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Beta-Adrenergic Blockade Decreases the Neuroimmune Changes in Mice Induced by Cohabitation with an Ehrlich Tumor-Bearing Cage Mate

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Stress · *d,l*-Propranolol · ICI-118.581 · Catecholamines · Serotonin · Cohabitation with a sick conspecific · Neutrophils · Behavior

Abstract
Objectives: Cohabitation with Ehrlich tumor-bearing (ETB) mice induced behavioral, neurochemical, hormonal, and immune effects in the conspecifics as a consequence of stress-induced activation of the sympathetic nervous system (SNS) with catecholamine release. In the current study, the non-specific β-AR blocker *d,l*-propranolol and the specific β_2-AR blocker ICI-118.551 were employed as pharmacological tools to assess the extent to which catecholamines participated in the effects induced by cohabitation with ETB mice. ***Methods:*** Two experiments were performed, 1 with *d,l*-propranolol treatment and the other with ICI-118.551. One mouse in the experimental group was called the "companion of the sick partner" (CSP) since it was forced to live in the same cage with 2 (experiment 1) or 1 (experiment 2) cage mate that had been i.p. injected with 5×10^6 Ehrlich tumor cells. ***Results:*** The *d,l*-propranolol treatment, but not the ICI-118.551 treatment, attenuated the effects of cohabitation with 2 ETB mice on both open-field behavior and the hypothalamic levels and turnover rate of norepinephrine. The 2 β-AR blockers were unable to change the serum corticosterone levels and adrenal weights of the CSP mice; however, these drugs abrogated the effects of cohabitation on neutrophil oxidative burst and phagocytosis. Finally, an increase in the 5-HT turnover rate was observed in the olfactory bulb of CSP mice compared to their respective controls, an effect that was not modified by β-AR blockade. ***Conclusion:*** These results confirm and strengthen our hypothesis that the SNS is involved in the effects induced by cohabitation with ETB mice and point towards β_2-AR participation in the immune effects analyzed.

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Introduction

Endogenous and exogenous stressors are known to modify innate and acquired immune system responses [1–3], the Th1 × Th2 cytokine profile [4–6] and animals' susceptibility and resistance to infections, tumors, and inflammatory diseases [2, 7]. Both the central nervous system (CNS) and immune system actively participate in responses to stressors by modulating behavior and immune system activity according to the type, duration, and intensity of the specific stressors [7]. When homeostasis is disturbed by stressors, the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis are activated. Under physiological conditions, catecholamines and glucocorticoids are released by adrenal glands in response to stressors [8]. The SNS also contributes to the stress response through the release of catecholamines by sympathetic nerves in lymphoid tissue. Hormones and neurotransmitters induced by stress bind specific receptors on immune cells and subsequently influence their activation and function [9]. Adrenaline and noradrenaline (NA) are known to mediate their effects on immune cells through G-protein-coupled β-AR (β-adrenergic receptors).

Cohabitation with an Ehrlich tumor-bearing (ETB) mouse was shown to produce relevant behavioral, neurochemical, endocrine, and immune effects [10]. As shown in our previous study, odor cues released by the ETB mice are aversive, suggesting that they are stressors for the companion animals [11]. Physical and psychological stressors are known to induce endocrine [12], neurochemical [13], and immune [8] changes similar to the changes currently reported in mice after cohabitation with a tumor-bearing cage mate. The lack of changes in the serum corticosterone levels in the companions of ETB mice [10, 11, 14] led us to suggest that the final neural link between the neuroimmune impairments observed in companions of the ETB mice involves catecholamine release and/or CNS catecholaminergic activation as a consequence of the psychological stress imposed by the forced housing condition. Changes in catecholamine levels after stress were reported to modify the cytokine network [15] and cytokines are known to modulate the activity of immune cells [16] and the animal's resistance to diseases [17]. For instance, companions of ETB mice presented decreased neutrophil [10] and macrophage [18] activities, and as components of innate immunity, these cells provide the first line of defense against invading bacteria via phagocytosis, superoxide generation, and the formation of oxygen radicals that play important microbicidal roles [6]. Catecholamines were reported to decrease the rate of superoxide formation in neutrophils [19], and studies have indicated an inhibitory role for β-AR stimulation in neutrophil phagocytosis and degranulation [20, 21].

Although the overall neuroimmune outcomes of cohabitation in companions of ETB mice have been extensively reported [10, 11, 14, 22, 23], we must determine whether catecholamine release and SNS activation are indeed related to the neuroimmune effects on the development of behavioral and neutrophil alterations reported to be caused by the housing condition. Therefore, the purpose of the present study was to determine the extent to which catecholamines participate in these effects by employing β-AR blocking agents as pharmacological tools. Treatment with *d,l*-propranolol prior to tail shock exposure decreased the immunomodulatory effects induced by the stressor [24] and the effects reported after social disruption-induced stress in mice [25]. Furthermore, we also aimed to analyze the levels and turnover rate of serotonin (5-hydroxytryptamine, 5-HT) in the olfactory bulbs of companions of ETB mice. Olfactory cues released by ETB mice are considered pivotal for the neuroimmune changes induced by cohabitation with ETB mice [11]. Broad spectrum 5-HT receptor agonists attenuate odor-evoked olfactory neuron activity in the olfactory bulb [26, 27].

Materials and Methods

Animals

Naïve Swiss female mice (aged 90–110 days) were used. Female mice were chosen based on the results from our previous studies [11, 28] and because they are less aggressive than males when housed in groups. The animals were housed under a controlled temperature (22–26°C) with artificial light (12-h light/12-h dark, lights on at 7:00 a.m.) and free access to rodent chow and water. Mice were transferred to a different (temperature-consistent) room and were acclimated for 10 days before the experiments. Animal maintenance and use were performed according to the recommendations of the Brazilian National Council on the Control of Animal Experimentation (CONCEA). All studies were performed after the study was approved by the Committee on the Care and Use of Animal Resources of the School of Veterinary Medicine, University of São Paulo, São Paulo, Brazil.

Drugs

d,l-Propranolol (Sigma®, St. Louis, MO, USA), a nonselective antagonist of β-AR, and ICI-118.551 (Tocris®, Ellisville, MO, USA), a selective antagonist of β_2-AR, were used at the doses and time schedules reported in the literature for their capacity to block β-AR [29–32].

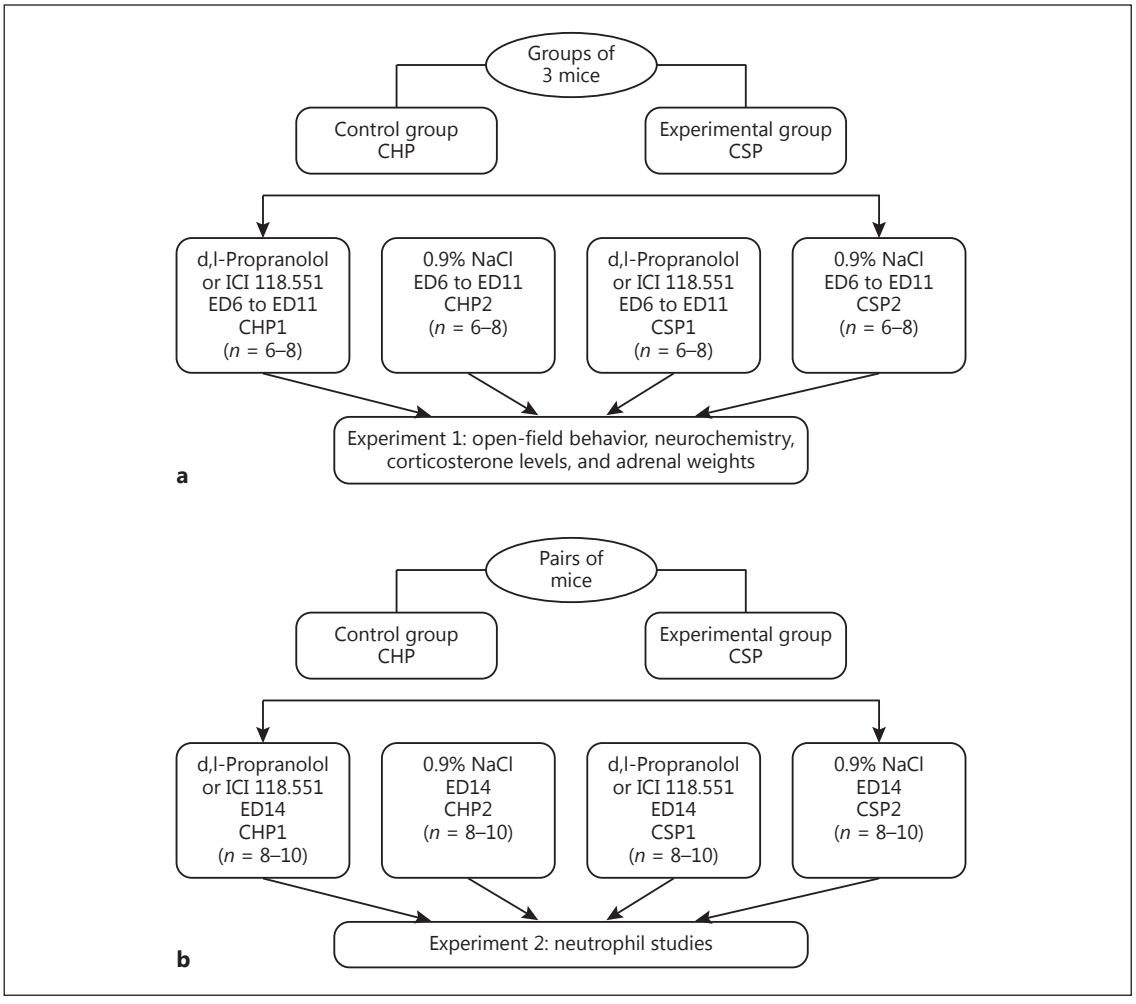

Fig. 1. Schematic diagram of the experimental design. **a** Mice were housed in groups of 3 according to weight, and subsequently separated into 1 control and 1 experimental group. Two animals from each experimental group (sick partners) were i.p. inoculated with 5×10^6 Ehrlich tumor cells; the other animal, the subject of this study, was undisturbed (CSP). Two mice from each control pair (healthy partners) were i.p. injected with 0.9% NaCl (0.1 mL/10 g); the other animal was undisturbed (CHP). Injections were administered on ED1. On ED6, the CHP and CSP mice were randomly separated into 2 groups: CSP_1 and CSP_2, and CHP_1 and CHP_2, respectively. From ED6 to ED11, the CSP_1 and CHP_1 mice were treated once daily (at 5:00 p.m.) with 20 mg/kg/day of *d,l*-propranolol (1st replicate) or 15 mg/kg/day of ICI-118.551 (2nd replicate). In both replicates, the CSP_1 and CHP_1 mice received 0.9% NaCl (0.1 mL/10 g/day) at the same time points for the same period of time (ED6 to ED11). The mice were removed from their cages on the morning of ED12 and analyzed within an open-field arena; blood was immediately collected to determine the corticosterone levels, and the animals were then euthanized for neurochemical studies and adrenal weight measurements. **b** The same experimental design was employed in the second experiment, except that the mice were housed in pairs according to weight and then distributed into 2 groups. As described above, the injections were administered on ED1. On ED14, the CSP and CHP mice were separated into 2 groups: CSP_1 and CSP_2, and CHP_1 and CHP_2, respectively. The CSP_2 mice were treated with a single 20-mg/kg dose of *d,l*-propranolol (1st replicate) or with a 15.0-mg/kg dose of ICI-118,551 (2nd replicate). The mice in the CSP_1 and CHP_1 groups received 0.1 mL/10 g of 0.9% NaCl at the same time points. Blood was drawn from the mice in these 4 groups 90 and 180 min after the administration of the *d,l*-propranolol or ICI-118.551 treatments, respectively, for the neutrophil studies.

Group Formation, Pharmacological Treatments, and Experimental Design

As presented in Figure 1, 2 independent experiments with 2 equal replicates each were performed: 1 for *d,l*-propranolol and the other for ICI-118.551 treatments. Respectively, 192, 192, 80, and 80 female mice were used. The experiments were conducted in accordance with good laboratory practice protocols and quality assurance methods.

In experiment 1 (Fig. 1a), the mice were weighed, housed in groups of 3 per home cage according to weight, and then subse-

quently allocated into 2 groups: 1 control and 1 experimental group. Seven days after grouping, 2 animals from each experimental group (the sick partners; SP) were intraperitoneally (i.p.) inoculated with 5×10^6 Ehrlich tumor cells. The other animal, the subject of this study, was undisturbed and was referred to as the CSP (companion of the SP). Two mice in each control pair (healthy partners; HP) were i.p. injected with 0.9% NaCl (0.1 mL/10 g), and the other animal was undisturbed (companion of the HP; CHP). In this experiment (experiment 1), the CSP mice were cohabitated with 2 ETB mice to increase the magnitude of the behavioral and neurochemical changes under analysis [33]. The day on which the injections were administered was experimental day 1 (ED1). On ED6, the CHP and CSP mice were randomly separated into 2 groups: CHP_1 and CHP_2 and CSP_1 and CSP_2, respectively. From ED6 to ED11, the CSP_1 and CHP_1 mice were treated once daily (5:00 p.m.) with 20 mg/kg/day of *d,l*-propranolol (1st replicate) or 15 mg/kg of ICI-188.551 (2nd replicate). In both replicates, the CSP_2 and CHP_2 mice received 0.9% NaCl (0.1 mL/10 g) at the same time points and for the same period of time (ED6 to ED11). The mice were removed from their cages on the morning of ED12 and analyzed in an open-field arena; blood was then immediately drawn, the animals were euthanized, and their brains and adrenal glands were removed. The blood was used to determine serum corticosterone levels. The brains were dissected to analyze hypothalamic NA and olfactory bulb 5-HT levels and turnover rates. Finally, the adrenals were removed to determine their relative weights.

The same experimental design was employed in the second experiment, with the exception that mice were housed in pairs according to weight and then subsequently separated into 2 groups: 1 control and 1 experimental group (Fig. 1b). One mouse from each control or experimental pair was i.p. injected with 0.1 mL/10 g of 0.9% NaCl (HP) or with 5×10^6 Ehrlich tumor cells (SP), respectively, as described above for experiment 1; the other mouse from the pair (CSP and CHP) was undisturbed. As described above, the injections were administered on ED1. Immediately after these treatments, CSP and CHP mice were separated into 2 groups: CSP_1 and CSP_2, and CHP_1 and CHP_2, respectively. On the morning of ED14, the CSP_1 and CHP_1 mice were treated with a single 20 mg/kg dose of *d,l*-propranolol (1st replicate) or a 15.0 mg/kg dose of ICI-118.551 (2nd replicate). *d-l*-Propranolol and ICI-118.551 treatments were administered for 90 and 180 min prior to blood collection, respectively. The mice in the CSP_1 and CHP_1 groups received 0.1 mL/10 g of 0.9% NaCl at the same time points. Blood collected from CHP_1, CHP_2, CSP_1, and CSP_2 mice was used to determine the serum corticosterone levels and neutrophil oxidative burst and phagocytosis.

Sick animals were analyzed in their cages for signs and symptoms of Ehrlich tumors, as proposed elsewhere [18]. 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.

The order of the mice undergoing the behavioral and immune analyses was alternated among CHP_1, CHP_2, CSP_1, and CSP_2 mice and was performed between 8:30 and 10:30 p.m. to minimize the influence of possible circadian changes. The open-field apparatus was wiped with ethanol (a 1% solution in water) before each mouse was tested to eliminate possible biasing effects due to odor cues left by other animals.

Behavioral Study

An open-field apparatus was used to analyze the effects of cohabitation with ETB mice treated or with or without *d,l*-propranolol or ICI118,551 on locomotor activity and anxiety levels. Within the context of this study, an "anxiety level" was operationally inferred using a previously described method [34], i.e., as the response to a situation in which behavior is influenced by 2 motivational forces (e.g., a natural curiosity to explore unexplored areas vs. an aversion to open areas). Mice were placed within the open-field arena and allowed to explore for 5 min. The following behavioral parameters were analyzed: total distance traveled within the apparatus (cm), average speed (cm/s) within the apparatus, and total time spent in the central open-field zone (s). 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.

Corticosterone Study

Serum corticosterone levels in CSP_1, CSP_2, CHP_1, and CHP_2 mice were assayed on ED12 (experiment 1). Blood samples (100–200 μL) were collected from each animal immediately after the behavioral observations to quantify the serum hormone levels. Serum corticosterone levels were measured using ELISA kits (DetectX®, Arbor Assays, Ann Abor, MI, USA) at an ambient temperature, according to the manufacturer's instructions. The data are expressed as nmol/mL.

Neurochemical Study

Hypothalamic NA and olfactory bulb 5-TH levels and respective turnover rates were evaluated in CSP_1, CSP_2, CHP_1, and CHP_2 mice on ED12 (experiment 1). Cohabitation with an ETB cage mate promotes hypothalamic noradrenergic activity; serotoninergic projections from the olfactory bulb regulate odor-evoked neuronal activity in this region [26, 27]. Immediately after blood sampling, the mice were decapitated and the brains were removed, washed in a cold 0.9% NaCl solution and subsequently dissected on ice to remove the hypothalamus and olfactory bulb. These tissues were stored at –80 °C until analysis. The NA, 3-methoxy-4-hydroxy-phenylethylene glycol (MHPG), 5-HT, and 5-hydroxyindole acetic acid (5-HIAA) levels in CSP_1, CSP_2, CHP_1, and CHP_2 mice were individually evaluated as described by Sider et al. [35]. Briefly, brain tissue samples were homogenized in perchloric acid by sonification, and the NA, MHPG, 5-HT, and 5-HIAA levels were measured using high-performance liquid-performance chromatography coupled with an electrochemical detector (HPLC-ED). The apparatus used for this experiment 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 10 ng for all of the substances analyzed, the recovery values were greater than 80%, the coefficients of variation were less than 15%, and curve linearity values were greater than 0.95. The turnover rates of NA in the hypothalamus and 5-HT in the olfactory bulb were expressed as MHPG/NA and 5-HIAA/5-HT ratios, respectively.

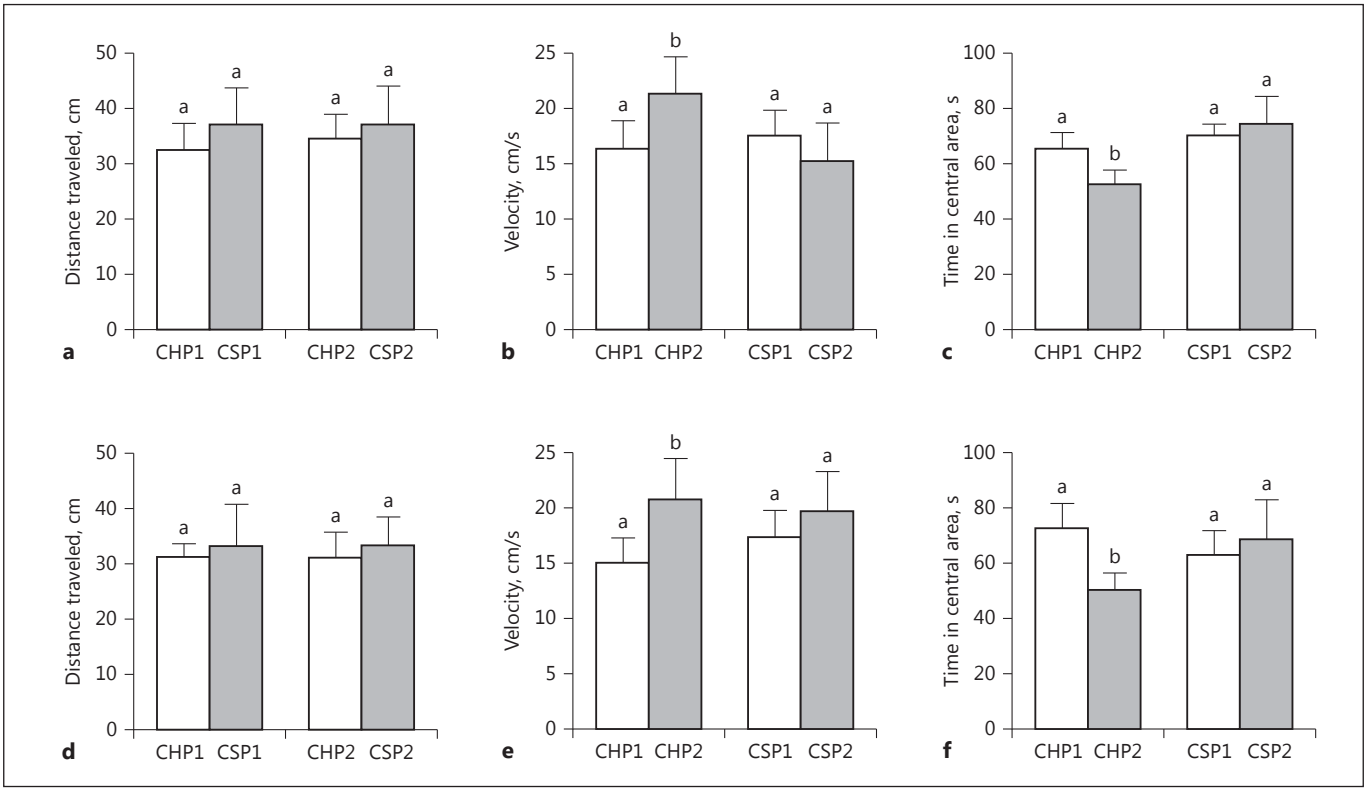

Fig. 2. Open-field behaviors of mice that were housed with 2 sick companions (CSP_1 and CSP_2) or with 2 healthy partners (CHP_1 and CHP_2) for 11 days and treated once daily with (CHP_2 and CSP_2) or without (CHP_1 and CSP_1) *d*,*l*-propranolol (20 mg/kg/day) or ICI-118.551 (15 mg/kg/day) from ED6 to ED11: data obtained from the *d*,*l*-propranolol-treated group (**a–c**) and from the ICI-118.551-treated group (**d–f**). **a**, **d** Total distance traveled. **b**, **e** Velocity within the arena. **c**, **f** Time spent within the open-field central zone. Data are presented as the means ± SD of 8 mice per group, but minor variations in *n* might have occurred for technical reasons. Different letters above the columns ($^{a, b}$) indicate significant differences at $p \leq 0.05$ (2-way ANOVA followed by the Tukey-Kramer test).

Adrenal Weight Study

Immediately after the brains were removed (ED12), the abdominal cavities of CSP_1, CSP_2, CHP_1, and CHP_2 mice were opened to remove the left and right adrenal glands (experiment 1). Individual adrenal weights were then determined using a precision scale. The data are presented as relative adrenal weights, i.e., adrenal weight/body weight.

Flow Cytometry Study

In experiment 2, neutrophil oxidative burst and phagocytosis were measured in CSP_1, CSP_2, CHP_1, and CHP_2 mice using a previously described method [36]. Briefly, blood was collected from the mice on ED14 and placed in lithium-heparin Vacutainer tubes (Becton Dickinson and Co., Franklin Lakes, NJ, USA); the animals were then immediately euthanized. Blood samples (100 µL) were used to assess the phagocytic activity and the oxidative burst of neutrophils. The substances used to trigger oxidative burst were phorbol myristate acetate (PMA; 100 ng) and *Staphylococcus aureus* (2.4×10^6 bacteria/mL). Briefly, 100 µL of whole blood (2×10^5 cells/100 µL) were mixed with 200 µL of 2′,7′-dichlorofluorescein diacetate (DCFH-DA, 0.3 nM) in PBS and 100 µL of either propidium iodide (PI)-labeled *S. aureus* or PMA in separate polypropylene tubes. The samples were incubated at 37°C for 20 min with agitation. The reactions were stopped by adding 2 mL of a cold EDTA solution (3 mM) to terminate phagocytosis. After centrifugation (250 *g* for 10 min), the erythrocytes were lysed in all samples using sterile 0.2% NaCl (2 mL/tube) for 20 s. Immediately afterwards, a 1.6% NaCl sterile 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, CA, USA) interfaced with a Macintosh G4 computer was used. Data were collected from 10,000 events 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; cells were mechanically sorted and then evaluated with a light microscope after staining with Giemsa stain. Fluorescence data were collected in log scale. Green fluorescence from DCFH-DA (Molecular Probes, Eugene, OR, USA) was measured at 530 ± 30 nm (FL1 detector) and red fluorescence from PI-labeled *S. au-*

reus (Sigma, St. Louis, MO, USA) was measured at 585 ± 42 nm (FL2). PI and DCFH fluorescence were analyzed after compensation to correct for possible cross-over between their signals. Direct measurements of the mean fluorescence of the green and red channels were recorded as oxidative burst and phagocytosis, respectively. Phagocytosis and oxidative burst were quantitatively estimated using a previously reported method [37] by calculating the mean PI and DCFH fluorescence/cell, respectively. The percentage of phagocytosis (percent of neutrophils with ingested bacteria) was expressed as the number of neutrophils with red fluorescence divided by the total number of cells (×100).

Statistics

The Bartlett test was performed to evaluate whether the obtained data were parametric or nonparametric. In the subsequent analysis, differences were assessed using 2-way analyses of variance (groups × treatments) followed by the Tukey-Kramer test for multiple comparisons. GraphPad® Prism 6 software (Windows, San Diego, CA, USA) was used for all analyses. For all comparisons performed, $p \leq 0.05$ was considered significant. The data are presented as means ± SD.

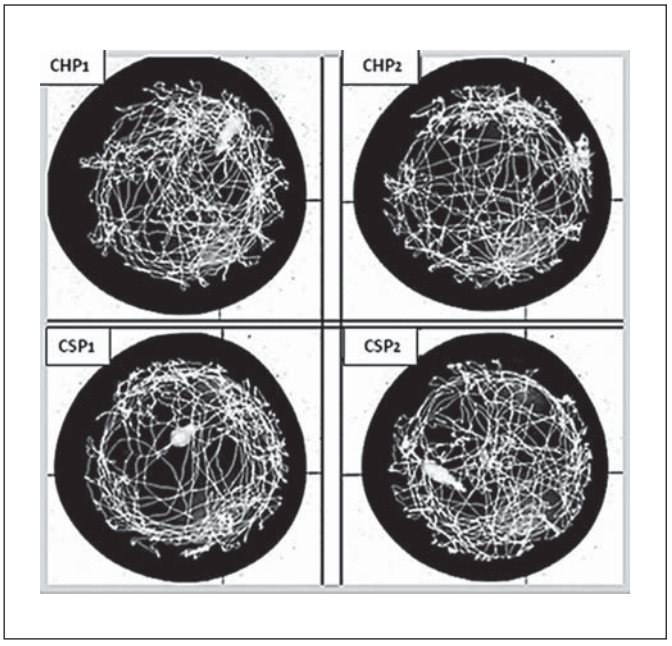

Fig. 3. Representative motion plots of mice within the open field. Lines within the arena represent the trails left by the animals after 5 min of observation. CHP_1: animal injected with 0.1 mL/10 g of 0.9% NaCl; CSP_1: mouse housed with 2 ETB mice; CHP_2: mouse injected with 0.1 mL/10 g of 0.9% NaCl that received 20.0 mg/kg of *d,l*-propranolol; CSP_2: mouse housed with 2 ETB mice that received 20.0 mg/kg of *d,l*-propranolol. The animals were treated with *d,l*-propranolol from ED6 to ED11.

Results

The injection of Ehrlich tumor cells induced behavioral changes in the sick animals. Behavioral alterations in all ETB mice were progressive and were characterized by the presence of lethargy, a reduced interest in their surroundings, and a decreased ability to respond to the companion mice. Experiments were performed on the morning of ED12 (experiment 1) and on ED14 (experiment 2), i.e., 1 and 3 days after the signs and symptoms indicative of a score of 4 were observed in the majority of the Ehrlich tumor cell-injected mice, respectively. A progressive increase in the activity of the CSP_1 mice compared to the CHP_1 mice within their home cages was observed from ED4 to ED11. Changes were not observed in cycling CHP_1, CHP_2, CSP_1, and CSP_2 females.

Experiment 1

Effects of *d,l*-Propranolol or ICI-118.551 Treatments on the Open-Field Behavior of Mice Housed with 2 ETB or Control Cage Mates

Figure 2a–f depicts the effects of daily *d,l*-propranolol (20 mg/kg/day) or ICI 118.551 (15 mg/kg/day) injections from ED6 to ED11 on the open-field behavior of the mice. As depicted in Figure 2a and d, statistically significant differences were not observed in the distance traveled within the open field on ED12 among the mice of the CHP_1, CHP_2, CSP_1, and CSP_2 groups; thus, both the *d,l*-propranolol ($F_{1, 3, 40} = 13.68$; $p > 0.05$) and ICI-118.551 ($F_{1, 3, 40} = 14.06$; $p > 0.05$) treatments were unable to modify this behavioral parameter. However, compared to the mice in the CHP_1 group, the *d,l*-propranolol treatment abrogated the effects of the housing condition on the velocity of the CSP_2 animals within the apparatus ($F_{1, 3, 40} = 9.29$; $p < 0.05$; Fig. 2b) and the time the CSP_2 mice spent in the central area of the open field ($F_{1, 3, 40} = 12.57$; $p < 0.05$; Fig. 2c). Figure 3 shows the locomotor activity of 1 mouse of each group in the open-field arena; cohabitation with ETB mice clearly decreased the locomotor activity of mice within the central area of the apparatus (left panel, bottom), an effect that was abrogated by the *d,l*-propranolol treatment (right panel, bottom). Interestingly, compared to the mice in the CSP_1 group, ICI-118.551 administration for the same period of time did not modify the velocity of the CSP_2 animals within the open-field arena ($F_{1, 3, 40} = 6.00$; $p < 0.05$; Fig. 2e) or the time they spent in the central zone of the apparatus ($F_{1, 3, 40} = 6.18$; $p < 0.05$; Fig. 2f). According to further analyses, the *d,l*-propranolol and ICI-118.551 treatments were unable to change the open field behaviors of mice that lived with HP ($CHP_1 \times CHP_2$; Fig. 2d–f).

Table 1. Effects of *d,l*-propranolol on NA and MHPG levels (ng/g) and the NA turnover rate (MHPG/NA) in the hypothalamus of mice that were (CSP_1 and CSP_2) or were not (CHP_1 and CHP_2) housed with 2 ETB cage mates for 11 days

	CHP_1	CHP_2	CSP_1	CSP_2
NA	1,273.8±20.6	1,185.4±102.3	1,035.3±16.2*	1,246.9±42.6
MHPG	728.7±22.6	636.9±35.9	1,211.9±86.8*	819.7±31.2
MHPG/NA	0.57±0.05	0.54±0.07	1.17±0.09*	0.65±008

Data are from animals that were treated once daily with 20 mg/kg/day of *d,l*-propranolol (CHP_2 and CSP_2) or with 0.1 mL/kg/day of 0.9% NaCl (CHP_1 and CSP_1) from ED6 to ED11. Values are the means ± SD of at least 6 mice/group. * $p < 0.05$ vs. CHP_1 (2-way ANOVA and Tukey-Kramer test).

Table 2. Effects of *d,l*-propranolol on 5-HT and 5-HIAA levels (ng/g) and the 5-HT turnover rate (5-HIAA/5-HT) in the olfactory bulb of mice that were (CSP_1 and CSP_2) or were not (CHP_1 and CHP_2) housed with 2 ETB cage mates for 11 days

	CHP_1	CHP_2	CSP_1	CSP_2
5-HT	2,810.5±154.6	2,713.8±310.5	2,499.5± 233.2	2,587.9±475.1
5-HIAA	1,336.0±215.0	1,391.4±197.0	1,564.0±198.6	1,557.0±168.5
5-HIAA/5-HT	0.43±0.02	0.39±0.02	0.75±0.02*	0.74±0.04*

For further information see legend to Table 1.

Effects of the *d,l*-Propranolol and ICI-118.551 Treatments on Hypothalamic NA Activity in Mice Housed with 2 ETB or Control Cage Mates

Table 1 shows the effects of *d,l*-propranolol on the hypothalamic NA levels and turnover rate of mice that lived with 2 ETB or control companions. Two-way ANOVA showed differences in the NA levels ($F_{1, 3, 40} = 21.79$; $p < 0.05$), MHPG levels ($F_{1, 3, 40} = 33.26$, $p < 0.05$; $p < 0.05$), and MHPG/NA ratio ($F_{1, 3, 40} = 23.07$; $p < 0.05$) among the groups and treatments. Cohabitation with 2 ETB mice decreased NA levels ($p < 0.05$) and increased MHPG levels ($p < 0.05$) and NA turnover rate ($p < 0.05$) in CSP_1 mice compared to CHP_1 mice. Furthermore, the *d,l*-propranolol treatment abrogated the effects induced by cohabitation; indeed, the NA and MHPG levels and NA turnover rates in CSP_2 mice were not different from the values measured in the CHP_1 and CHP_2 animals ($p > 0.05$). Significant differences in the NA data were not observed between the mice in the CHP_1 and CHP_2 groups. The ICI-181.551 treatment was unable to change hypothalamic NA activity of mice that were housed with (CSP_2) or without (CSP_1) 2 ETB mice compared to the CHP_2 and CHP_1 mice, respectively (data not shown).

Effects of the *d,l*-Propranolol and ICI-118.551 Treatments on Olfactory Bulb 5-HT Levels in Mice Housed with 2 ETB or Control Cage Mates

The olfactory bulb NA and 5-HT levels and turnover rates are presented in Table 2. Although 5-HT levels tended to be lower in mice in the CSP_1 group compared to the CHP_1 group, no differences in the 5-TH ($F_{1, 3, 40} = 4.65$; $p > 0.05$) and 5-HIAA ($F_{1, 3, 40} = 5.63$; $p > 0.05$) levels were observed among the CHP_1, CHP_2, CSP_1, and CSP_2 groups. However, the 5-HT turnover rate was higher in CSP_1 mice than in CHP_1 and CHP_2 animals ($F_{1, 3, 40} = 22.89$; $p < 0.05$), an effect that was not abrogated by the *d,l*-propranolol treatment (20 mg/kg/day from ED6 to ED11). The ICI-188.551 treatment (15 mg/kg/day from ED6 to ED11) did not induce changes in the olfactory bulb 5-HT, 5-HIAA levels, and 5-HIAA/5-HT ratios in mice in the CHP_2 and CSP_2 groups (data not shown).

Effects of the *d,l*-Propranolol Treatment on Serum Corticosterone Levels and Adrenal Weights of Mice Housed with 2 ETB or Control Cage Mates

No differences in the serum corticosterone levels ($CHP_1 = 15.03 \pm 3.35$ ng/mL; $CHP_2 = 14.24 \pm 5.35$ ng/mL; $CSP_1 = 15.35 \pm 7.10$ ng/mL, and $CSP_2 = 15.26 \pm 8.28$ ng/

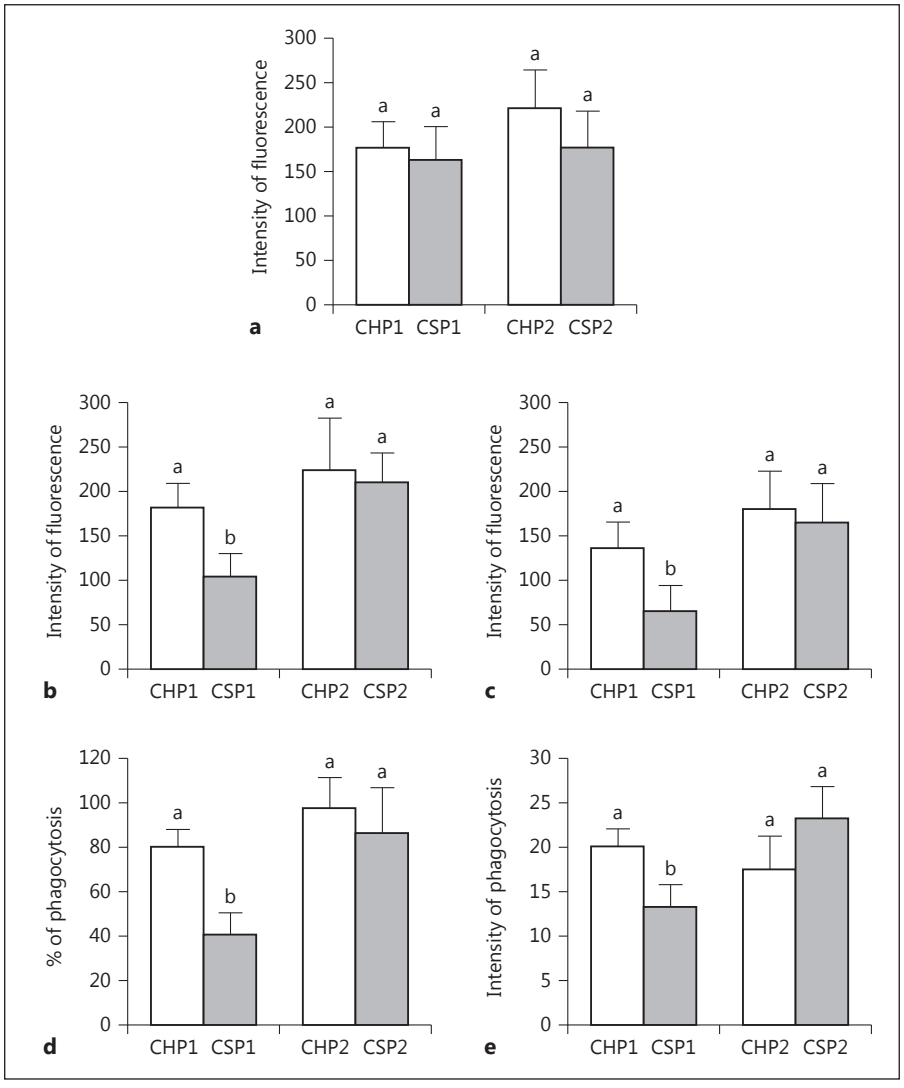

Fig. 4. Neutrophil activity in mice that were housed with 1 sick companion (CSP_1 and CSP_2) or with 1 healthy partner (CHP_1 and CHP_2) and treated with (CHP_2 and CSP_2) or without (CHP_1 and CSP_1) 20.0 mg/kg of *d,l*-propranolol 90 min prior to evaluation. The animals were treated with *d,l*-propranolol on ED14, 90 min prior to blood collection. The graphs show the basal neutrophil oxidative burst (**a**), the bursts observed after PMA (**b**) or *S. aureus* (**c**) inductions, the percentage of neutrophils performing phagocytosis (**d**), and the intensity of phagocytosis (**e**). Data are presented as the means ± SD of 10 mice per group, but minor variations in *n* might have occurred for technical reasons. Different letters above the columns ($^{a, b}$) indicate significant differences at $p \leq 0.05$ (2-way ANOVA followed by the Tukey-Kramer test).

mL) and relative adrenal weights ($CHP_1 = 0.41 \pm 0.09$ mg; $CHP_2 = 0.42 \pm 0.05$ mg; $CSP_1 = 0.38 \pm 0.08$ mg, and $CSP_2 = 0.42 \pm 0.03$ mg) were observed in mice that were housed with or without 2 ETB mice or treated with or without *d,l*-propranolol (20 mg/kg/day from ED6 to ED11). Further analyses did not reveal differences in the serum corticosterone levels and adrenal weights among control and experimental mice treated with or without 15.0 mg/kg of ICI-118.551 (data not shown).

Experiment 2

Effects of a Single *d,l*-Propranolol Treatment on Neutrophil Activity in Mice Housed with 1 ETB or Control Cage Mate

Figure 4 shows the neutrophil activity in mice that were housed with 1 ETB companion (CSP_1 and CSP_2) or with 1 HP (CHP_1 and CHP_2) for 14 days and treated with or without a single 20 mg/kg dose of *d,l*-propranolol administered 90 min prior to the evaluation. Statistically significant differences in the basal neutrophil oxidative burst were not observed between groups ($F_{1, 3, 23} = 6.00$; $p > 0.05$; Fig. 4a). However, significant differences in the PMA- ($F_{1, 3, 20} = 17.6$; $p \leq 0.05$; Fig. 4b) and *S. aureus*-induced ($F_{1, 3, 23} = 28.00$; $p \leq 0.05$; Fig. 4c) neutrophil oxidative burst, the number of bacteria phagocytosed ($F_{1, 3, 20} = 17.00$; $p \leq 0.05$; Fig. 4d) and the intensity of phagocytosis ($F_{1, 3, 18} = 19.00$; $p \leq 0.05$; Fig. 4e) were observed between the groups and treatments; a significant group × treatment interaction was also observed. In all cases, values for the CSP_1 mice were less than the values for the CHP_1 and CHP_2 groups. Furthermore, as shown in Figure 4b–e, administration of the *d,l*-propranolol

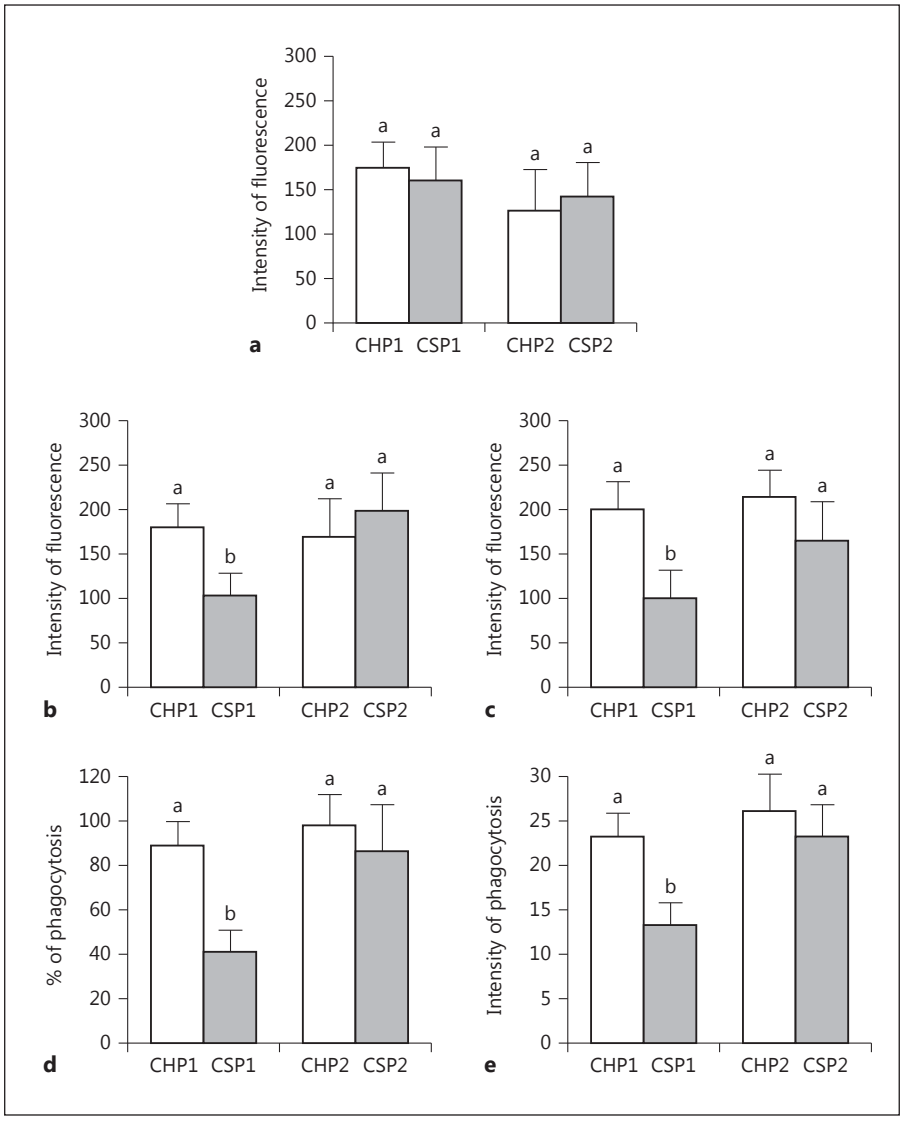

Fig. 5. Neutrophil activity in mice that were housed with 1 sick companion (CSP_1 and CSP_2) or with 1 healthy partner (CHP_1 and CHP_2) and treated with (CHP_2 and CSP_2) or without (CHP_1 and CSP_1) 15.0 mg/kg of ICI-118.551. ICI-118.551 was administered on ED14 at 180 min prior to blood collection. **a–e** The experimental details are the same as described in the legend to Figure 4. Data are presented as the means ± SD of 10 mice per group, but minor variations in *n* might have occurred for technical reasons. Different letters above the columns ($^{a, b}$) indicate significant differences at $p \leq 0.05$ (2-way ANOVA followed by the Tukey-Kramer test).

treatment prior to the neutrophil studies attenuated the effects of cohabitation on PMA- and *S. aureus*-induced neutrophil oxidative burst and the intensity and percentage of *S. aureus* phagocytosis.

Effects of a Single ICI-118.551 Treatment on Neutrophil Activity in Mice Housed with 1 ETB or Control Cage Mate

Figure 5 shows the neutrophil activity of mice that were housed with a sick companion (CSP_1 and CSP_2) or with an HP (CHP_1 and CHP_2) for 14 days and treated with or without a single 15 mg/kg dose of ICI-118.551 180 min before the evaluation. No differences ($F_{1, 3, 16} = 6.90$; $p > 0.05$; Fig. 5a) in the basal (DCFH) neutrophil oxidative burst data were observed among the 4 groups. However, significant differences in the PMA- ($F_{1, 3, 16} = 22.0$; $p \leq 0.05$; Fig. 5b) and *S. aureus*-induced ($F_{1, 3, 20} = 19.40$; $p \leq 0.05$; Fig. 5c) neutrophil oxidative burst and the percent ($F_{1, 3, 21} = 35.00$; $p \leq 0.05$; Fig. 5d) and intensity of neutrophil phagocytosis ($F_{1, 3, 17} = 18.30$; $p \leq 0.05$; Fig. 5e) were observed between the groups and treatments; a significant group × treatment interaction was also observed. Once again, cohabitation with ETB mice decreased ($p < 0.05$) PMA- and *S. aureus*-induced neutrophil oxidative burst and the intensity and percentage of neutrophil phagocytosis ($CSP_1 \times CHP_1$). Furthermore, the ICI-

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118.551 (CSP_2) treatment attenuated and/or abrogated the PMA- and *S. aureus*-induced neutrophil oxidative burst and the percent and intensity of *S. aureus* phagocytosis compared to mice in the CSP_1 group.

Discussion

Cohabitation with ETB mice was reported to increase hypothalamic norepinephrine (NE) activity and the plasma A and NA levels, but not serum corticosterone levels [10, 36]. These findings led us to hypothesize that the final neural link between the neuroimmune changes observed in companions of ETB mice involves peripheral catecholamine release and/or CNS activation as a consequence of the stress imposed by the forced housing condition [11]. The current data obtained after β-adrenergic receptor blockade confirm and strengthen this hypothesis. Indeed, the *d,l*-propranolol treatment attenuated the effects of the forced housing condition on open-field activity and the hypothalamic levels and turnover rate of NA. Furthermore, the *d,l*-propranolol and ICI-118.551 treatments abrogated the effects of cohabitation on peripheral neutrophil oxidative burst and phagocytosis. According to studies from other laboratories, stress generated in a social disruption paradigm also induces significant neuroimmune alterations that are attenuated by a *d,l*-propranolol treatment [25].

Cohabitation with ETB mice increases the locomotor activity of their cage mates within their home cages and the open field [18, 33]. The current data are consistent with and reinforce this assumption, since increased activity within animals' home cages and a tendency towards an increase in the distance traveled and a significant increase in the animals' velocity within the open field were observed in CSP_1 mice compared to the CHP_1 group. However, these data provide new information and showed that cohabitation with 2 ETB mice also induces anxiety-like behaviors, an effect that was not reported previously for companions of 1 ETB mouse. This anxiety-like behavior observed in mice in the CSP_1 group was reduced by the *d,l*-propranolol treatment, but not by the ICI-118.551 treatment. Specifically, cohabitation with 2 ETB mice reduced the time the CSP_1 animals spent within the central zone of the open field ($CSP_1 \times CHP_1$) and the administration of *d,l*-propranolol, but not ICI-18.551, abrogated these effects ($CSP_2 \times CHP_2$).

Stressors and/or anxiogenic drugs increase the amount of time rodents spent in the peripheral zone of the open field, simultaneously decreasing the time spent in the central open-field zones of the apparatus [34, 38]. Thus, is seems feasible to assume that CSP_1 mice were posed to an anxiogenic situation within their home cages. Indeed, a reduction in the total locomotor activity within the central zone of the open-field apparatus is considered a good index of anxiety levels and the stress response [34, 39, 40]. Tumors produce volatile organic compounds that are released into the atmosphere [41, 42], and odor, but not visual or olfactory cues, released by ETB mice were shown to be aversive and to induce psychological stress in their conspecifics [11]. Furthermore, companion mice are continuously subjected to a behavioral conflict in their home cages: the natural need for social interaction and the simultaneous and opposite natural drive to avoid the odor cues of their SP [11]. Similar behavioral conflict paradigms are considered good models of anxiety and stress [34, 39, 43]. Notably, based on data from the open-field test, the *d,l*-propranolol and ICI-118.551 treatments had no effects on the locomotor parameters analyzed in the control mice ($CHP_1 \times CHP_2$), suggesting that the effects of *d,l*-propranolol on anxiety-like behaviors in CSP_2 mice were not a consequence of an overall impact on the animals' activity levels.

The hypothalamic NA levels in CSP_1 mice are consistent with and reinforce the observations in the open-field test. Indeed, similar to previous reports [36], companions of ETB mice presented decreased levels and an increased turnover of hypothalamic NA compared to their respective controls ($CSP_1 \times CHP_1$), an effect that was attenuated by the daily *d,l*-propranolol treatment from ED6 to ED11 but not by ICI-118.551 treatments ($CSP_2 \times CHP_2$). Areas of the limbic system, such as the hypothalamus, hippocampus, and amygdala, display a rapid and significant increase in NA turnover in the presence of anxiety and stress [44, 45]. Accordingly, stress induced by a communication box was reported to activate catecholaminergic neurotransmission within the CNS [45]. Cold stress [46] and immobilization stress [47] increase NE release and turnover in the hypothalamus. This increased turnover is considered a response to increased tyrosine hydroxylase activity [48]. Most research data support the hypothesis that an intact or augmented central NA function is required to successfully cope with a variety of stress responses, including locomotion and anxiety [49, 50].

The currently reported effects of the *d,l*-propranolol treatment on open-field behavior and NA activity in companions of ETB mice, however, does not seem to be directly related to the specific β_2-AR blockade; administration of ICI-118.551, a specific β_2-AR antagonist, was

unable to modify these CNS outcomes in the companions of ETB mice. Although the current data does not allow further examination, the effects of *d,l*-propranolol on the CNS have been related to: (1) its actions on β-AR present in NE pathways [51, 52], (2) a stabilizing and direct effect on the NE neuronal membranes [49, 50] and, not less importantly, (3) an effect exerted outside the CNS by reducing the effects of stressors [53, 54]. Interestingly, the β_1-AR antagonist metoprolol does not exert antianxiety effects on humans and the polar antagonist nadolol, which does not cross the blood/brain barrier, produces effects on humans similar to the effects reported for *d,l*-propranolol [55].

In addition to the reported increase in sympathetic activity in mice that were housed with ETB conspecifics [10, 11], the current data showed that vehicle-, *d,l*-propranolol-, and ICI-118.551-treated mice did not exhibit significant differences in serum corticosterone levels and adrenal weights, which support and strengthen previously reported data for companions of ETB mice [28, 33]. Notably, even the presence of 2 ETB mice within the home cage, i.e., a higher level of cohabitation-induced stress, was unable to modify serum corticosterone levels and adrenal weights of the cage mates ($CSP_1 \times CHP_1$). A lack of changes in serum corticosterone levels in mice in response to social disruption stress has been reported by Hanke et al. [25]. Based on these data, the NE response that signals through the β-AR does not significantly influence the activation of the HPA axis, indicating that attenuation of the observed stress-induced immune and behavioral changes induced by cohabitation with ETB mice is in fact due to changes in SNS activity and not altered activation of the HPA axis. It seems reliable to suggest that cohabitation-induced stress is not strong enough to change the HPA axis activity. The effects of stress depend on the type of stressor, its duration and frequency, the temporal relationship between stressor application and the moment of analysis and, among many other variables, the subject's ability to control or escape from stressors.

Smell is the most behaviorally relevant sensory modality in rodents. Serotoninergic fibers originating in the brainstem raphe nuclei target not only SNC regions, such as the cortical sensory areas, but also peripheral regions, such as the olfactory bulb [56, 57]. The current data showed a trend toward lower 5-HT levels and a significant increase in the 5-HT turnover rate in the olfactory bulb of companions of ETB mice ($CSP_1 \times CHP_1$), an effect that was not abrogated by the *d,l*-propranolol or ICI-118.551 treatments ($CSP_1 \times CHP_2$). The application of broad-spectrum 5-HT receptor agonists was reported to attenuate odor-evoked olfactory receptor neuron activity [58]. Conversely, 5-HT receptor antagonists increase glomeruli activation [26, 58]. These data and other findings prompted the hypothesis that 5-HT neurons present in the olfactory glomerular layer suppress olfactory bulb inputs as 5-HT signals might have a role in olfactory inputs during the acquisition of olfactory information [58]. Thus, although the current and preliminary 5-HT data do not allow further examinations, the increased olfactory bulb 5-HT activity currently being reported in the CSP_1 and CSP_2 mice might represent a response to allow the mice to adjust their sensory gain to the arousal state imposed by the odors continuously released by their tumor-injected cage mates. Indeed, increased 5-HT activity has been reported to regulate the gain of olfactory inputs during intense arousal states, an effect that is mediated by $5\text{-}HT_3$ receptor activation [26].

Flow cytometry data for the basal and activated neutrophils in the peripheral blood of CHP_1 and CSP_1 mice are consistent with and expand previously reported results for neutrophils and macrophages [10, 18]. Neutrophils are key players in immunity, and their activities are essential for the resolution of infectious diseases. Indeed, neutrophils, which, along with macrophages, comprise the professional phagocytes, are endowed with a unique capacity to engulf and thereby eliminate pathogens, tumor cells, and cell debris. Similar to our previous works [33, 36], no differences were observed among the 4 different groups of mice regarding the basal neutrophil oxidative burst in the current study. However, a significant decrease was noted in neutrophil oxidative burst and phagocytosis in mice in the CSP_1 group compared to the CHP_1 group after either PMA or *S. aureus* inductions. Interestingly, these effects were attenuated by the *d,l*-propranolol and ICI-118.551 treatments ($CSP_1 \times CHP_2$), clearly suggesting that the β_2-AR is involved in the effects induced by cohabitation with ETB mice. Many in vivo and in vitro studies suggest that stress, catecholamine exposure, and β_2-AR stimulation play an important role in the generation and maintenance of a normal immune response [1, 9, 59]. Thus, the current data on neutrophil activity after β-adrenergic receptor blockade also support and strengthen our working hypothesis that the effects of the housing condition rely on SNS activation and catecholamine release. Interestingly, the *d,l*-propranolol and ICI-118.551 treatments did not influence the neutrophil activity of CHP_2 mice compared to the CSP_1 group, suggesting that the observed effects are a consequence of the stress imposed by housing with the ETB cage mates, i.e., in the group of mice that was previously shown to exhibit

changes in neutrophil activity and increased plasma catecholamine level.

The neutrophil surface is complex, with myriads of folds, crevices, and sites that allow the neutrophil to interact with its surroundings [60]. Critical surface receptors that facilitate phagocytic movement and particle ingestion through pathways affecting cytoskeletal reorganization are located in these sites [61]. PMA- and *S. aureus*-induced neutrophil activation were lower in the companions of ETB mice compared to controls ($CHP_1 \times CSP_1$). These stimuli use different mechanisms to trigger oxidative burst and phagocytosis. *S. aureus* is a phagocytic stimulus that binds to receptors on the cell surface, which, in turn, activate NADPH oxidase and myeloperoxidase independent of protein kinase C activity [62]. In contrast, PMA indirectly stimulates NADPH oxidase by activating protein kinase C [63], but it is only a weak trigger of myeloperoxidase release [64]. Since the effects of both PMA and *S. aureus* on neutrophil activity were both decreased by the housing condition ($CHP_1 \times CSP_1$), these effects likely occurred before PMA and *S. aureus* administration, most likely during the forced cohabitation within the animals' home cage. Perhaps the *d,l*-propranolol and ICI118.581 treatments, which were administered to CSP_2 mice 90–180 min before they were euthanized in the current experiments, abrogated the effects induced by cohabitation.

Stimulation of the β_2-AR has been shown to regulate lymphopoiesis, lymphocyte homing, the immune cell surface phenotype, mature cell function, and cytokine production [3]. β_2-AR agonists have been shown to increase the production of the anti-inflammatory cytokine IL-10 and decrease the production of the proinflammatory cytokines TNF-α, IL-12, and IL-6 by lipopolysaccharide-stimulated macrophages in vitro [5, 65]. In vivo intraperitoneal administration of salbutamol has been shown to decrease IL-12 production and increase IL-10 production by peritoneal macrophages [66]. Activation of β_2-AR in murine macrophages with the specific agonist salmeterol was recently shown to upregulate IL-1β and IL-6 mRNA and protein levels [4]. Administration of *d,l*-propranolol 30 min prior to tail-shock exposure attenuated plasma IL-1β and IL-6 levels [24]. Notably, cohabitation with ETB mice also decreases peritoneal macrophage activity [18].

Cytokine production was analyzed in spleen cells from CSP and CHP mice stimulated with lipopolysaccharide in vitro [67]. Compared to the CHP mice, cohabitation for 11 days with ETB mice decreased the levels of IFN-α and IL-6 produced by neutrophils from CSP mice. These preliminary results extend the effects of cohabitation with ETB mice to cytokines and suggest that they might be involved with the currently reported effects of the *d,l*-propranolol and ICI-118.551 treatments. Indeed, increased levels of IL-6, TNF-α, and MCP-1 have been reported in mice submitted to social disruption stress, an effect that was abrogated by pretreatment with *d,l*-propranolol [25]. A shift in the Th1 × Th2 cytokine response toward a Th2 profile was reported to be an effect induced by cohabitation with ETB mice [67] and the application of psychological stressors [15, 68]. Stimulation of the β_2-AR with NE has been reported to influence Th1 effector cell function in vivo [69]. Mice deficient in dopamine-β-hydroxylase, an enzyme required for NE production, produce lower levels of Th1 cell cytokines than control mice, whereas the level of the Th2 cytokines remains similar to the control [70]. Effective immune function against tumor cells and bacteria requires cooperation between neutrophils, macrophages, NK cells, and T and B lymphocytes. Therefore, suppression of 1 of these components may compromise immunocompetence.

Interestingly, the proinflammatory cytokine IL-6 is associated with alterations in rodent behaviors [71], and our group recently observed decreased IL-1β and IL-6 levels in the olfactory bulb of CSP mice compared to the CHP group [unpubl. data]. Cytokines are reported to serve as messengers to the brain from the activated immune cells that are responding to an external or internal threat [72].

In conclusion, the current data on the effects of *d,l*-propranolol on open-field behavior and hypothalamic NE activity and the *d,l*-propranolol and ICI-118.551 treatments on neutrophil activity in companions of ETB mice strongly suggest that the SNS participates in the process via catecholamines and β_2-AR stimulation. Possible roles for cytokines in behavior, immune cell activation, and catecholamine sensitivity in companions of ETB mice also seem plausible. Indeed, engagement of the β_2-AR activates a cascade of signaling intermediates, including cAMP and protein kinases, which induces the phosphorylation of cellular proteins, including transcriptional factors that mediate gene expression [3]. Ongoing studies might help researchers understand the neuroimmunomodulatory mechanisms of cohabitation-induced stress. After a careful extrapolation of the data from animal models to humans, the current data generated with β-AR blockers might inspire and/or support the development of new therapeutic approaches for the management of stress.

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Disclosure Statement

No potential conflicts of interest are disclosed.

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