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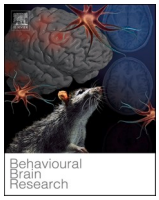

Research report

Chronic social instability stress down-regulates IL-10 and up-regulates CX3CR1 in tumor-bearing and non-tumor-bearing female mice

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ABSTRACT

Extensive literature has reported a link between stress and tumor progression, and between both of these factors and mental health. Despite the higher incidence of affective disorders in females and the neurochemical differences according to sex, female populations have been understudied. The aim of this study was therefore to analyze the effect of stress on tumor development in female OF1 mice. For this purpose, subjects were inoculated with B16F10 melanoma cells and exposed to the Chronic Social Instability Stress (CSIS) model. Behavioral, neurochemical and neuroendocrine parameters were analyzed. Female mice exposed to CSIS exhibited reduced body weight and increased arousal, but there was no evidence of depressive behavior or anxiety. Exposure to CSIS did not affect either corticosterone levels or tumor development, although it did provoke an imbalance in cerebral inflammatory cytokines, decreasing IL-10 expression (IL-6/IL-10 and TNF-α/IL-10); chemokines, increasing CX3CR1 expression (CX3CL1/CX3CR1); and glucocorticoid receptors, decreasing GR expression (MR/GR). In contrast, tumor development did not alter body weight and, although it did alter behavior, it did so to a much lesser extent. Tumor inoculation did not affect corticosterone levels, but increased the MR/GR ratio in the hippocampus and provoked an imbalance in cerebral inflammatory cytokines and chemokines, although differently from stress. These results underscore the need for experimental approaches that allow us to take sex differences into account when exploring this issue, since these results appear to indicate that the female response to stress is mediated by mechanisms different from those often proposed in relation to male mice.

1. Introduction

Chronic stress (CS) has been shown to be a primary precipitating factor in mental and/or physical illnesses, such as depression and cancer. The most common CS in humans and other social animals is that emerging from social interactions, with Chronic Social Stress (CSS) being the type most strongly associated with depression [105,106,47]. Moreover, a large body of work has shown that chronic psychosocial stress affects the development of cancer and increases the mortality rate associated with this pathology [17,56,74]. The high prevalence of depressive disorder among cancer patients, together with findings from tumor-bearing animals [13,58,84], suggest a relationship between these two pathologies [109,16,95], although the underlying physiological mechanism remains unknown.

The inflammatory response produced by neuroendocrine changes induced either by CS or the presence of the tumor itself [107,24,3,90,93] is currently thought to be a possible mechanism. A large body of evidence has shown that inflammatory processes alter the activity of the Hypothalamus Pituitary Adrenal (HPA) axis, neurotransmission, neuroplasticity and neurotoxicity, all of which are involved in the pathophysiology of mood disorders [90]. These changes occur through the release of proinflammatory cytokines, chemokines, enzymes, and second messengers, which are produced through the activation of glial cells, mainly the microglia [108]. Along with others, these mediators trigger a series of chain reactions that enable the internal changes necessary to adapt the organism to external (social stress) and internal

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(tumor) demands. This allostatic process alters the neurochemical and neuroendocrine balance [110], while causing behavioral and physiological changes that contribute to the survival of the individual. Anti-inflammatory cytokines released in response to inflammation help ensure that this inflammatory response is transient. However, when the inflammatory imbalance is prolonged over time, allostatic load appears, together with the negative effects of prolonged inflammatory activation. In this regard, while pro-inflammatory cytokines have been associated with neuronal damage, the anti-inflammatory activity of cytokines such as IL-10 has been associated with neuroprotection [111,96]. For example, it has been observed that IL-10 supports neurogenesis in the hippocampus of adult animals [82]. On the other hand, chemokines play a fundamental role in the communication between the Nervous System and the Immune System. In this context, CX3CL1 (neuron released) - CX3CR (microglia-localized receptor) signaling is the best characterized axis of neuron-microglia interaction [89]. This axis controls numerous homeostatic processes [4,80] that can be profoundly affected by psychosocial stress [112,49,60,83], including anti-tumor immune activity [28,72] and the neurochemical balance associated with depressive-like behavior [85].

Although it has been well documented that CSS increases inflammation [5,100], and that inflammation is elevated in both depression ([27,37]) and tumor progression [1,53], significant variability has been found across studies [2,27]. Firstly, the sex differences observed in prevalence, symptomatology, and treatment response [54,8] contribute greatly to depression heterogeneity [104,12,68,79]. And secondly, sex differences have also been observed in the incidence, development and mortality associated with different types of cancer [69,9]. These sex differences in physical and mental health could be explained, at least in part, by sexual dimorphism in the immune activity [48,61,94,98,99].

In addition to immune sexual dimorphism, sex differences have also been observed in response and susceptibility to different types of social stressors, probably due to disparities in the perception of social threat, which may contribute to differences in vulnerability and/or resilience to environmental challenges. Since both glucocorticoids and catecholamines regulate the immune function [87], a differential physiological stress response may contribute to the divergence in the inflammatory profile observed between the two sexes, accentuating sex differences in brain networks and pre-existing vulnerability factors [57]. This highlights the importance of studying the response to a variety of stressors also in females [120,65]. Despite existing evidence regarding sex differences in the neural, immune and behavioral response to CSS, most of the models developed for the study of its effects in rodents, such as the social defeat model, work optimally in males [45,52], but are not suitable for inducing CSS in females, as they do not reveal territorial aggression [31,78]. Consequently, knowledge of the specific mediators involved in the possible negative effects of CSS in females is very limited. Considering the social nature of females, the model of chronic social instability stress (CSIS) may be more appropriate and have a greater ethological validity for this population. Although the results are not always consistent, when applied to female mice, the CSIS model has been associated (in our laboratory also) with anxious-depressive-like behavioral changes [22,55,7,91], accompanied by physiological changes such as high corticosterone levels [36,39,55,7,97], reduced IL-10 levels and hippocampal inflammatory imbalance [55], which may indicate an increased vulnerability to new challenges that is characteristic of females [29].

In light of the above, the aim of the present study is to analyze the effects of the CSIS on tumor development among female mice, and the effects of both factors (stress and tumor) on behavior, as well as to evaluate the underlying neuroendocrine, neurochemical and neuroinflammatory activity. To this end, we have induced tumors using B16F10 melanoma cells; a group of animals was submitted to CSIS and after that, behavioral parameters were analyzed. Next, neuroendocrine activity was analyzed through serum corticosterone levels and its receptors' mRNA expression levels in the hypothalamus and hippocampus. Neuroinflammatory activity was measured through pro-inflammatory and anti-inflammatory cytokine expression in the hippocampus and striatum, as well as through the neuronal control of microglia activity in response to immune activation in the hippocampus, striatum and prefrontal cortex.

2. Material and methods

2.1. Subjects and husbandry

Ninety-one eight-week-old OF1 outbred female mice (Janvier Labs, France) were housed in groups of three in transparent plastic cages measuring 24.5 × 24.5 × 15 cm. Food and water were available ad libitum. The holding room was maintained at a constant temperature of 24 °C with a 12-hour inverted light/dark cycle (white lights on from 20:00 h to 08:00 h), including 20 min of progressively increasing light (dawn, 7:40–8:00 am) and 20 min of progressively decreasing light (dusk, 7:40–8:00 pm). The reversal of the light cycle allowed the manipulation of mice in their active phase (under dim red light), avoiding applying the tests in their resting phase. All procedures involving mice were performed in accordance with the Directive 2010/63/EU regarding the protection of animals used for scientific purposes. The Gipuzkoa Provincial Council (PRO-AE-SS-062) and the Animal Welfare Ethics Committee of the University of the Basque Country (CEEA-UPV/EHU) controlled and approved all procedures used in this experiment.

2.2. Experimental procedure

The experiment began after a 10-day adaptation period, after which a 4-day basal measuring period was initiated (Fig. 1). On day − 4, animals (n = 91) were weighted and a basal blood sample was taken (via submandibular vein puncture). On day 0, two groups were randomly established: tumor-bearing mice (T), inoculated with B16F10 melanoma tumor cells (n = 49) and non-tumor (NT) mice, which received a physiological saline solution injection (n = 42). Immediately after, each group was separated into two subgroups, based on stress condition, resulting in four experimental subgroups: non-stressed-non-tumor (NS/NT) (n = 18), stressed-non-tumor (S/NT) (n = 24), non-stressed-tumor (NS/T) (n = 21) and stressed-tumor (S/T) (n = 28). The S groups were subjected to the CSIS model for 28 days, and NS groups remained in the same housing conditions as during the adaptation period. At the end of the CSIS period, Sucrose Preference Test (SPT) was performed, followed by Sociality Test (ST), Open Field test (OFT) and, finally, Novel Object Recognition Test (NORT). On day 31, the animals were weighed and blood was collected by submandibular puncture. They were then sacrified by cervical dislocation. The rest of the blood was collected by cardiac puncture. Lungs were removed to analyze tumor development. Whole hypothalamus, hippocampi, prefrontal cortices and striata were dissected under sterile conditions with stereomicroscopic observation with reference to the mouse brain atlas [81] and were stored at − 80 °C for biological determinations.

2.3. Stress procedure

Animals in the stressed group (both inoculated and non-inoculated) were exposed to the CSIS stress model, which was adapted and modified from a protocol described by Haller et al. [31] in rats and by Schmidt et al. [97] in mice. The mice were exposed to a highly unstable social situation with alternating phases of isolation (1, 2 or 3 days) and crowding (4 subjects per cage, during 1, 2 or 3 days) over a 28-day period. During each crowding phase, we ensured that four different mice that had no previous contact were placed together in a new clean cage; control mice were meanwhile allocated to stable groups of 3 mice.

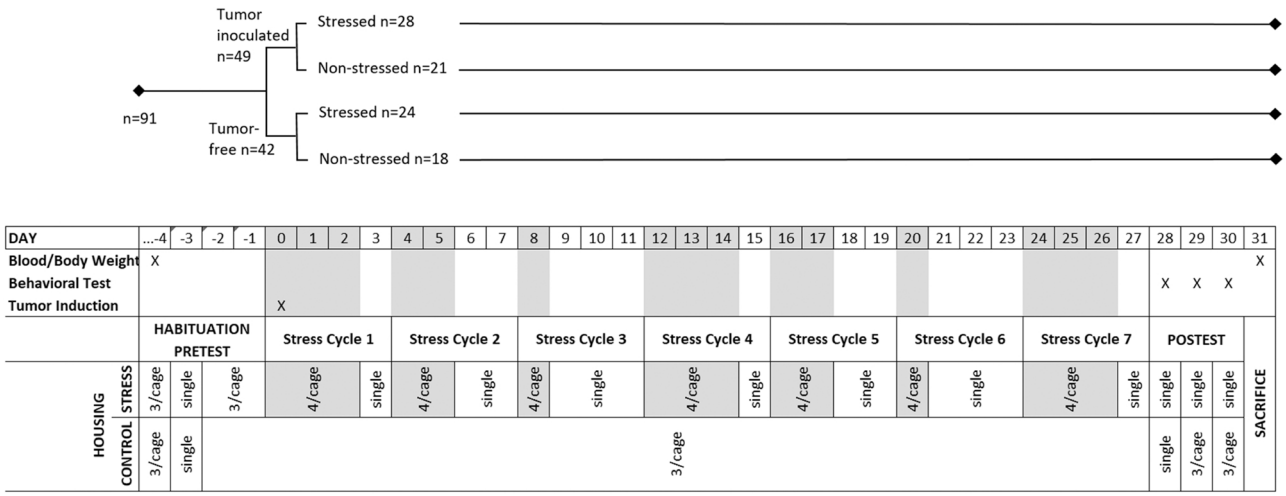

Fig. 1. Experimental procedure.

2.4. Experimental tumor induction

Tumors were induced by inoculating mice with B16F10 murine melanoma cells. These cells arrest in the lung following intravenous injection, which makes them an ideal choice for studying lung-specific metastasis in mice [14]. The B16F10 cells were maintained in vitro by subculturing the tumor cells at 37 °C in a humidified atmosphere of 5 % CO^2 in 75-cm^3 cell culture flasks (Corning Inc., Corning, NY, USA) with RPMI-1640 culture medium supplemented with HEPES and L-glutamine (Lonza, Basel, Switzerland) at a density of 10^5 cells/ml. Adherent B16F10 cells were detached by incubation with 0.02 % EDTA for 5 min and subsequently washed in RPMI-1640 medium. Mice that had been pre-anesthetized via intraperitoneal injection of Nembutal (sodium pentobarbital, 60 mg/kg) were inoculated with 5×10^4 viable B16F10 cells in 0.1 ml of medium via the lateral tail vein using a 30.5-gauge needle, after the tail had been previously heated with a thermal pillow.

2.5. Behavioral assessment

2.5.1. Sucrose preference test (SPT)

All mice were individually housed for 24 h with two different bottles; one contained a 1 % sucrose solution, and the other one, water. To prevent the possible effect of a side preference, the position of the bottles was counterbalanced. The animals were not deprived of food or water before the test. Consumption of each solution was measured by weighing the bottles at the beginning and end of the test. Each value was then divided by the mouse's body weight. Sucrose preference discrimination index was calculated as: (sucrose consumption - water consumption)/ total consumption.

2.5.2. Open field test (OFT)

This test was performed to assess anxiety-like behavior. Mice were placed in a black Plexiglass arena (40 × 40 × 30 cm) and allowed to explore for 5 min. Time spent in the center and in the peripheral zone of the arena was analyzed, along with latency to the first entry to the center and locomotor activity (distance traveled, and mobile time).

2.5.3. Sociability test (ST)

This test reflects the preference of a mouse for spending time with another mouse compared to the time spent alone or enclosed to an object during 5 min. The behaviors assessed were time mobile and the time spent close to the other mouse (subject area) and the object area. The discrimination index was calculated as: (time with subject-time with object)/(time with subject + time with object).

2.5.4. Novel object recognition test (NORT)

Mice were subjected to a training session in which they were placed in a black Plexiglass-open-field box (40 × 40 × 30 cm) with 2 identical objects (4 cm diameter plastic caps) and were allowed to investigate for 5 min, after which they were returned to their home-cage. Twenty-four hours later, the mice were returned to the same box, but one of the familiar objects (F) was replaced by a novel object (steel triangle; N). The total time exploring both objects was recorded during 5 min. Recognition memory was measured in terms of discrimination index: (time with N - time with F)/(time with N + time with F). Time mobile, time spent in the F and NF areas and the mean distance from them were also analyzed.

Behavior was recorded with video cameras (GZ-MG773; JVC, Yokohama, Japan) for subsequent assessment using the ANY-maze© version 4.96 video-tracking software (Stoelting Europe, Dublin, Ireland). All experiments were conducted between 9:00 am and 12:00 pm and arenas and objects were cleaned between trials with a solution of 0.5% acetic acid.

2.6. Physiological determinations

2.6.1. Determination of pulmonary tumor area and foci

After several days of incubation in Bouin's solution, the 5 lobes of the lung were separated, and digital images were obtained. Each photograph was captured with the same exposure conditions and included a standard-sized reference circle (9.62 mm^2). The tumor area was determined with the public domain ImageJ software program developed at the U.S. National Institutes of Health (ImageJ, U.S. NIH, Bethesda, MD, USA; available at http://rsbweb.nih.gov/ij/). The number of metastatic foci was also determined using an Olympus SZ30 Zoom Stereo Microscope (Olympus, Tokyo, Japan).

2.6.2. Blood collection and plasma isolation

Blood was collected in heparinized containers by submandibular vein or cardiac puncture between 9:00 and 10:00 a.m. Samples were centrifuged at 1800g for 15 min at 4 °C, and the resulting plasma was collected and stored at − 80 °C to determine corticosterone levels.

2.6.3. Determination of plasma corticosterone concentrations

Plasma corticosterone levels were quantified using an enzyme immunoassay (Corticosterone Elisa Kit, Enzo Life Sciences), in accordance with the manufacturer's recommendations, and a Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Data were analyzed by means of a 4-parameter logistic curve fit using MyAssays (Data Analysis Tools and Services for Bioassays; available at

https://www.myassays.com/). The sensitivity of the assay was 27.0 pg/ml, and the intra and inter-assay variation coefficients were between 7% and 8%.

2.6.4. Real-Time RT–PCR measurements of mRNA gene expression in the hypothalamus, hippocampus, prefrontal cortex, and striatum

The total RNA of each structure was isolated using the NucleoSpin RNA Plus kit (Macherey Nagel, Germany). A spectrophotometric analysis was performed at 260 nm to determine RNA concentrations, while the 260:280 absorbance ratio was utilized to assess nucleic acid purity (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA). The total RNA was then reverse-transcribed using the PrimeScript RT reagent kit (Takara Bio Inc., Madrid, Spain). The resulting cDNA was quantified by SYBR Green-based (SYBR®Premix Ex TaqTM, Takara Bio Inc., Madrid, Spain) real-time PCR, and the formation of PCR products was monitored using the 7500 Real-Time PCR System (Applied Biosystems, Madrid, Spain). Both hypoxanthine phosphoribosyl transferase (HPRT) and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) were used as reference genes. Primer sequences were designed using Primer Express Software v3.0 (Applied Biosystems, Madrid, Spain), and obtained from Applied Biosystems (Supplementary material Appendix 1, Table A.1). The relative gene expression was determined using the $2^{-D\Delta t}$ method [64].

2.7. Statistical analysis

All statistical analyses were performed using the SPSS 28.0 for Windows software package (SPSS Inc., Chicago, IL, USA), with the level of significance set at $p < 0.05$. Normality and homogeneity criteria were respected, and outlier values were adjusted in accordance with the boxplot outlier labeling rule [113]. Behavioral and physiological variables were analyzed with one-way or two-way ANOVA; When the stress x tumor interaction reached significance level, specific comparisons were carried out using a post hoc Tukey test. Cohen's d test for effect size was performed to estimate the strength of the effects between two groups ("d" values > 0.8 are considered indicative of large effects, values between 0.5 and 0.8 are considered indicative of moderate effects, and values < 0.5 are considered to indicate small effects). A partial eta-square (η2) test for effect size was used for analyses with more than two groups and interactions (η2 = 0.01, small; η2 = 0.06, moderate; and η2 = 0.14, large effects).

3. Results

3.1. Model characterization

3.1.1. Body weight (BW) and sucrose preference test (SPT)

We observed a BW reduction after CSIS in S animals ($F [1,87] = 4.316$, $p = 0.041$, $\eta2 = 0.047$) but no differences in the SPT (Fig. 2).

3.1.2. Behavioral assessment

In the OFT, both S groups traveled a greater distance ($F [3,87] = 8.799$; $p = 0.004$; $\eta2 = 0.092$), and spent more time in the center ($F [3,87] = 4.628$; $p = 0.035$; $\eta2 = 0.058$) than their NS counterparts (Fig. 3a, b). No differences were observed between groups in the ST discrimination index (Fig. 3c). Nor were any between-group differences observed in any of the variables studied, either stratified in accordance with the presence of a tumor or in relation to the interaction between stress and tumor, indicating that tumor presence was not a factor that influenced behavior. In the NORT, the S/T group showed a greater preference for the N object than the NS/NT group ($F [3,87] = 4.384$; $p = 0.039$; $\eta2 = 0.048$) (Fig. 3d), although no differences were observed for the stress x tumor interaction. Interestingly, S mice spent more time mobile than NS mice ($F [3,87] = 8.217$; $p = 0.005$; $\eta2 = 0.086$).

3.1.3. Effect of stress on tumor development

No differences were found between groups in the total number and area of metastatic foci, indicating that CSIS did not affect tumor development (Fig. 4).

3.2. Biological assessment

3.2.1. Neuroendocrine effects

3.2.1.1. Corticosterone serum levels. The three-way ANOVA (time x stress x tumor) with repeated measures revealed significant differences in corticosterone levels for the time factor ($F [3,85] = 4.247$; $p = 0.042$; $\eta2 = 0.048$), and tumor factor ($F [3,85] = 6.647$; $p = 0.012$; $\eta2 = 0.073$) decreasing corticosterone levels in both tumor groups (Fig. 5). Stress resulted in a significant increase in corticosterone levels on day 31, but only in animals without tumors ($p = 0.009$, Cohen's $d = 1.015$).

3.2.1.2. Hypothalamic and hippocampal GR and MR mRNA relative gene expression. Differences were observed in GR and MR expression levels, as well as in the ratio between the two receptors. In both the hypothalamus and the hippocampus, GR expression levels were lower in the stressed group ($F [3,85] = 35.213$; $p < 0.001$; $\eta2 = 0.293$ and $F [3,80] = 35.005$; $p < 0.001$; $\eta2 = 0.304$, respectively), and the MR/GR ratio

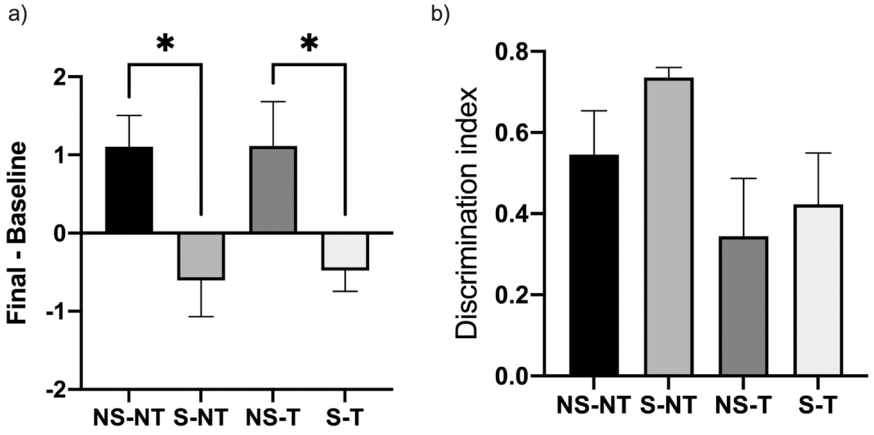

Fig. 2. a) Final-Baseline BW index in grams and b) SPT discrimination index. Data are expressed as mean ± SEM. *p < 0.05.

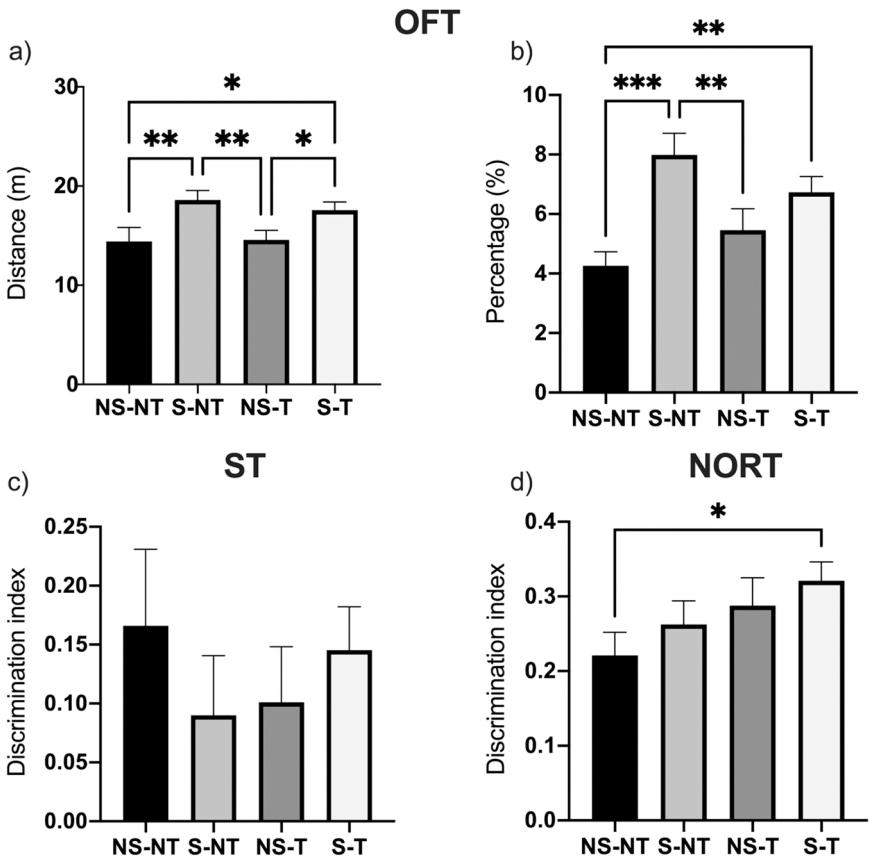

Fig. 3. a) Distance traveled and b) percentage of time spent in the center in the OFT, c) ST and d) NORT discrimination indexes. Data are expressed as mean ± SEM. *p < 0.05 **p < 0.01 and ***p < 0.001.

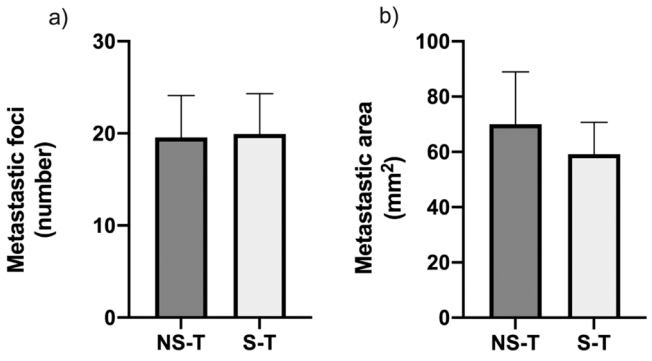

Fig. 4. a) Mean of the pulmonary metastatic foci, and b) total area of the metastatic foci observed. Data are expressed as the mean ± SEM.

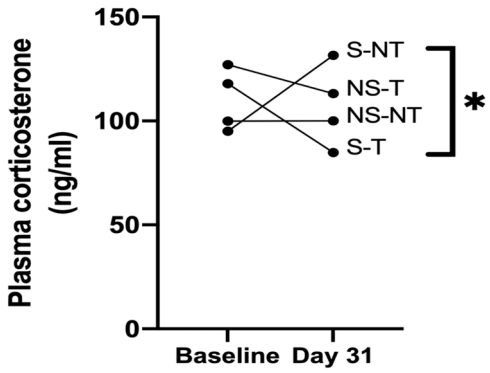

Fig. 5. Plasma corticosterone levels (ng/ml) at days − 4 (baseline) and 31.

was higher (F [3,85] = 10.765; p = 0.002; η2 = 0.112 and F [3,80] = 19.511; p < 0.001; η2 = 0.196, respectively); although the stressed group had lower MR expression levels in the hypothalamus (F [3,85] = 7.861; p = 0.006; η2 = 0.085). Moreover, tumor-bearing mice had lower GR and MR expression levels, although no differences were observed in the ratio between the two receptors in the hypothalamus (F [3,85] = 26.572; p < 0.001; η2 = 0.238; F [3,85] = 42.951; p < 0.001; η2 = 0.336; F [3,85] = 0.004; p = 0.947; η2 = 0.000); they also had higher MR expression levels as well as a higher ratio between the two receptors in the hippocampus (F [3,80] = 14.944; p < 0.001; η2 = 0.157 and F [3,80] = 7.230; p = 0.009; η2 = 0.083, respectively) (Fig. 6a, b, c, d). The interaction between stress and tumor was only significant for MR expression levels and for the MR/GR ratio in the hypothalamus (F [3,85] = 9.665; p = 0.003; η2 = 0.102 and F [3,85] = 5.983; p = 0.017; η2 = 0.066, respectively) (Supplementary material Appendix 1, Table A.2) and for GR expression levels in the hippocampus (F [3,80] = 5.064; p = 0.027; η2 = 0.060) (Supplementary material Appendix 1, Table A.3).

3.2.2. *Proinflammatory and anti-inflammatory cytokine mRNA relative gene expression*

3.2.2.1. Hippocampus. The stressed mice presented lower IL-6 (F [3,80] = 5.032; p = 0.028; η2 = 0.059), IL-1β (F [3,80] = 43.841; p < 0.001; η2 = 0.354), TNF-α (F [3,80] = 4.217; p = 0.043; η2 = 0.050) and IL-10 (F [3,80] = 7.689; p = 0.007; η2 = 0.088) expression levels (Fig. 7a) in the hippocampus. With regard to ratios, they had a lower IL-1β/IL-10 ratio (F [3,80] = 4.889; p = 0.030; η2 = 0.058) (Fig. 6b) than their non-

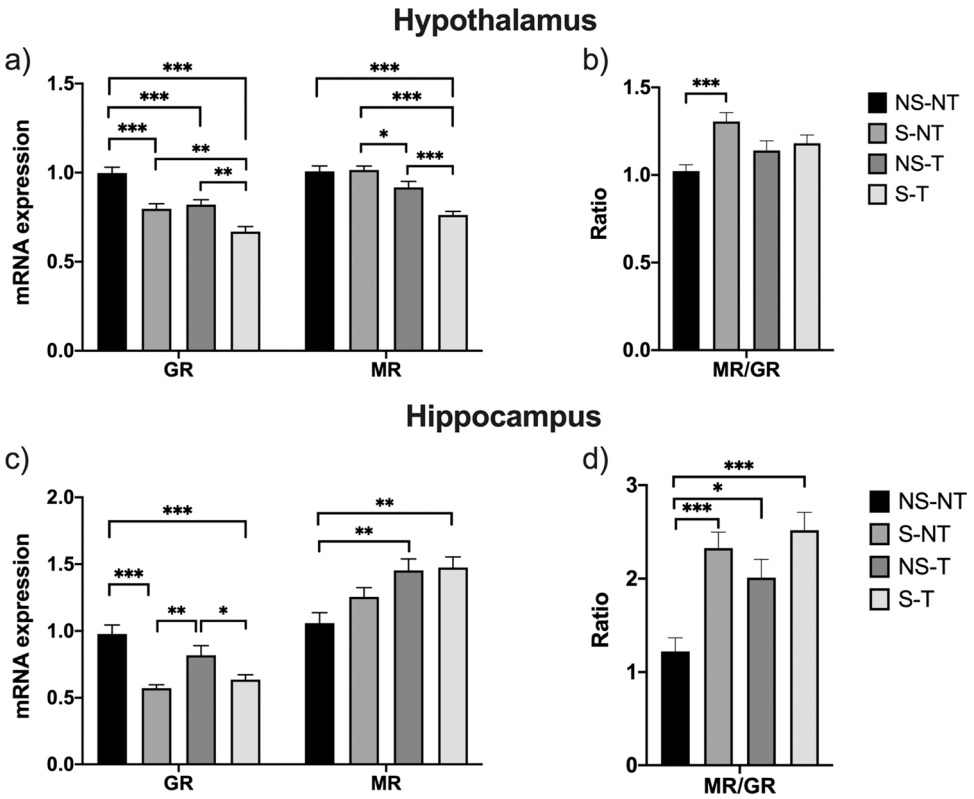

Fig. 6. a) Hypothalamic GR and MR mRNA expression levels, b) MR/GR ratio in the hypothalamus, c) Hippocampal GR and MR mRNA expression levels, and d) MR/GR ratio in the hippocampus. Data are expressed as the mean ± SEM. *p < 0.05, * *p < 0.01 and * **p < 0.001.

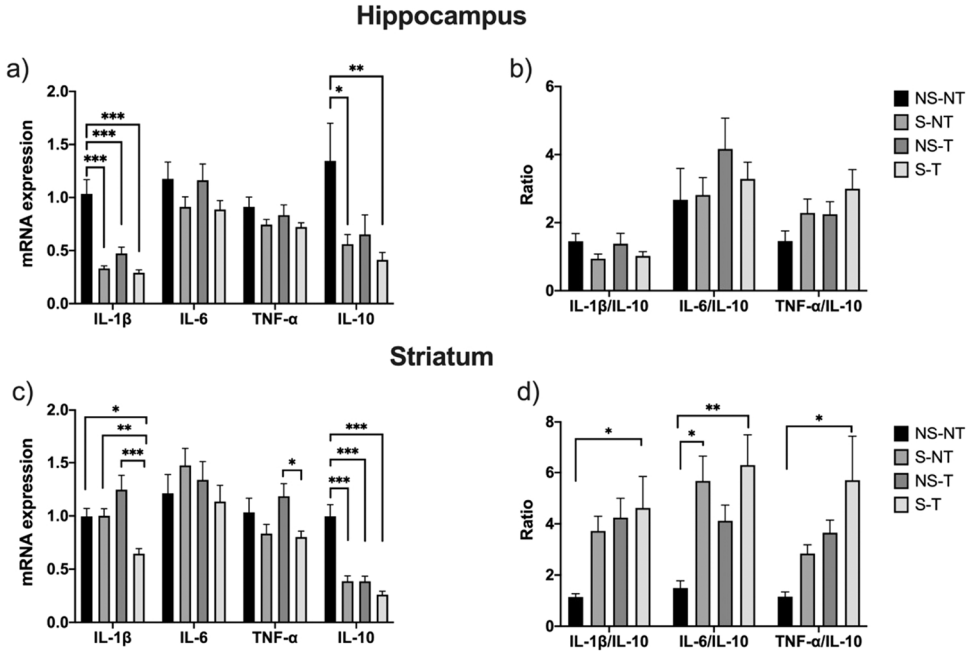

Fig. 7. a) IL-1β, IL-6, TNF-α and IL-10 mRNA expression levels in the hippocampus, b) the pro-inflammatory versus anti-inflammatory cytokine ratios in the hippocampus, c) IL-1β, IL-6, TNF-α and IL-10 mRNA expression levels in the striatum, and d) the pro-inflammatory versus anti-inflammatory cytokine ratios in the striatum. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001.

stressed counterparts. For its part, the tumor group had lower IL-1β (F [3,80] = 20.278; p < 0.001; η2 = 0.202), IL-10 (F [3,80] = 5.203; p = 0.025; η2 = 0.061) (Fig. 7a). The interaction between stress and tumor was significant for IL-1β (F [3,80] = 15.137; p < 0.001; η2 = 0.159) (Supplementary material Appendix 1, Table A.4).

3.2.2.2. Striatum. The stress group had lower IL-1β, TNF-α and IL-10 expression levels (F [3,79] = 12.714; p = 0.001; η2 = 0.139; F [3,79] = 9.103; p = 0.003; η2 = 0.103 and F [3,79] = 37.221; p < 0.001; η2 = 0.320, respectively) (Fig. 7c) and higher IL-6/IL-10 ratio (F [3,79] = 11.673; p = 0.001; η2 = 0.129) (Fig. 7d). Similarly, the tumor group

also had lower IL-10 levels (F [3,79] = 37.439; $p < 0.001$; $\eta 2 = 0.322$) (Fig. 7c) and a higher ratio between IL-1β and IL-10 (F [3,79] = 2.948; $p = 0.090$; $\eta 2 = 0.036$) (Fig. 7d). Finally, the tumor-stress interaction had an effect on IL-1β and on IL-10 expression levels (F [3,79] = 13.219; $p < 0.001$; $\eta 2 = 0.143$ and F [3,79] = 16.146; $p < 0.001$; $\eta 2 = 0.170$, respectively) (Supplementary material Appendix 1, Table A.5).

3.2.3. CX3CR1 and CX3CL1 mRNA relative gene expression

3.2.3.1. Hippocampus. Stressed mice had higher CX3CR1 expression levels (F [3,80] = 72.078; $p < 0.001$; $\eta 2 = 0.474$) (Fig. 8a) and a lower CX3CL1/CX3CR1 ratio (F [3,80] = 24.924; $p < 0.001$; $\eta 2 = 0.238$) (Fig. 8b). For its part, the tumor group had lower CX3CL1 expression levels (F [3,80] = 24.270; $p < 0.001$; $\eta 2 = 0.233$) (Fig. 8a), higher CX3CR1 expression levels (F [3,80] = 49.560; $p < 0.001$; $\eta 2 = 0.383$) (Fig. 8a) and a lower CX3CL1/CX3CR1 ratio (F [3,80] = 77.842; $p < 0.001$; $\eta 2 = 0.493$) (Fig. 8b) than the non-tumor group. Finally, the interaction between stress and tumor was significant for CX3CL1 (F [3,80] = 4.568; $p = 0.036$; $\eta 2 = 0.054$) levels and for the CX3CL1/CX3CR1 ratio (F [3,80] = 19.389; $p < 0.001$; $\eta 2 = 0.195$) (Supplementary material Appendix 1, Table A.4).

3.2.3.2. Striatum. As in the hippocampus, stressed mice had higher CX3CR1 expression levels (F [3,79] = 63.784; $p < 0.001$; $\eta 2 = 0.447$) (Fig. 8c) and a lower CX3CL1/CX3CR1 ratio (F [3,79] = 16.372; $p < 0.001$; $\eta 2 = 0.172$) (Fig. 8d). Moreover, tumor-bearing mice had

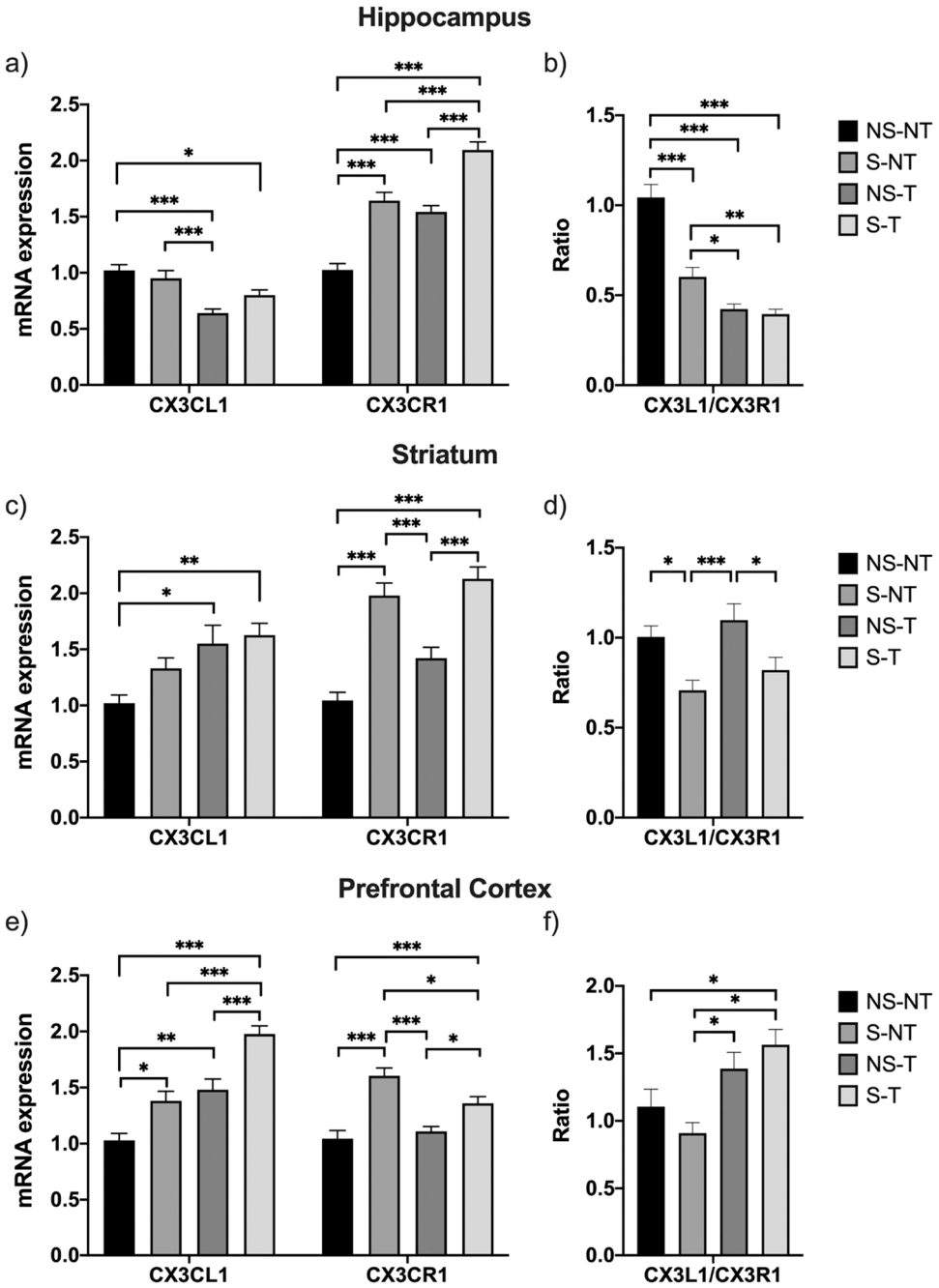

Fig. 8. a) CX3CL1 and CX3CR1 mRNA expression levels and b) chemokine ratio in the hippocampus; c) CX3CL1 and CX3CR1 mRNA expression levels and d) chemokine ratio in the striatum; e) CX3CL1 and CX3CR1 mRNA expression levels and f) chemokine ratio in the PFC. The data are expressed as the mean (± SEM). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

higher CX3CL1 and CX3CR1 expression levels (F [3,79] = 13.103; p = 0.001; η2 = 0.142 and F [3,79] = 6.564; p = 0.012; η2 = 0.077, respectively) (Fig. 8c).

3.2.3.3. Prefrontal cortex. The stressed group had higher CX3CR1 and CX3CL1 levels (F [3,83] = 40.991; p < 0.001; η2 = 0.331 and F [3,83] = 27.123; p < 0.001; η2 = 0.246, respectively) and the tumor group had higher CX3CL1 expression levels (F [3,83] = 41.368; p < 0.001; η2 = 0.333) (Fig. 8e), as well as a higher CX3CL1/CX3CR1 ratio (F [3,83] = 17.056; p < 0.001; η2 = 0.170) (Fig. 8f). Moreover, the interaction between the tumor and stress factors had an effect on CX3CR1 expression levels (F [3,83] = 6.039; p = 0.016; η2 = 0.068) (Supplementary material Appendix 1, Table A.6).

4. Discussion

4.1. Chronic social instability stress in tumor-bearing and non-tumor-bearing female mice: Specific effects on behavior, neuroendocrine activity and tumor development

The results of this study show that, when applied during 4 weeks, CSIS reduces body weight, increases locomotor activity, and modifies the neuroinflammatory response, but does not produce the expected depressive-like behavior, nor any changes in tumor development in OF1 female mice.

Although substantial evidence suggests that stressful life events predispose individuals to depression and anxiety-like behaviors [18,34], in our work the CSIS model did not reveal anhedonia, a key index of depressive-like behavior [118]. The application of this stress paradigm has revealed positive [36] and negative [59,77] anhedonic effects, probably due to methodological (stress or anhedonia protocol, light or dark phase behavioral testing) and individual differences (species, sex, stress coping strategies, females estrous cycle stage). However, stressed female mice did engage in more locomotor activity (greater distance traveled in OFT and NORT), although no anxiety-like behavior was observed, since they exhibited less thigmotaxis in the OFT (shorter latency to enter the center and more time spent in this central zone) and greater social exploration when subjected to the ST, indicating lower anxiety towards novel conspecifics, according to Koolhaas et al. [51]. Consistently with that observed previously in our laboratory [55], in this study, the active behavioral profile of female mice exposed to CSIS was indicative of higher arousal, as indeed observed by other authors also [22]. However, these behavioral results do not enable us to rule out the possibility of this being indicative of anxiety-like behaviors, since the application of traditional paradigms has not yet been sufficiently validated in females and may not reflect the same emotional states in both sexes [50,101]. In this regard, the specific hyper-activation observed in females following emotional stress has been interpreted as a transitional phase towards a pathological stress response [6], or alternatively as an adaptive coping strategy designed to manage and regulate pressures, demands, and emotions in response to stress [46]. On the other hand, although previous work in our laboratory found no effect of the estrous cycle on behavior following social stress due to instability [55] this analysis was not considered on this occasion, and we cannot rule out some effect of the estrous cycle and the estrogen levels on this active behavioral profile observed in female mice exposed to CSIS [40,73].

Consistently with that reported by other authors, stress did not alter corticosterone levels during exposure to CSIS [36,7]. Nevertheless, the higher cortisol levels observed in stressed and tumor-free subjects after the end of the CS period does not rule out the involvement of glucocorticoids in the decreased body weight growth and behavioral reactivity observed after social stress. Similarly, the exposure of female mice to CSIS did not generate the expected results in terms of glucocorticoid receptor expression. Rather, social instability stress increased the MR/GR ratio, decreasing GR levels in both the hypothalamus and the hippocampus, in the latter case, even despite the significant decrease in MR expression. Although stress has been commonly associated with a decrease in MR receptors relative to GR and a reduction in MR functionality, numerous studies have also observed sex differences in the physiological response to stress and its regulation [33]. For example, acute stressors have been found to upregulate GR and MR mRNA in the hypothalamus of male, but not female rats [43]. Sex differences in GR function also appear to make females more susceptible to dysregulation after a stressful event [117]. Following HPA axis activation, GRs are critical to the negative feedback process that inhibits additional glucocorticoid release. Thus, the significant reduction in GR expression previously observed in our laboratory in female mice [55] may be attenuating negative feedback in response to a situation of chronic stress, and may explain the increase in corticosterone levels observed at the end of the CSIS among non-tumor stressed subjects. These changes have also been associated with stress-related disorders [86].

In contrast to that reported by other authors, as well as to our previous results with male mice [115], exposure to CSIS did not affect tumor development in female mice. Although it may be that the CSIS model applied was not sufficient to cause allostatic overload and significant alterations, the evidence linking chronic psychosocial stress and increased tumor development is equivocal. The differences observed in the literature regarding the effects of social stress on tumor development may be attributed to methodological (type of stressor, chronicity, tumor model, timing of stress exposure in tumor progression) or individual differences (sex, strain, coping strategies) [103,42]. In female mice, Dawes et al. [25] have recently demonstrated psychosocial stress-induced tumor inhibition in a preclinical mouse model of breast cancer, mediated by β-AR activation. In this sense, our results also highlight the need to take sex differences into account in the study of the effects of social stress on tumor progression.

In contrast to social stress, tumor development did not alter body weight, and the only significant effect in terms of behavior was a greater preference for the novel object in the NORT among stressed, tumor-bearing subjects, which a priori rules out any deleterious effect of any of either factor on memory. Although it has been shown that tumor-bearing females maintain their food intake and lose a smaller percentage of body mass than male mice [21], most studies point out that tumor growth increases neuroinflammation, cognitive impairment and depressive-like behavior in both males and females [114,119,75]. Although previous work carried out in our laboratory with male mice inoculated with the same experimental tumor resulted in the appearance of sickness behavior [114,115], when the same tumor model was used in C57BL/6 mice, significantly less tumor volume was found over 14 days in female mice compared to male mice [23]. The reduced lung metastatic development observed in female mice after 28 days of tumor development may explain the normal body weight gain observed and the absence of cognitive impairment and sickness behavior. Although this circumstance may also explain why the tumor had no effect on corticosterone levels at the end of the experiment, we cannot rule out the possibility that the timing of the analysis may have masked an effect, as tumor development was not found to generate a significant decrease in corticosterone levels over time. It has been observed that transplantation of tumor cells causes early inflammatory changes within 16–48 h, which in turn significantly alters the endocrine balance of the host [11,76], increasing corticosterone levels and eliciting a systemic anti-inflammatory response to control this inflammatory effect. Therefore, tumor inoculation may have caused some alteration of the HPA axis prior to our analysis, which was performed 28 days after tumor inoculation, and may mediate the observed interaction with social stress after the end of the experiment. In this regard, the results indicate that the presence of a tumor reduces corticosterone levels and hypothalamic MR expression in stressed subjects, and stress increases corticosterone levels in non-tumor-bearing subjects. Regardless of stress, the tumor increased the MR/GR ratio in the HC by increasing the expression of MR receptors and having no effect on GR receptor expression. MR expression is well

documented in the hippocampus, where it has previously been shown to mediate memory consolidation [26,30] and provide neuroprotection against different insults, including apoptosis upon glucocorticoid depletion [66,70]. Although the results obtained in tumor-bearing subjects support this idea (better discrimination in the NORT), we cannot claim that this result reflects a deleterious effect of tumor development in the HC. Further research is required into the neuroendocrine changes caused by tumor development in females and possible sex differences in sickness behavior.

4.2. Chronic social instability stress in tumor-bearing and non-tumor-bearing female mice: Specific effects on inflammatory neurochemistry

Consistently with the results reported by other authors, as well as with those obtained in our laboratory [55,116] CSIS in female mice was found to trigger a significant decrease in IL-10 in both the striatum and the hippocampus, together with a significant decrease in pro-inflammatory cytokines. Although this decline in both pro- and anti-inflammatory cytokines may indicate the absence of an inflammatory response, the greater decrease observed in IL-10 points to a relative increase in inflammatory activity in the striatum (higher IL-6/IL-10, IL1-B/IL-10 and TNF-α/IL-10) and in the hippocampus (higher TNF-α/IL-10), even though these ratios did not always reach significance level.

The calculation of the ratio between pro and anti-inflammatory cytokines is considered a key analysis that may clarify whether or not the inflammatory process is controlled [92]. Nevertheless, in light of our behavioral results, the observed downregulation of both pro- and anti-inflammatory cytokines, rather than a shift in the cytokine profile may indicate a transitional phase towards a pathological stress response and constitutes immune response that is different from the one traditionally reported in males. Ex vivo LPS administration in depressed patient samples revealed a positive association with proinflammatory cytokine production only in males, whereas in females, this association was negative in terms of both proinflammatory cytokine and IL-10 production [67], indicating that changes in IL-10 levels affected females more deeply than males [71]. Several studies have also reported sex differences in the levels of several microglia-linked immune factors, such as IL-10 mRNA and IL-1β protein, with higher levels in the female parietal cortex and hippocampus [38,98].

Inflammatory cytokines are required for the induction of critical mediators of inflammation-induced mood disorders [10,19], and may also explain the behavioral results observed, which provided no evidence of depressive-like behavior.

Similarly, after CSIS, an imbalance in the CX3CL1/CX3CR1 axis was only observed in the striatum and hippocampus, with this ratio decreasing as a function of stress due to the significant increase in the expression of CX3CR1 in the three structures analyzed (prefrontal cortex, striatum, and hippocampus). It is well known that neurons express high levels of CX3CL1, whereas CX3CR1 is found almost exclusively in microglia [15,41], suggesting that heightened CX3CR1 expression in females may indicate greater neuron-microglia cross talk and, potentially, a greater need for neuronally expressed CX3CL1 in the regulation of microglial activation. In this regard, the elevated levels of CX3CL1 observed only in the prefrontal cortex probably regulate the microglia overproduction of inflammatory mediators and the glutamate-mediated neurotransmission tone in this structure [102]. In contrast, the lower CX3CL1/CX3CR1 ratio observed, due to elevated CX3CR1 levels in the striatum and hippocampus, may explain the inflammatory imbalance in these structures [44]. The well-known CX3CR1-mediated inhibition of the proinflammatory capacity of microglia may explain the reduced levels of IL-6, IL-1β and TNF-α observed following CSIS. The reduction in proinflammatory cytokine expression, together with the observed increase in CX3CR1 expression after CSIS in female mice, supports the relevance of the role played by the CX3CL1/CX3CR1 axis in the regulation of the microglia function, as well as in the development of stress-induced depressive behavior [35]. Interestingly, the generalized reduction in IL-10 levels observed may prevent CX3CR1 downregulation [88], thereby helping to mitigate neuroinflammation and mood disorders after CSIS in female mice. Although the current conflicting observations do not provide a coherent picture of the role of the CX3CL1/CX3CR1 axis in depressive disorders following exposure to chronic stress [35,63,72,20], these results nevertheless support the idea that neuron-microglia communication via the CX3CL1/CX3CR1 pathway may attenuate the effects of CSIS on depressive-like behavior and cognitive impairment in female mice.

Tumor development also affected the balance of the CX3CL1/CX3CR1 axis, but differently from stress and differently also in the various brain structures analyzed. While in the striatum, the significant increase in CX3CL1 and CX3CR1 expression did not alter the axis balance, in the PFC, the CX3CL1/CX3CR1 ratio was increased by the increase in CX3CL1 expression, and in the hippocampus, the tumor reduced the CX3CL1/CX3CR1 ratio by increasing CX3CR1 expression and decreasing CX3CL1 expression. This decrease in CX3CL1 expression, which was only observed in the hippocampus, may indicate a loss of neuronal control of microglia in the tumor-associated hippocampus [28,62], and may explain the lack of a proinflammatory ratio in this structure. However, the interaction between the two factors studied shows that this effect only occurs in non-stressed subjects, and that the greatest imbalance in the CX3CL1/CX3CR1 axis occurs in stressed subjects inoculated with the experimental tumor in the hippocampus, where the most negative ratio is observed. However, and despite growing interest in the involvement of the CX3CL1/CX3CR1 axis in different brain disorders, the results remain controversial, and further research is required. Sex differences may again be one of the factors that help explain the diversity of results due to the dimorphism that exists between males and females in microglial-induced inflammation [32]. In this sense, tumor development in female mice only generated a pro-inflammatory imbalance in the striatum (higher IL-1β/IL-10 ratio, and higher TNF-α/IL-10 ratio), mediated by a significant decrease in IL-10, as in the case of social stress. The absence of significant effects of this anti-inflammatory interleukin in the hippocampus eliminates the inflammatory profile in this structure, where a significant decrease in IL-1β (hippocampus) was observed. Interestingly, the interaction between the two factors analyzed shows that this decrease in cytokine expression in tumor-bearing mice is especially acute in stressed subjects (IL-1β in the HC and IL-10 in the striatum).

Although social instability stress and tumor development have been found to have independent effects on behavior and neuroendocrine activity, these effects on inflammatory neurochemistry do not rule out a possible synergistic effect of both factors on neuroimmunomodulatory activity. The results presented here highlight the complexity of the mechanisms underlying the effects of social stress on the course of tumor development and vice versa, and underscore the need for experimental approaches that allow us to take sex differences into account when exploring this issue.

CRediT authorship contribution statement

Alina Díez-Solinska: investigation, formal analysis, data curation, visualization, writing-original· **Andrea Lebeña:** investigation, methodology, writing-original· **Larraitz Garmendia:** conceptualization, validation, writing-original, funding acquisition· **Ainitze Labaka:** supervisión, writing-review· **Garikoitz Azkona:** writing-original, visualization, supervision· **Joana Perez-Tejada:** supervisión, writing-review· **Oscar Vegas:** conceptualization, investigation, resources, writing, supervision, project administration, funding acquisition.

Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2022.114063.

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