect ($p < 0.05$). #Significantly different from corticosterone levels in the morning ($p < 0.05$).

($F_{(1,55)} = 7.72$; $p < 0.0074$). The post hoc comparisons indicated that the animals with LEW alleles at *Ofil1* showed stress-induced analgesia as compared with their baseline hot-plate latencies ($p < 0.0002$). Moreover, they showed higher stress-induced analgesia than the animals with SHR alleles at *Ofil1* ($p < 0.0011$). No differences were found for *Ofil1* ($F_{(1,55)} = 0.69$; $p = 0.41$), *Ofil2* ($F_{(1,55)} = 0.01$; $p = 0.95$), gender ($F_{(1,55)} = 2.09$; $p = 0.15$), and for the interactions between *Ofil1* versus *Ofil2* ($F_{(1,55)} = 2.03$; $p = 0.16$), *Ofil2* versus gender ($F_{(1,55)} = 0.08$; $p = 0.78$), *Ofil1* versus time ($F_{(1,55)} = 1.51$; $p = 0.22$), *Ofil2* versus time ($F_{(1,55)} = 0.07$; $p = 0.79$), *Ofil1* versus *Ofil2* versus gender ($F_{(1,55)} = 0.45$; $p = 0.51$), *Ofil1* versus *Ofil2* versus time ($F_{(1,55)} = 0.29$; $p = 0.59$), *Ofil2* versus gender versus time ($F_{(1,55)} = 0.18$; $p = 0.68$) and *Ofil1* versus *Ofil2* versus gender versus time ($F_{(1,55)} = 0.34$; $p = 0.56$).

Regarding the influence of *Ofil1* and *Ofil2* on corticosterone levels (Fig. 2(b)), the four-way ANOVA revealed a significant effect of gender ($F_{(1,55)} = 135.27$; $p < 0.0001$), of time ($F_{(2,110)} = 178.71$; $p < 0.0001$) and an interaction between these two factors ($F_{(2,110)} = 43.55$; $p < 0.0001$). The post hoc comparisons indicated that the corticosterone levels in males were higher after stress exposure compared to the baseline levels, i.e. in the morning ($p < 0.0002$) and late afternoon ($p < 0.002$). In females, the following rank order for corticosterone levels was observed: morning < late afternoon < TMT odor ($p < 0.0002$). Moreover, the corticosterone levels, in baseline and post-stress conditions, were higher in females than in males ($p < 0.0002$). No differences were found for the factors *Ofil1* ($F_{(1,55)} = 1.23$; $p = 0.27$), *Ofil2* ($F_{(1,55)} = 2.30$; $p = 0.13$), and for the interactions between *Ofil1* versus *Ofil2* ($F_{(1,55)} = 0.21$; $p = 0.65$), *Ofil1* versus gender ($F_{(1,55)} = 2.84$; $p = 0.10$), *Ofil2* versus gender ($F_{(1,55)} = 0.81$; $p = 0.37$), *Ofil1* versus time ($F_{(2,110)} = 1.66$; $p = 0.19$), *Ofil2* versus time ($F_{(2,110)} = 2.72$; $p = 0.07$), *Ofil1* versus *Ofil2* versus gender ($F_{(1,55)} = 2.35$; $p = 0.13$), *Ofil1* versus *Ofil2* versus time ($F_{(2,110)} = 0.02$; $p = 0.98$), *Ofil1* versus gender versus time ($F_{(2,110)} = 0.86$; $p = 0.43$), *Ofil2* versus gender versus time ($F_{(2,110)} = 2.01$; $p = 0.14$) and *Ofil1* versus *Ofil2* versus gender versus time ($F_{(2,110)} = 0.13$; $p = 0.87$).

In the present study, the LEW and SHR inbred rat strains, which are known to differ in several behavioral tests of anxiety/emotionality [16,21], showed similar levels of analgesia and corticosterone induced by stress. Moreover, the loci *Ofil1* and *Ofil2*, two female-specific QTLs that modulate an anxiety-related behavior [17,21], did not affect either stress-induced analgesia or plasma corticosterone levels in female rats. In males, however, the locus *Ofil1* significantly affected the stress-induced analgesia, which represents the first identification of a QTL affecting this type of behavioral response in rats.

A growing body of evidence suggests that the exposure of rats to TMT elicits a variety of autonomic and behavioral

changes [7] that have been interpreted as indicative of aversion, fear and/or anxiety. For example, TMT exposure induces unconditioned freezing [23], conditioned flavor avoidance [14], enhanced startle, inhibition of appetitive behavior [6], analgesia [10,22] and release of corticosterone in rats [3,4]. It was also reported that TMT exposure increases c-fos expression in several brain regions which are presumed to control defensive behaviors and HPA axis activity [3,14]. Thus, the aforementioned evidence suggests that TMT induces stress-related responses. Because motivational processes such as anxiety play an important role in stress reactivity [9], it could be expected that two rat strains that differ in anxiety levels would respond differently when exposed to predator odors. Herein, LEW and SHR male and female rats, which are known to display high and low anxiety-related behaviors, respectively [16,21], showed both a significant increase in the latency to nociception in the hot-plate test (i.e. stress-induced analgesia) and an enhanced plasma corticosterone level after exposure to the TMT odor. These findings corroborate the hypothesis that the TMT odor is highly and innately aversive for rats. However, no strain differences were observed in either measure, despite the well-known difference in basal nociception between LEW and SHRs in the hot-plate [20]. These findings suggest that the anxiety-related differences previously observed between LEW and SHR rats do not modulate the stress responses to TMT odor.

In agreement with previous studies conducted in our laboratory [5], basal corticosterone levels were not different between SHR and LEW rats. However, one somewhat surprising result found in the present study was that LEW and SHR rats showed similar levels of corticosterone following TMT exposure. There is evidence indicating that LEW rats show lower corticosterone levels after stress produced by tail shocks [8] and after exposure to a novel environment or forced exercise (but not immobilization) [5] compared to SHR rats. In the study by Berton et al. [2], however, no differences were found in the corticosterone levels between these strains when exposed to social defeat stress. Differences in the type of measure, procedure, apparatus and/or sub-strains used may underlie these discrepancies across studies.

The salient result of the present study was that the locus *Ofil1* affected significantly stress-induced analgesia in male rats. However, no genetic effects were observed on corticosterone levels of either sex. Male recombinant rats (F3) carrying two LEW alleles at the locus *Ofil1* showed higher levels of stress-induced analgesia (with no change in baseline nociception latencies) than males carrying two SHR alleles. This finding represents the first evidence of a QTL controlling analgesic responses induced by stress in rats. It is important to remember that the QTLs *Ofil1* and *Ofil2* [17,21] were found to be female-specific and had a specific effect on central open-field locomotion, a putative measure of anxiety. The fact that these QTLs did not modulate any effects in females in the present study provides further genetic evidence that the open-field and the predator-odor tests are likely to reflect different psychological traits controlled by different genetic mechanisms. It is remarkable that basal levels of nociception in both male and female F3 rats were quite similar to those showed by LEW rats, thus suggesting a dominance of the LEW genotype on this trait. In addition, it is clear that the stress-induced analgesia was lower in female F3 rats (also in males S4/S7 and S4/L7) compared to the purebred strains LEW and SHR. Some possibilities may be advanced to explain this type of outcome. Whereas the inbred strains LEW and SHR are genetically homozygous for all the genome, the recombinant lines are mostly heterozygous, which might cause, in the latter, heterosis and/or new epistatic interactions. Furthermore, corticosterone levels were not affected by either *Ofil1* or *Ofil2* in male and female rats. One recent study reported a QTL controlling late afternoon corticosterone levels [15] localized approximately in the same region as *Ofil1* on chromosome 4 in rats. The effect of this genomic region on corticosterone levels was not confirmed in the present study using a different rat strain in comparison with the LEW strain.

Concerning sex differences, similar levels of stress-induced analgesia were observed in the present study for male and female LEW and SHR rats. On the other hand, the analgesic effect produced by stress in the recombinant lines appears to be more pronounced in males than in females. The literature examining sex differences in stress-related responses is highly controversial. There is evidence showing that stress-induced analgesia is more pronounced in males than in females [18]. Other studies, however, indicate that stress-induced analgesia is equipotent or even more pronounced in females than in males [19,20]. The reasons for these differences remain unknown, but hormonal factors must be considered in attempting to explain the sex differences in the magnitude of stress-induced analgesia, because both castration and ovariectomy significantly reduced stress-induced analgesia in rats [18]. Overall, herein female rats showed higher corticosterone levels than male rats. Atkinson and Waddell [1] reported marked differences in the levels of corticosterone across the estrous cycle. In that study, the corticosterone value in male rats was not different from that of estrous females, but it was lower than that of females at all other stages. Further studies are needed to investigate the role of hormonal influences on the phenotypes studied.

The results of the present study should be interpreted with caution for a number of reasons. First, because control groups that did not experience any stressful olfactory stimulation were not used in the present study, it is difficult to determine whether or not the phenotypic differences observed herein are specific to the predator-odor stress. Second, it is possible that the relatively high dose of TMT used in the present study induced a ceiling effect on the physiological responses, thus masking possible genetic effects. Third, because the estrous cycle was not monitored, hormonal influences on the observed results cannot be fully appreciated.

In conclusion, the expected behavioral and neuroendocrine differences between the LEW and SHR strains were not found in the present paradigm. Further behavioral and pharmacological evaluation of these rats in tests using predator odors as well as in other types of emotionality tests should improve the understanding of their psychological profile. In addition, the effect of *Ofil1* on behavioral responses induced by stress represents a starting point in the search for genes involved in stress. Further dissection of this locus should advance the understanding of genetic susceptibility to stressful events as well as of the neuropsychi-

atric disorders associated to stress. Studies aiming to breakdown the effect of this region are currently underway.

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