Physiology & Behavior 169 (2017) 82–89

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Cohabitation with an Ehrlich tumor-bearing cagemate induces immune but not behavioral changes in male mice

Thalita R.M. Machado [a,b], Glaucie J. Alves [a,c], Wanderley M. Quinteiro-Filho [a,b], João Palermo-Neto [a,b,*]

[a] Neuroimmunomodulation Research Group, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil
[b] Department of Pathology, School of Veterinary Medicine, Universidade de São Paulo, São Paulo, SP, Brazil
[c] Nuclear and Energy Research Institute-IPEN-CNEN/SP, Brazil

HIGHLIGHTS

- This article shows the effects of cohabitation with Ehrlich tumor-bearing mice on the open-field behavior.
- The hypothalamic NA levels and turnover rate.
- Host resistance to Ehrlich tumor growth and corticosterone studies of Swiss male mice.

ARTICLE INFO

Article history:
Received 29 March 2016
Received in revised form 5 October 2016
Accepted 19 November 2016
Available online 22 November 2016

Keywords:
Ehrlich tumor
Neutrophil
Open-field behavior
Noradrenaline
Stress
Gender
Neuroimmunomodulation

ABSTRACT

Cohabitation with Ehrlich ascitic tumor-injected conspecifics induces behavioral, neurochemical, endocrine and immune changes indicative of stress and immune impairment in female mice. The present work analyzed the effects of similar cohabitation in Swiss and Balb/C male mice. At least 12 pairs of male mice were divided into a control group and an experimental group. On experimental day 1 (ED1), one animal within each experimental pair was inoculated with 5×10^6 Ehrlich tumor cells intraperitoneally (i.p.); the other animal was kept undisturbed and was referred to as the CSP (companion of a sick partner). One male mouse of each control pair was treated i.p. with 0.9% NaCl (1 mL/kg); the other animal (the CHP, companion of a healthy partner) was kept undisturbed. Cohabitation with a sick partner for 11 days did not induce any behavioral, hypothalamic noradrenergic, corticosterone or adrenal weight changes in the Swiss CSP male mice compared to those of the Swiss CHP group. However, impairments in neutrophil phagocytosis and oxidative burst as well as increased levels of catecholamines were observed in Swiss and Balb/C CSP mice relative to CHP male animals of the same strains on ED11 and ED14, respectively. Moreover, after a challenge with 5×10^6 Ehrlich tumor cells on ED11 of cohabitation, the number and concentration of tumor cells found in the ascitic fluid were higher in the Swiss CSP male mice than in the CHP mice. These data suggest that the immune changes observed in Swiss and Balb/C male CSP mice after cohabitation with a sick cagemate might, ultimately, depend on the changes induced by catecholamines, as previously reported for CSP female mice. However, contrary to that reported in Swiss CSP female mice, changes in behavioral and hypothalamic noradrenaline activity were not found in the Swiss CSP male mice analyzed in this work. This fact suggests that male and female CSP mice might use similar immune but different CNS strategies against the threats posed by the tumor-bearing animals.

1. Introduction

Animals do not function in isolation in their environment; they exhibit a variety of social systems that form the basis of behaviors essential to the proper functioning of pair or group living in most social species.

One of the major costs of social behavior is the increased risk of exposure to diseases. Acute illness not only reduces the expression of social behavior by sick rodents but also can lead to avoidance responses when detected by healthy partners. Cohabitation with an Ehrlich tumor-bearing mouse increased the locomotor activity of female mice and decreased the host's resistance to tumor growth [1,2]. Similar cohabitation with a sick cagemate was shown in female mice to (1) decrease the levels and increase the turnover rate of hypothalamic noradrenaline (NA) [3], (2) decrease neutrophil and macrophage oxidative

* Corresponding author at: School of Veterinary Medicine, USP, Av. Prof. Dr. Orlando Marques de Paiva, nº 87, CEP 05508-000 São Paulo, SP, Brazil.
E-mail address: jpalermo@usp.br (J. Palermo-Neto).

http://dx.doi.org/10.1016/j.physbeh.2016.11.022
0031-9384/

burst after *Staphylococcus aureus* induction, (3) decrease the percentage of neutrophil and macrophage phagocytosis [1–5], and (4) modify dendritic cell phenotype [6]. Since corticosterone plasma levels were not changed by cohabitation [3,5,11], the final neural link between the neuroimmune changes observed in companions of Ehrlich tumor-bearing mice was reported to involve catecholamine release and/or central nervous system (CNS) activation as a consequence of the psychological stress imposed by the forced housing condition [2]. Studies from other laboratories have shown that psychological stress generated in a social disruption stress paradigm also induced significant neuroimmune alterations [7].

Odor but not visual or auditory cues were taken to be pivotal for the neurochemical and immune changes induced in female mice by cohabitation with an Ehrlich tumor conspecific [4,5]. Olfaction is a fundamental sense through which most animals perceive the external world. The olfactory system detects odors via specialized sensory organs such as the main olfactory epithelium and the vomeronasal organ [8]. Tumors produce volatile organic compounds that are released into the atmosphere in breath, sweat and urine [9,10]. We have shown that odor cues released by Ehrlich tumor-bearing mice are aversive to female mice [11]. This avoidance-induced behavior was interpreted as being an adaptive evolutionary response designed to limit the spread of infection among conspecifics. Odorant cues released by rodents play a key role in mate preference and selection [12]. Animals can recognize specific odorants that convey information regarding conspecific health condition, which in turn plays a significant role in regulating social communication and social and non-social behavior [11–14]. This pattern of social behavior is taken to be adaptive, helping prevent the spread of disease in otherwise highly social animals, and can be observed across a variety of rodent species [15–18]. Gender differences were reported to exist in the strategies used by males and females against the risks posed by diseases [15]. Sexual dimorphism has been reported in the genes that encode neuropeptides involved in the recognition and avoidance of odors of infected individuals [17] and also in the locus coeruleus arousal system during stress [18]. To date, however, our studies on cohabitation with a sick partner have focused only on female mice because they are less aggressive than males when grouped; thus, we designed the present work to analyze the effects induced by cohabitation with an Ehrlich tumor-bearing cagemate on male mice.

2. Materials and methods

2.1. Animals

Naïve Swiss and naïve Balb/C male mice, aged 90–100 days at the beginning of the experiments, were used. The animals were housed under conditions of controlled temperature (22 ± 2 °C) and artificial light (12-hour light/12-hour dark, lights on at 7:00 a.m.). An electricity generator was used to protect the whole system against power failures. Mice were transferred to a different (temperature-consistent) room and were acclimated for 20 days before beginning the experiments. The maintenance and use of the animals followed the recommendations of the National Council on the Control of Animal Experimentation (CONCEA). All studies were performed after the approval of the Committee on Care and Use of Animal Resources of the School of Veterinary Medicine, University of São Paulo, Brazil.

2.2. Group formation and experimental design

As presented in Fig. 1, five independent experiments were performed with 15, 12, 15 and 15 pairs of Swiss and 16 pairs of Balb/C mice, respectively. They were conducted in accordance with good laboratory practice protocols and quality assurance methods.

In all experiments, male mice were weighed, paired according to weight, and then subsequently divided into 2 groups: 1 control and 1 experimental group. Swiss and Balb/C male mice from the same litter and cage were paired to avoid the presence of aggressive episodes and threats. Pairs were discharged from the experiments in the case of aggressive behavior or the presence of wounds. Ten days after pairing, 1 male animal from each experimental pair (the SP, sick partner) was inoculated with 5×10^6 Ehrlich tumor cells intraperitoneally (i.p.). The other male, the subject of this study, was kept undisturbed and was referred to as the CSP (companion of the sick partner). One male mouse of each control pair (the HP, healthy partner) was i.p. treated with 0.9% NaCl (1 mL/kg), and the other male was kept undisturbed (CHP, companion of the healthy partner). The day on which the injections were given was experimental day 1 (ED1). In the first experiment, CSP and CHP Swiss mice were removed from their cages on ED11 and analyzed within an open-field arena; immediately after that, they were killed, and their brains were removed and dissected for analysis of NA levels and turnover rate. In the second experiment, blood was taken from

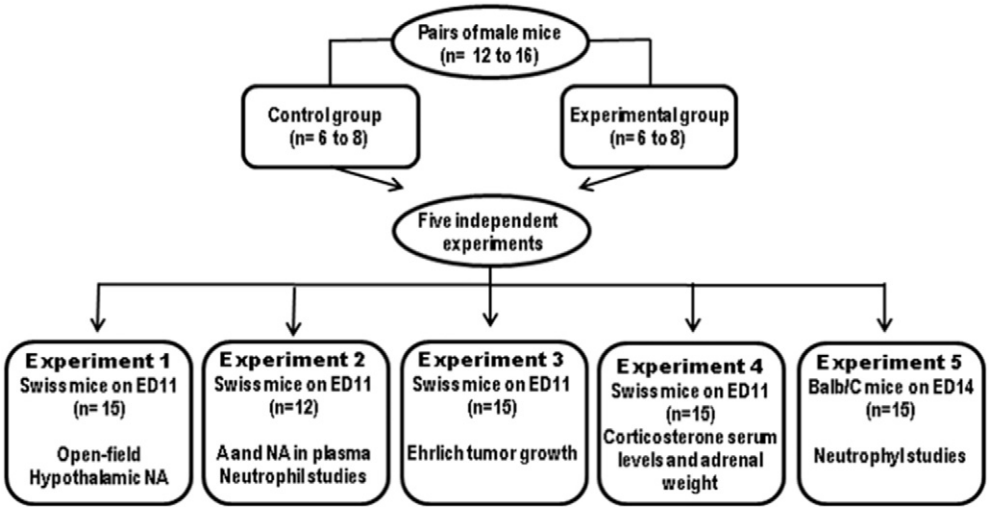

Fig. 1. Schematic diagram of the experimental design. At least 12 pairs of Swiss and Balb/C male mice were distributed into a control and an experimental group used to perform 5 independent experiments. On Experimental Day (ED) 1, one male mouse from each pair of experimental mice (the SP) was inoculated with 5×10^6 Ehrlich tumor cells i.p.; the other, the subject of this study, was kept undisturbed (the CPS). One male mouse from each control pair (the HP) was i.p. treated with 0.9% NaCl (1 mL/kg); the other animal (the CHP) was kept undisturbed. Studies with Swiss mice (experiments 1 to 4) were performed on ED11, and those with Balb/C mice (experiment 5) on ED14.

the CSP and CHP Swiss male mice on ED11 to determine the plasma levels of adrenaline (A) and NA and neutrophil oxidative burst and phagocytosis. In the third experiment, CSP and CHP Swiss mice were injected i.p. on ED11 with 5×10^6 Ehrlich tumor cells and subsequently analyzed for tumor growth. In the fourth experiment, corticosterone serum levels were assayed in CSP and CHP Swiss mice on ED5, ED7, ED9 and ED11; immediately after that, the animals were killed to assess adrenal weight. Finally, in the last and fifth experiment, we repeated the neutrophil studies using male Balb/C mice. Balb/C mice were used not only because they are thought to be more prone to immune challenges [19,20] but also to confirm and straighten the immune data from the Swiss strain. Isogenic strains of mice such as Balb/C and C57/B16 are commonly known to provide more consistent immune data than an outbred mouse strain such as the Swiss strain, i.e., data from these mice are less variable and more reproducible. Blood was taken from these CSP and CHP Balb/C male mice on ED14 to analyze neutrophil oxidative burst and phagocytosis.

Swiss and Balb/C sick animals were analyzed in their cages for Ehrlich tumor signs and symptoms as described elsewhere [1]. Briefly, the following scoring system was employed: 0 = predominantly active with normal feeding and the presence of rough hair; 2 = active, normal feeding, rough hair and the presence of a small increase in abdominal volume; 3 = active, normal feeding, rough hair and a mild increase in abdominal volume; and 4 = an absence of activity, anorexia, dyspnea, rough hair and a severe increase in abdominal volume. ED11 and ED14, the days chosen for the CSP and CHP Swiss and Balb/C studies, were the days on which the vast majority of Ehrlich tumor-bearing mice presented a score of 4. Genetically inbred Balb/C mice were shown to be more resistant to stressor effects [19], such as that generated by the forced housing condition [11].

To minimize the influence of possible circadian changes, the order of the mice undergoing the behavioral analysis within the open field and subsequent brain removal was alternated between CHP and CSP Swiss mice, being performed between 8:00 and 10:00 a.m.; blood was always collected at the same time of day, i.e., between 8:00 and 9:00 a.m. The open field used was wiped with ethanol (1% solution in water) before the mice were introduced to eliminate possible bias effects due to odor cues left by other animals.

2.3. Behavioral study

The open-field apparatus was used to analyze the effects of cohabitation with tumor-bearing male mice on locomotor activity and anxiety levels. The elevated plus-maze was not used to test anxiety levels in this work because CSP female mice presented with a dramatic increase in locomotor activity around ED11 and onward [1,4,5], a fact that impairs the discriminatory power of the apparatus as it induces an anxiety-like profile [1,20]. Within the context of this study, an "anxiety level" was operationally inferred as previously suggested [21], i.e., as the response to a situation in which behavior is influenced by two motivational forces (e.g., a natural curiosity to explore unexplored areas vs. an aversion to open areas). Male animals were kept within the open-field arena for 5 min. The following behavioral parameters were analyzed: total distance travelled within the apparatus (cm), total time spent in the peripheral and central open-field zones (s), frequency of crossings in the peripheral and central open-field zones (n), total immobility time (s) and average speed (cm/s) within the apparatus. The behavioral data were collected using a camera mounted vertically above the apparatus and were analyzed by Ethovision System software (Noldus® Information Technology, Leesburg, VA, USA) installed on a compatible IBM® computer.

2.4. Hypothalamic NA study

NA levels and turnover rates were evaluated in CSP and CHP Swiss male mice on ED11 immediately after the behavioral analysis. The brains of CSP and CHS male mice were removed immediately after decapitation, washed in cold 0.9% NaCl solution and subsequently dissected on ice to remove the hypothalamus, which was kept at −80 °C until analysis. In CSP and CHP male mice, NA and 3-methoxy-4-hydroxyphenolglycol (MHPG) were individually evaluated as described elsewhere [22]. Briefly, brain tissue samples were homogenized in perchloric acid by sonification, and NA and MHPG were measured by high-performance liquid chromatography with an electrochemical detector (HPLC-ED). The apparatus used was an HPLC System (model 6A, Shimadzu, Kyoto, Japan) equipped with a C-18 column (Shimpak, ODS, Kyoto, Japan) and an electrochemical detector (model 6A Chromatopac, Shimadzu). The limit of detection was 0.2 ng for both substances; the recovery values were higher than 80%; the coefficients of variation were smaller than 15%; and curve linearity (r) was higher than 0.95. The turnover rate of NA is expressed as the MHPG/NA ratio.

2.5. Plasma catecholamine study

Plasma levels of NA and A were evaluated in Swiss CSP and CHP male mice. Blood was taken from the mice on ED11 and placed into lithium–heparin Vacutainer tubes (Becton Dickinson and Co., Franklin Lakes, NJ, USA) immediately after each animal was euthanized; blood sampling took no >1 min/animal. Blood combined with 10 μL of heparin was kept on ice before centrifugation at 2500*g* for 20 min at 4 °C. The plasma samples were stored at −80 °C prior to analysis. Plasma concentrations of catecholamines were measured by ELISA kits (Thermo Fischer Scientific Inc., Rockford, IL, USA) at ambient temperature [23]. All samples were analyzed in duplicate and using a single assay. The analytical sensitivity was 7 pg/mL (3.82×10^{-5} nmol/mL) for A and 35 pg/mL (5.90×10^{-3} nmol/mL) for NA. The intra-assay coefficient of variation was 12.6 and 6.8% for A and NA, respectively. The data are expressed as nmol/mL.

2.6. Flow cytometry study

Two independent experiments were conducted: one with Swiss and the other with Balb/C male mice. Neutrophil oxidative burst and phagocytosis were measured as described elsewhere [3–5]. As depicted in Fig. 1, the blood taken on ED11 from the same Swiss CSP and CHP mice used for the quantification of plasma catecholamines was used for neutrophil oxidative burst and phagocytosis determination (second experiment). Blood from naïve Balb/C CSP and CHP male mice was taken on ED14 (fifth experiment). In both cases, the removed blood was placed into lithium–heparin Vacutainer tubes (Becton Dickinson and Co., Franklin Lakes, NJ, USA) immediately after each animal was euthanized. The blood samples (100 μL) were used to assess the phagocytic activity and the oxidative burst of neutrophils. PMA (100 ng) and *S. aureus* (2.4×10^6 bacteria/mL) were used to trigger the oxidative burst. Briefly, 100 μL of whole blood (2×10^5 cells/100 μL) was mixed with 200 μL of 2′,7′ dichlorofluorescein diacetate (DCFH-DA, 0.3 nM) in PBS and with 100 μL of either propidium iodide (PI)-labeled *S. aureus* or PMA in separate polypropylene tubes. Samples were incubated with agitation at 37 °C for 20 min. The reactions were stopped by the addition of 2 mL of cold EDTA solution (3 mM) to terminate phagocytosis. After centrifugation (250 g for 10 min), erythrocytes were lysed from all of the samples with sterile 0.2% NaCl (2 mL/tube) for 20 s. Immediately afterwards, sterile 1.6% NaCl solution (2 mL) was added to each sample to restore isotonicity. The samples were then centrifuged (250*g* for 10 min), and the pellets were resuspended in 1 mL of cold EDTA (3 mM) for flow cytometry.

A flow cytometer (FACSCalibur®, Becton Dickinson Immunocytometry Systems, San Jose, California, USA) interfaced with a Macintosh G4 computer was used. Data from 10,000 events were collected in list mode and analyzed with CellQuest software (Becton Dickinson Immunocytometry Systems). Cell populations were identified based on their properties of forward scatter versus side scatter plots, mechanically

sorted and then evaluated with light microscopy after Giemsa staining. Fluorescence data were collected in log scale. Green fluorescence from DCFH-DA (Molecular Probes, Eugene, Oregon, USA) was measured at 530 ± 30 nm (FL1 detector); red fluorescence from PI-labeled *S. aureus* (Sigma, St Louis, California, USA) was measured at 585 ± 42 nm (FL2). PI and DCFH fluorescence were analyzed after compensation to correct for possible signal crosstalk. Direct measurements of the mean fluorescence of the green and red channels were recorded as the oxidative burst and phagocytosis, respectively. Quantification of phagocytosis and the oxidative burst was performed as previously described [24], i.e., using the mean PI and DCFH fluorescence/cell, respectively. The percentage of phagocytosis (percentage of neutrophils with ingested bacteria) is expressed as the number of neutrophils with red fluorescence divided by the total number of cells (×100).

2.7. Ehrlich tumor growth study

On ED11, CSP and CHP Swiss male mice were challenged for their host resistance to tumor growth; for that, Swiss male mice were injected with 5×10^6 Ehrlich tumor cells for tumor growth evaluation. Injected animals were returned to their home cages where they stayed for 11 days; then, they were killed. The ascitic fluid was removed from individual CSP and CHP mice to analyze the following parameters: ascitic fluid volume, total number of tumor cells in the ascitic fluid and number of tumor cells/mL of ascitic fluid. These measures have been reported to be a reliable measure for Ehrlich tumor growth [25].

2.8. Corticosterone study

Corticosterone serum levels were assayed in Swiss CSP and CHP male mice on ED5, ED7, ED9 and ED11. On each of those days, samples of 100 to 200 μL of blood were taken from individual animals through the submandibular vein for serum hormone quantification; blood sampling within each day took no >1 min/animal. Corticosterone serum levels were quantified using ELISA kits (DetectX®, Arbor assays, Michigan, USA) at ambient temperature, as proposed by the manufacturer. The data are expressed as nmol/mL.

2.9. Adrenal weight study

The same Swiss male animals used for corticosterone studies were used here. Immediately after the last blood collection (ED11), CSP and CHP mice were killed, and their abdominal cavities were opened to remove their left and right adrenal glands. Individual adrenal weights were determined afterwards using a precision scale. The data are presented as relative adrenal weights, i.e., adrenal weight/body weight.

2.10. Statistics

The Bartlett test was performed to evaluate whether the obtained data should be handled as parametric or nonparametric. In subsequent analyses, differences were assessed using Student's *t*-test or two-way ANOVA followed by Tukey-Kramer tests for multiple comparisons. GraphPad® Prism 6 and Sigma Stat 3.0® software were used throughout. For all comparisons performed, $p \leq 0.05$ was considered significant. The data are presented as the mean plus standard deviation.

3. Results

Episodes of aggressive behavior were not observed within the Swiss and Balb/C male mice home cages over the course of the experiments; wounds were also not observed in the CSP and CHP Swiss and Balb/C mice as well as in their tumor-bearing cagemates. Dominance/submissive postures were seldom observed within the animals' cages, and significant differences were not observed in the number and duration of their expression between the CHP and CSP Swiss and Balb/C male mice (data not shown).

Ehrlich tumor cell injection induced behavioral changes in the Swiss and Balb/C sick animals. These alterations arose progressively and were characterized by the presence of lethargy, a reduced interest in their surroundings, and a decreased ability to respond to their companion mouse. We performed the experiments with Swiss and Balb/C CSP mice on ED11 and ED14, respectively i.e., when the Ehrlich tumor signs were given a rating score of 4 in the vast majority of the sick animals.

3.1. Open-field behavior

Table 1 shows the effects of cohabitation with Ehrlich tumor-bearing mice on the open-field behavior of Swiss male mice. The parameters analyzed were not different between the CSP and CHP animals ($p > 0.05$). Thus, differences were not found for the total time spent by the CSP and CHP mice in the peripheral and central zones of the open field or for the frequency of crossings they displayed in these areas. The total immobility time and the average speed were also similar between the CSP and CHP male mice ($p > 0.05$). As a consequence, differences in anxiety levels between CSP and CHP mice could not be inferred.

Interestingly, Swiss and Balb/C CSP male mice displayed few behavioral changes within their home cages around ED11 and ED14, respectively, a time at which their tumor-injected partners remained in one of the corners of the cage, frequently under the wood shavings (data not shown).

3.2. Hypothalamic NA levels and turnover rate

Fig. 2 depicts the effects of cohabitation with a sick cagemate on brain NA and MHPG levels and on NA turnover in the Swiss CSP and CHP male mice. As seen here, no changes were found in the hypothalamic NA and MHPG levels or in the NA turnover rate.

3.3. Plasma levels of A and NA

The plasma levels of A and NA measured in the Swiss CSP and CHP male mice on ED11 are depicted in Fig. 3. As seen here, significant differences were found in the mice for both A and NA; the levels of both catecholamines were higher ($p < 0.05$) in the CSP mice than in the CHP mice.

3.4. Neutrophil oxidative burst and phagocytosis

Table 2 shows the data regarding neutrophil oxidative burst and phagocytosis in the CSP and CHP Swiss male mice. Significant differences were found in Swiss mice for both PMA- and *S. aureus*-induced oxidative bursts ($p < 0.05$); the oxidative bursts were smaller in the Swiss CSP mice than in the CHP mice. Differences were not detected for the basal oxidative burst or for the percentage and intensity of neutrophil phagocytosis in the Swiss male mice ($p > 0.05$).

Table 1
Effects of cohabitation for 11 days with an Ehrlich tumor-bearing companion on open-field behavior of Swiss male mice.

Open-field parameters[a]	CHP	CSP
Total distance travelled (cm)	3186.0 ± 399.0	3312.0 ± 549.0
Average speed (cm/s)	10.66 ± 13.44	11.08 ± 18.36
Time in peripheral zone (s)	265.66 ± 11.46	255.1 ± 14.67
Time in central zone (s)	133.46 ± 31.44	132.56 ± 41.22
Crossings in peripheral zone (n)	30.50 ± 12.26	25.10 ± 14.67
Crossings in central zone (n)	29.63 ± 12.12	36.20 ± 13.22
Total immobility time (s)	233.70 ± 27.31	247.30 ± 29.12

Differences were not detected (*t*-test) at $p < 0.05$.
[a] Mean ± SD of 15 mice.

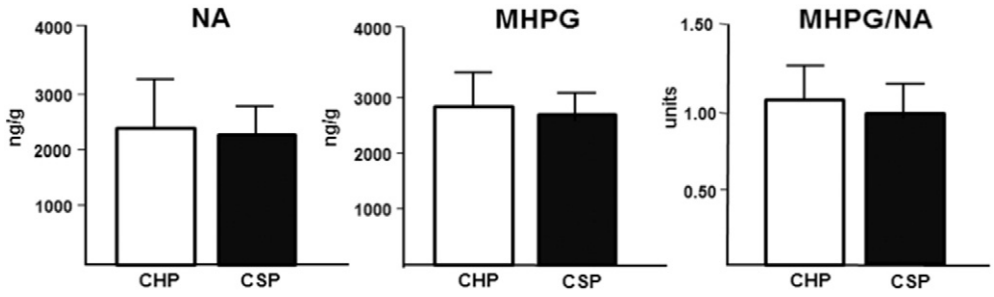

Fig. 2. Effects of cohabitation for 11 days with Ehrlich tumor-bearing companions on the hypothalamic noradrenaline (NA) and MHPG (B) levels and on the NA turnover rate (C) in the Swiss male mice. Data are the mean ± SD. $N = 15$ animals per group.

As depicted in Fig. 4, significant differences were found between CSP and CHP Balb/C male mice for neutrophil oxidative burst and phagocytosis, replicating the data reported above for the Swiss strain. Indeed, although differences were not found between the CHP and CSP data regarding the basal oxidative burst, a decrease ($p < 0.05$) was found in this parameter in mice of the CSP group compared to those of the CHP group after either PMA or *S. aureus* induction. A significant decrease ($p < 0.05$) was also found in the percentage and intensity of phagocytosis in mice of the CSP group compared to those of the CHP group.

3.5. Host resistance to Ehrlich tumor growth

The data regarding host resistance to Ehrlich tumor growth in the Swiss CSP and CHP male mice are presented in Fig. 5. Differences were not found in the ascitic fluid volume between the CSP and CHP animals. However, both the total number of tumor cells found in the ascitic fluid and the number of tumor cells/mL of ascitic fluid were higher in the CSP mice than in the CHP mice ($p < 0.05$).

3.6. Corticosterone serum levels and adrenal weights

Table 3 shows the corticosterone serum levels of the Swiss CSP and CHP male mice on ED5, ED7, ED9 and ED11. Two-way ANOVA showed significant differences among the groups and days as well as a significant interaction between the days and groups ($F(3,26) = 62.85$; $p < 0.05$). An increase in corticosterone serum levels over the experimental days was found in both the CHP and CSP male animals ($p < 0.05$); the corticosterone serum levels of the CHP and CSP mice were higher on ED11 than on ED5, ED7 and ED9. However, differences were not found between the corticosterone serum levels of the CHP and CSP mice on any of the experimental days.

Additionally, no differences were found between the relative adrenal weights of the Swiss CSP and CHP male mice (CHP = 0.22 mg ± 0.06 and CSP = 0.17 mg ± 0.05).

4. Discussion

The present data show that cohabitation with Ehrlich tumor-bearing conspecifics produced no changes in the behavior of Swiss male mice in the open field and in their home cages. Indeed, no differences were found between Swiss CHP and CSP male mice for locomotor activity and anxiety levels within the open field. Similarly, differences were not perceived in the behavior of the Swiss and Balb/C CSP male mice within their home cages throughout the period of cohabitation; this fact was observed even on ED11 and ED14, the days on which the vast majority of the Ehrlich tumor-bearing Swiss and Balb/C mice presented a sickness score of 4, respectively. Accordingly, differences were not found in this work between Swiss CSP and CHP male mice for hypothalamic NA levels and turnover rate. Contrary to these findings, we had previously reported that Swiss CSP female mice, i.e., female mice that lived with Ehrlich tumor-bearing female partners showed the following results in relation to CHP female animals: (1) increased activity within their home cages with clear attempts to escape from the cages, mainly around ED10 i.e., the point at which the tumor-injected female mice started to present sickness behavior and stopped responding to their partner's interaction requests [5,6], (2) a huge increase in locomotor activity within the open field on ED11 [5,6], and (3) decreased hypothalamic NA levels and increased hypothalamic NA turnover rate [3].

One of the major costs of social behavior is the increased risk of exposure to diseases. The avoidance of sick conspecifics is presumably an adaptive evolutionary response designed to limit the spread of the disease among conspecifics. However, male and female individuals exhibit very different levels of investment and strategies in response to environmental cues, such as during mating. Thus, by avoiding sick mates, choosy females can reduce their risk of contracting contagious diseases as they potentially spend more parental investment than the males [17,26]. From an evolutionary perspective, the ability to detect environmental cues from sick individuals and to display an appropriate behavioral (avoidance) response would be highly beneficial for females. On the

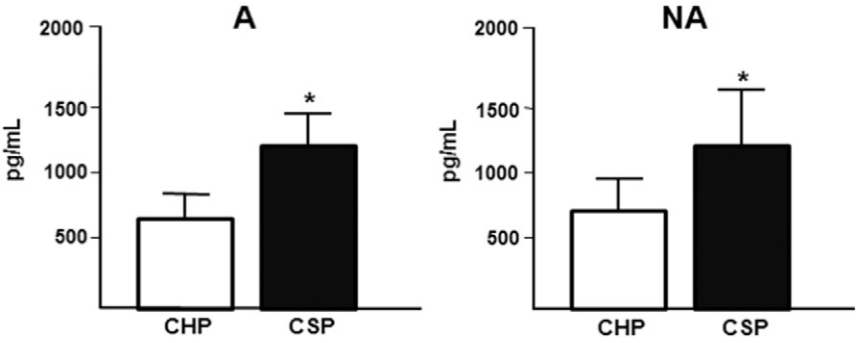

Fig. 3. Effects of cohabitation for 11 days with Ehrlich tumor-bearing companions on the plasma levels of adrenaline (A) and noradrenaline (NA) in the Swiss and Balb/C male mice. Data are the mean ± SD. $n = 12$ Swiss animals per group. *$p < 0.05$ (t-test).

Table 2
Effects of cohabitation for 11 days with an Ehrlich tumor-bearing companion on the neutrophil activity and phagocytosis of Swiss male mice.

Neutrophil activity[a]	CHP	CSP
Oxidative burst		
Basal	82.38 ± 39.42	77.07 ± 32.56
PMA-induced	334.80 ± 83.34	220.10 ± 69.71*
S. aureus-induced	378.60 ± 142.30	184.3 ± 78.63*
S. aureus-induced phagocytosis		
Intensity	58.77 ± 10.57	56.97 ± 11.18
Percentage	80.94 ± 54.00	79.47 ± 10.66

[a] Mean ± SD.
* $p < 0.05$ (*t*-test).

other hand, males have a lower energy investment in the mating process and might even gain advantages in the presence of a sick male conspecific. In fact, it was shown that subordinate male mice in the presence of dominant cagemates displaying sickness behavior showed aggressive behavior toward the dominant male and, in some cases, took the opportunity to become the dominant within the cage [27]. Thus, considering the undisputable hypothalamic NA and behavioral changes reported elsewhere for female mice when paired with Ehrlich tumor-injected female conspecifics [1–5] and the absence of changes in open-field behavior and hypothalamic NA activity reported in the male Swiss CSP mice here, it seems feasible to suggest that male and female mice present different vulnerabilities to the odor cues released by the Ehrlich tumor-injected partners. As discussed above for mating, a sexual dimorphism has already been reported in the recognition and avoidance of odors of infected individuals [17].

Odor but not visual or auditory cues released by the sick companions were considered pivotal for the neuroimmune effects induced by cohabitation with tumor-bearing mice [10]. This finding is in agreement with and reinforces previously reported findings for the mammary tumor virus [9]. Tumors have been reported to produce volatile organic compounds that are released into the atmosphere through urine, breath and sweat [10,28]. Recently, we have shown that the odor cues released by Ehrlich tumor-bearing female mice are aversive [11]. We know that the vomeronasal system (VNS) is a sexually dimorphic chemosensory structure [28] that is deeply involved with the different behavioral outcomes induced by odor cues in males and females [29]. Thus, although the present observed data does not directly allow for this conclusion, it is tempting to suggest that male and female mice differently sense and/or respond to the odor cues released by their Ehrlich tumor-bearing partners. However, interestingly, the changes in immune and plasma catecholamines induced by cohabitation in male mice were similar to those reported elsewhere in females [3–5]. This fact is relevant because upon encountering a sick individual, it would be advantageous for both genders to mount an immune response when a possible disease is encountered. When activated, the catecholaminergic system can provide the body with a needed "boost" to combat the immediate threat of the disease.

Physical and psychological stressors are known to induce endocrine [30] and immune [31,32] changes similar to those currently reported in Swiss and Balb/C male mice after cohabitation with a tumor-bearing cagemate. As reported previously for female mice [3–6], a decrease was found in this study for the neutrophil oxidative bursts in the Swiss and Balb/C male mice of the CSP group compared to those of the CHP group after either *S. aureus* or PMA induction. A decrease in the percentage and intensity of neutrophil phagocytosis was also found in this work in male Balb/C CPS mice in relation to those of the CHP group on ED14. Balb/C animals are thought to be more prone to immune challenges [19,20].

In recent years, significant insights into the progressive states of tumor growth have arisen from basic research dealing with cell biology

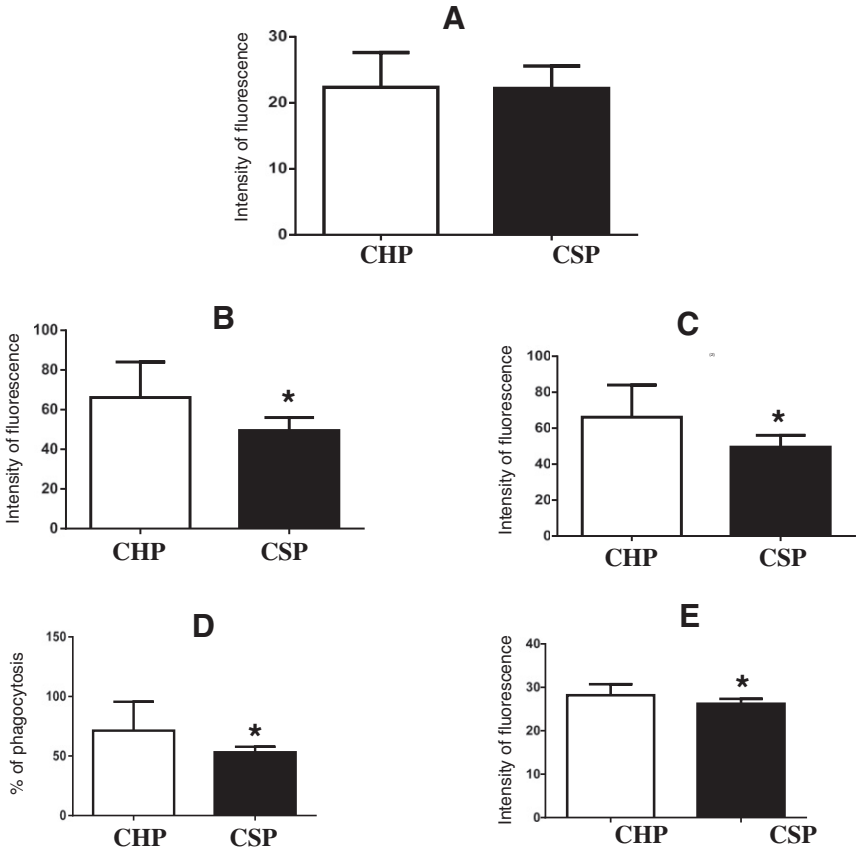

Fig. 4. Effects of cohabitation for 14 days with Ehrlich tumor-bearing companions on the neutrophil activity and phagocytosis in Balb/C male mice. A: basal oxidative burst; B: *S. aureus*-induced oxidative burst; C: PMA-induced oxidative burst; D: percent of phagocytosis and E: intensity of phagocytosis. Data are the mean ± SD. $N = 16$ animals per group. *$p < 0.05$ (*t*-test).

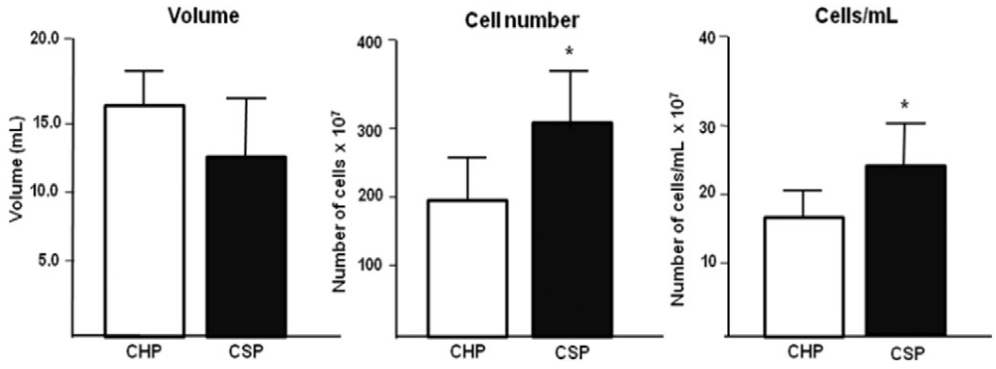

Fig. 5. Effects of cohabitation for 11 days with an Ehrlich tumor-bearing companion on the host resistance to tumor growth of the Swiss male mice. Data are the mean ± SD of the ascitic fluid volume, the total number of tumor cells/animal and the number of tumor cells/mL of ascitic fluid. $n = 15$ animals per group, $^{*}p < 0.05$ (t-test).

and immunology. Because innate immunity operates at the tumor interface, it may critically influence tumor growth. A decreased host resistance to Ehrlich tumor growth was found in the Swiss CSP male mice studied in this work, i.e., the number of tumor cells/mL of ascitic fluid was higher in the male CSP mice, as was the total number of tumor cells/animal; similar findings have also been reported in female mice [1–3]. As reported previously for female mice [2,11], cohabitation with a tumor-bearing cagemate did not change adrenal weights or the serum levels of corticosterone in the CSP male mice. The increased levels of corticosterone currently observed in Swiss CSP and CHP male mice over the experimental days (ED5, ED7, ED9 and ED11) seems more related to the stress imposed by the successive blood sampling procedure than to the cohabitation with the sick cagemate per se. As a matter of fact, the avoidance response of healthy rats to odor was not correlated with changes in plasma corticosterone concentrations from odor-donor animals injected with LPS, suggesting that the induced inflammatory cascade is likely to mediate aversive properties in odors that function to signal illness state to conspecifics [33]. The lack of corticosterone changes observed in this work suggests that the immune changes imposed in the CSP male mice by the stress of cohabitation with the tumor-bearing cagemate do not rely solely on the activity of the HPA-axis. In fact, it was shown that different stressors does not always elicit the same biochemical and physiological changes [34,35]. Thus, a factor other than corticosterone might be accountable for the immune effects imposed by the housing conditions in the CSP male mice; plasma catecholamines seem to be good candidates.

Swiss CSP male mice presented increased levels of serum catecholamines, as already described in Swiss CSP female mice [2,11] and CSP Balb/C male mice [37]. The autonomic nervous system is known to contribute to the stress response through the release of catecholamines by sympathetic nerves and lymphoid tissues; catecholamines then mediate their effects on immune cells through G-protein coupled β-adrenergic receptors [31]. Catecholamines are known to change neutrophil activity [31,36]. Changes in catecholamines after stress were reported to modify the cytokine network [37], and cytokines are known to modulate the activity of immune cells [7,37]. A possible shift in the TH1/TH2 cytokine response toward a TH2 profile was recently suggested in female mice that lived with a sick partner [2,36]. Th1 cells are known to secrete a specific profile of cytokines, including IFN-γ and TNF-α, that favor the cellular immune response [38]; this cytokine profile change might explain the deceased host resistance to Ehrlich tumor growth found in the CSP male mice in this work. Indeed, neutrophils and macrophages are important components of natural immunity involved in inhibition of tumor growth and destruction of tumor cells [39], and a decrease in peritoneal macrophage and blood neutrophil activities was already reported in female CSP mice [1–4,11]. Thus, it seems feasible to suggest that the immune changes now being reported in male mice after cohabitation with a sick cagemate might, ultimately, depend on the changes induced by catecholamines on the cytokine network. If this is so, then the plasma catecholamine and immune changes induced in male and female mice by cohabitation with a sick cagemate are similar.

Overall, the results from this comprehensive study allow for at least two conclusions. First, as previously reported for female CSP mice, cohabitation with an Ehrlich tumor-bearing partner increased the levels of plasma catecholamines and decreased the immune response in CSP male mice, as assessed by (1) decreased neutrophil activity and (2) decreased host resistance to Ehrlich tumor growth. Second, and contrary to that reported by our group in female mice, cohabitation with a sick partner induced no changes in behavioral or hypothalamic NE activity in CSP male mice. Thus, it seems feasible to suggest that differences between the responses of male and female mice to cohabitation with Ehrlich tumor-bearing conspecifics might be a consequence of different behavioral strategies used by the animals against the threats posed by the diseased companion. This sexual dimorphism would be a consequence of a differential activation of the CNS structures involved in the psychological stress response imposed by the housing condition, such as the brain catecholamine pathways and the SNS. Sexual dimorphism has been reported in the vomeronasal system and in some limbic brain structures that receive vomeronasal input [29]. The current findings may have relevant implications for how disease contributes to brain, behavior and immune status of healthy conspecifics across the lifespan.

Table 3
Effects of cohabitation for 11 days with an Ehrlich tumor-bearing conspecific on the serum corticosterone levels of Swiss male mice.

Strain	Experimental Days	CHP	CSP
Swiss	ED5	$87.2 \pm 67.3^{(a)}$	$142.1 \pm 153.0^{(a)}$
	ED7	$210.9 \pm 135.1^{(b)}$	$263.8 \pm 168.4^{(b)}$
	ED9	$272.9 \pm 141.2^{(b)}$	$268.2 \pm 150.2^{(b)}$
	ED11	$425.4 \pm 219.1^{(c)}$	$411.9 \pm 207.1^{(c)}$

Data are the mean ± SD of 15 animas/group; different letters mean statistically significant differences at $p < 0.05$ (two-way ANOVA followed by Tukey-Kramer test).

Conflict of interest statement

No potential conflicts of interest are disclosed.

Acknowledgements

This research, which is part of the MS dissertation presented by Thalita R. M. Machado to the Experimental and Comparative Pathology Graduate Program of the Department of Pathology, School of Veterinary Medicine of the University of São Paulo, was supported by the FAPESP (Nos. 2009/51886-3; 2009/52487-5; 2011/23391-0 and 2011/50481-0) and by CNPq (Nos. 470776/2009-9 and 300764/2010-3), to which the authors express their gratitude.

References

[1] M.S. Morgulis, D. Stankevicius, L.C. Sá-Rocha, J. Palermo-Neto, Cohabitation with a sick cage mate: consequences on behavior and on Ehrlich tumor growth, Neuroimmunomodulation 11 (2004) 49–57.

[2] J. Palermo-Neto, G.J. Alves, Neuroimmune interactions and psychologycal stress induced by cohabitation with a sick partner: a review, Curr. Pharm. Des. 20 (2014) 4629–4641.

[3] G.J. Alves, L. Vismari, J.C. Florio, J. Palermo-Neto, Cohabitation with a sick cage mate: effects on noradrenaline turnover and neutrophil activity, Neurosci. Res. 56 (2006) 172–179.

[4] G.J. Alves, L. Vismari, R. Lazzarini, J.L. Merusse, J. Palermo-Neto, Odor cues from tumor-bearing mice induces neuroimmune changes, Behav. Brain Res. 214 (2010) 357–367.

[5] G.J. Alves, A. Ribeiro, J. Palermo-Neto, The neuroimmune changes induced by cohabitation with an Ehrlich tumor-bearing cage mate rely on olfactory information, Brain Behav. Immun. 26 (2012) 32–39.

[6] M.Y. Tomiyoshi, M. Sakai, R.B. Baleeiro, D. Stankevicius, C.O. Massoco, J. Palermo-Neto, J.A. Barbuto, Cohabitation with a b16f10 melanoma-bearer cage mate influences behavior and dendritic cell phenotype in mice, Brain Behav. Immun. 23 (2009) 558–567.

[7] M.L. Hanke, N.D. Powell, L.M. Stiner, M.T. Bailey, J.F. Sheridan, Beta adrenergic blockade decreases the immunomodulatory effects of social disruption stress, Brain Behav. Immun. 26 (2012) 1150–1159.

[8] T.S. Nakahara, L.M. Cardozo, X. Ibarra-Soria, A.D. Bard, V.M. Carvalho, G.Z. Trintinalia, D.W. Logan, F. Papes, Detection of pup odors by non-canonical adult vomeronasal neurons expressing an odorant receptor gene is influenced by sex and parenting status, BMC Biol. 14 (2016) 12.

[9] K. Yamazaki, E.A. Boyse, J. Bard, M. Curran, D. Kim, S.R. Ross, G.K. Beauchamp, Presence of mouse mammary tumor virus specifically alters the body odor of mice, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 5612–5615.

[10] M. Phillips, R.N. Cataneo, A.R. Cummin, A.J. Gagliardi, K. Gleeson, J. Greenberg, R.A. Maxfield, W.N. Rom, Detection of lung cancer with volatile markers in the breath, Chest 123 (2003) 2115–2123.

[11] G.J. Alves, J. Palermo-Neto, Odor cues released by Ehrlich tumor-bearing mice are aversive and induce psychological stress, Neuroimmunomodulation 22 (2015) 121–129.

[12] C. Willis, R. Poulin, Preference of female rats for the odours of non-parasitised males: the smell of good genes? Folia Parasitol. 47 (2000) 6–10.

[13] L.K. Takahashi, B.R. Nakashima, H. Hong, K. Watanabe, The smell of danger: a behavioral and neural analysis of predator odor-induced fear, Neurosci. Biobehav. Rev. 29 (2005) 1157–1167.

[14] H. Arakawa, K. Arakawa, D.C. Blanchard, R.J. Blanchard, A new test paradigm for social recognition evidenced by urinary scent marking behavior in c57bl/6j mice, Behav. Brain Res. 190 (2008) 97–104.

[15] R. Avitsur, R. Yirmiya, The immunobiology of sexual behavior: gender differences in the suppression of sexual activity during illness, Pharmacol. Biochem. Behav. 64 (1999) 787–796.

[16] C. Loehle, Social barriers to pathogen transmission in wild animal populations, Ecology 76 (1995) 326–335.

[17] M. Kavaliers, E. Choleris, D.W. Pfaff, Recognition and avoidance of the odors of parasitized conspecifics and predators: differential genomic correlates, Neurosci. Biobehav. Rev. 29 (2005) 1347–1359.

[18] R.J. Valentino, E. Van Bockstaele, Convergent regulation of *locus coeruleus* activity as an adaptative response to stress, Eur. J. Pharmacol. 583 (2008) 194–203.

[19] J.A. Burket, J. Mastropaolo, R.B. Rosse, S.I. Deutsch, Genetically inbred Bal/C mice are more sentitive to an effect of Flurazepan and more resistant to an effect of stress than a genetically outbred mouse strain, Epilepsy Behav. 16 (2016) 415–417.

[20] G.R. Dawson, S.P. Crawford, N. Collinson, S.D. Iversen, M.D. Tricklebank, Evidence that the anxiolytic-like effects of chlordiazepoxide on the elevated plus maze are confounded by increases in locomotor activity, Psychopharmacology 118 (1995) 316–323.

[21] S. Pellow, P. Chopin, S.E. File, M. Briley, Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat, J. Neurosci. Methods 14 (1985) 149–167.

[22] L.H. Sider, E.E. Hucke, J.C. Florio, L.F. Felicio, Influence of time of day on hypothalamic monoaminergic activity in early pregnancy: effect of a previous reproductive experience, Psychoneuroendocrinology 28 (2003) 195–206.

[23] E. Marana, A. Russo, S. Colicci, L. Polidori, F. Bevilacqua, D. Viviani, E. Di Stasio, Desflurane versus seroflurane; a comparison on stress response, Minerva Anestesiol. 79 (2013) 7–14.

[24] M. Hasui, Y. Hirabayashi, Y. Kobayash, Simultaneous measurement by flow cytometry of phagocytosis and hydrogen peroxidase production of neutrophils in whole blood, J. Immunol. Methods 117 (1989) 53–58.

[25] M.L.Z. Dagli, M. Soma, J.L. Guerra, P.H.N. Saldiva, Lymphatic dissemination in neoplasia: determination of nuclear volume and DNA content of primitive and regional lymph node Ehrlich tumor cells, Braz. J. Med. Biol. Res. 29 (1992) 267–271.

[26] K.D. Ehman, M.E. Scott, Female mice mate preferentially with non-parasitized males, Parasitology 125 (2002) 461–466.

[27] D.W. Cohn, D. Kinoshita, J. Palermo-Neto, Antidepressants prevent hierarchy destabilization induced by lipopolysaccharide administration in mice: a neurobiological approach to depression, Ann. N. Y. Acad. Sci. 1262 (2012) 67–73.

[28] K. Matsumura, M. Opiekun, H. Oka, A. Vachani, S.M. Albelda, K. Yamazaki, G.K. Beauchamp, Urinary volatile compounds as biomarkers for lung cancer: a proof of principle study using odor signatures in mouse models of lung cancer, PLoS One 5 (2010), e8819.

[29] S. Segovia, A. Guillamón, Sexual dimorphism in the vomeronasal pathway and sex differences in reproductive behaviors, Brain Res. Brain Res. Rev. 18 (1993) 51–74.

[30] C.V. Masini, H.E. Day, T. Gray, L.M. Crema, T.J. Nyhuis, J.A. Babb, S. Campeau, Evidence for a lack of phasic inhibitory properties of habituated stressors on HPA axis responses in rats, Physiol. Behav. 105 (2012) 568–575.

[31] I.J. Elenkov, R.L. Wilder, G.P. Chrousos, E.S. Vizi, The sympathetic nerve an integrative interface between two supersystems: the brain and the immune system, Pharmacol. Rev. 52 (2000) 595–638.

[32] A.P. Kohm, V.M. Sanders, Norepinephrine: a messenger from the brain to the immune system, Immunol. Today 21 (2000) 539–542.

[33] H. Arakawa, K. Arakawa, T. Deak, Oxytocin and vasopressin in the medial amygdala differentially modulate approach and avoidance behavior toward illness-related social odor, Neuroscience 171 (2010) 1141–1151.

[34] A. Armario, N. Daviu, C. Muñoz-Abellán, C. Rabasa, S. Fuentes, X. Belda, H. Gagliano, R. Nadal, What can we know from pituitary-adrenal hormones about the nature and consequences of exposure to emotional stressors? Cell. Mol. Neurobiol. 32 (2012) 749–758.

[35] S. Hayley, T. Borowski, Z. Merali, H. Anisman, Central monoamine activity in genetically distinct strains of mice following a psychogenic stressor: effects of predator exposure, Brain Res. 892 (2001) 293–300.

[36] E.K. Hamasato, A.P. de Lima, A.P. de Oliveira, A.L. dos Santos Franco, W.T. de Lima, J. Palermo-Neto, Cohabitation with a sick partner increases allergic lung inflammatory response in mice, Brain Behav. Immun. 42 (2014) 109–117.

[37] K.C. Torres, L.R. Antonelli, A.L. Souza, M.M. Teixeira, W.O. Dutra, K.J. Gollob, Norepinephrine, dopamine and dexamethasone modulate discrete leukocyte subpopulations and cytokine profiles from human PBMC, J. Neuroimmunol. 166 (2005) 144–157.

[38] T.H. Nguyen, T.B. Casale, Immune modulation for treatment of allergic disease, Immunol. Rev. 242 (2011) 258–271.

[39] L.P. Ruco, N.S. Meltzer, Macrophage activation for tumor cytotoxicity: increased lymphokine responsiveness of peritoneal macrophages during inflammation, J. Immunol. 120 (1978) 1051–1062.