Universidad del País Vasco
Euskal Herriko Unibertsitatea

Doctoral Thesis

Effects of Chronic Social Stress on neuroendocrine and neurochemical function, immune response and behavior: insights from experimental models in male and female mice

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San Sebastián, July 2024

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San Sebastián-Donostia, Julio de 2024

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Esta Tesis ha sido realizada gracias al contrato de Investigador Predoctoral en Formación PIFG20/04, disfrutado de octubre de 2020 a agosto de 2022, y a la ayuda económica de la Beca Predoctoral para la Formación de Personal Investigador (PIF) PIF22/192 de la Universidad del País Vasco/Euskal Herriko Unibertsitatea, disfrutada desde abril de 2023 hasta julio de 2024.

A mi familia

ACKNOWLEDGEMENTS

La *etapa Tesis,* estoy segura, es y ha sido uno de los periodos más enriquecedores tanto a nivel profesional como personal. No sólo han sido los conocimientos formales que he adquirido, sino también todas las experiencias que he tenido y todas las personas que he conocido, tanto dentro como fuera del doctorado, las que han hecho de esta etapa un aprendizaje continuo. Ahora que ya me encuentro en la fase final de escritura y de dar forma a todo el trabajo de estos años, es cuando echo la vista atrás y valoro tanto el haber contado con todas las personas que han formado parte de esto. Y es que, muchas veces, es inevitable flaquear, llegando a sentir la Tesis como un camino solitario, y, por ello, es fundamental contar con personas que nos motiven a seguir adelante. Afortunadamente, yo he podido contar con esas personas.

En primer lugar, me gustaría agradecer a mis Directores de Tesis, Oscar y Garikoitz, por su apoyo y ayuda constantes. A ti, Oscar, por haber estado ahí desde mucho antes de comenzar la Tesis; por siempre aconsejarme, muy acertadamente, en mi presente y futuro profesional; por motivarme, desde que era estudiante, a adentrarme en el mundo de la investigación; y por siempre atender y escuchar mis dudas e inquietudes. A ti, Gari, por haber aparecido en uno de los momentos de más intenso trabajo de la Tesis, por haberme enseñado a ver más allá de los datos, a trabajar y reflexionar sobre ellos, y porque, gracias a tu siempre presente buen humor, el trabajo ha sido mucho más llevadero. A los dos, por haberme incitado a estudiar, investigar y escribir, por haber hecho del laboratorio y del pasillo lugares de trabajo en equipo. Gracias por haber conseguido que haya disfrutado tantísimo de esta etapa. También quisiera

agradecerte de manera especial a ti, Zuri, por acceder a ser mi supervisora internacional en la Universidad de California, Monterey. Gracias por tu cálida acogida, por integrarme en la vida estadounidense, por todos tus consejos, por implicarte tanto en mi formación y orientarme en mi futuro, y por enseñarme otras maneras de hacer ciencia. Hertfelt thanks, Zuri. Por supuesto, a mis compañeras de trabajo y amigas Ibane, Nora y Ania, por haber sido un apoyo imprescindible durante la Tesis, por todos los momentos y éxitos que hemos compartido después de tanto esfuerzo, y por todo lo que nos llevamos de esta etapa. También a los demás doctorandos, Yeray, Jara, Borja, Paula… por ser la extensión del pasillo, por haber compartido momentos y confidencias, porque la Tesis nos ha unido y porque mejor tarde que nunca. Agradecer también a todo el equipo de *Psicobio,* por el buen ambiente y las buenas palabras que siempre he recibido de vosotros y vosotras, por haberme enseñado paciente y cariñosamente, por las comidas y las pausas café, y por haber estado presentes durante todo el proceso. *Mila esker.*

Fundamental ha sido siempre mi familia, y no lo ha sido menos desde que empecé los estudios. A mi madre y a mi padre, sin cuyo apoyo y sacrificio no habría podido lograr nada de lo que he conseguido. Gracias por apoyarme incondicionalmente, por el cariño, por la paciencia, por darme siempre la mejor educación posible, por enseñarme los valores de la constancia y la perseverancia, por confiar ciegamente en mí y en mi carrera profesional, y, en definitiva, por estar siempre. Por todo ello, y por mucho más, dedicaros estos años de trabajo doctoral es mi modesta forma de expresar mi más profundo agradecimiento. A mi hermano también gracias. Porque no hacen falta palabras

para entendernos, porque también me has apoyado en este proceso y porque sé que puedo contar contigo. Gracias, familia.

Por supuesto, no podría dejar de agradecer a todas las personas, conocidos, amigos y amiguísimos que habéis estado ahí, siendo partícipes de alguna forma, de todo o de algún momento de la Tesis. A mis amigas de Burgos, Mar, Lisa, Paula C, Carla, Paula A, Yoanna, por servirme de paréntesis siempre que he vuelto a casa, por acompañarme cuando lo he necesitado y por servirme de confidentes, de respiro y de desconexión. A Alba y Enara, porque habéis estado ahí desde que empezó una de las mejores etapas de nuestras vidas en San Sebastián. Por todos los momentos que tanto han servido a mi crecimiento personal. Por todo lo que he aprendido y disfrutado de y con vosotras y por todo lo que nos queda. A mis amigos de *El Secreto*, Ali, Carla, María, Álvaro, David, Nacho, Joserra, Guille y Rafa, por haberos conocido, por todo el cariño con el que guardo Bélgica y todas nuestras escapadas, porque con vosotros celebré el primer paper y por las relaciones de amistad a distancia. A Adri y Erlaitz, porque no podría haber tenido mejores compañeros de piso. Por todas las cenas y series hemos compartido, por vuestro sentido del humor, por la increíblemente fácil y siempre agradable convivencia que hemos tenido y porque siempre que piense en esta etapa pensaré en vosotros con tantísimo cariño. Por último, a tantos otros amigos que habéis estado ahí Andreea, Naia, Nacho, Álvaro, David, Manu, Juan… gracias por vuestro afecto y vuestra presencia a lo largo de esta etapa.

Gracias de corazón a todos y todas. Eskerrik Asko.

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ACRONYMS

5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-HydroxyTryptamine, serotonin
3-HK	3-HydroxyKynurenine
α-AR	Alpha Adrenergic Receptor
β-AR	Beta Adrenergic Receptor
A	Adrenaline
AA	Active-Aggressive
ACTH	Adrenocorticotropic hormone
ANS	Autonomic Nervous System
APC	Antigen Presenting Cell
AR	Adrenergic Receptor
AVP	Arginine Vasopressin
BBB	Blood Brain Barrier
BCR	B Cell Receptor
CD4+ T helper	Cluster of Differentiation 4 positive T helper cell
CD8+ T cell	Cluster of Differentiation 8 positive T cell
CNS	Central Nervous System
CORT	Corticosterone/cortisol
CRH	Corticotropin-releasing hormone
CS	Chronic Stress
CSDS	Chronic Social Defeat Stress
CSIS	Chronic Social Instability Stress
CSS	Chronic Social Stress
CX3CL1	C-X3-C motif chemokine ligand 1, Fractalkine

CX3CR1	Fractalkine Receptor
DA	Dopamine
DC	Dendritic Cell
DOPAC	3,4-dihydroxyphenylacetic acid
GAS	General Adaptation Syndrome
GC	Glucocorticoid
GR	Glucocorticoid Receptor
HC	Hippocampus
HPA	Hypothalamic-Pituitary-Adrenal (axis)
HPG	Hypothalamic-Pituitary-Gonadal (axis)
HPT	Hypothalamic-Pituitary-Thyroid (axis)
HS	High Sociable
HT	Hypothalamus
IL-1β	Interleukin-1 Beta
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-4	Interleukin-4
IL-6	Interleukin-6
INF	Interferon
iNOS	inducible Nitric Oxide Synthase
IS	Immune System
Kyn	Kynurenine
Kyna	Kynurenic Acid
LS	Low Sociable

mCX3CL1	Membrane CX3CL1 form
ME	Median Eminence
MHC	Major Histocompatibility Complex
MHPG	3-methoxy-4-hydroxyphenylglycol
MR	Mineralocorticoid Receptor
NA	Noradrenaline
NK	Natural Killer
NO	Nitric Oxide
NORT	Novel Object Recognition Test
OFT	Open Field Test
PFC	Prefrontal Cortex
Phe	L-Phenylalanine
PNS	Peripheral Nervous System
PR	Passive-Reactive
PSNS	Parasympathetic Nervous System
PVN	Paraventricular Nucleus of the Hypothalamus
ROS	Reactive Oxygen Species
SAM	Sympathetic Adreno Medullar (axis)
sCX3CL1	Soluble CX3CL1 form
SIT	Social Interaction Test
SNS	Sympathetic Nervous System
SPT	Sucrose Preference Test
ST	Striatum
TCR	T Cell Receptor
Th	T helper

TNF-α	Tumor Necrosis Factor alpha
Tryp	Tryptophan
Tyr	L-Tyrosine

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ABSTRACT

In the scientific field, chronic stress refers to a sustained state of physiological and psychological activation due to continuous input of environmental demands, commonly referred to as stressors, which can negatively affect individuals' health. Nowadays, it represents a significant public health concern given its potential impact on individuals of all ages, sexes or ethnicities. Consequently, its prevalence has increased among the population in the recent years. The study of stress, its nature, development and health implications, is essential for developing interventions with the aimed at preventing or reducing its negative effects on physical and mental well-being. The study of individual differences is also crucial, incorporating not only biological differences, such as sexual dimorphism, but also behavioral ones. Thus, investigating both men and women, along with their intrinsic behavioral traits, is essential for comprehending how stress affects individuals based on their specific characteristics rather than studying stress as a phenomenon that affects the entire population equally. Animal models are particularly relevant to achieve this objective, as acquiring a representative sample of both males and females and investigating their physiological and behavioral differences is comparatively more feasible than in human research. Therefore, this thesis presents the results obtained in four experimental studies, two in males and two in females, which analyze the different physiological and behavioral consequences of chronic stress in mice taking into account sex and individual behavioral differences.

Laboratory male mice are usually housed individually in between the experimental procedures. However, this isolation can be a source of stress in

sociable animals like mice. Thus, the first study aimed to examine the physiological consequences of social isolation on male mice, to determine if this condition could affect the results of the following experimental studies on chronic stress. Indeed, isolation induced significant changes in the level of white blood cells, alongside with alterations in fecal corticosterone, thus revealing that isolation can induce alterations in physiological variables and, therefore, must be considered when studying stress in mice.

In the subsequent study, male mice were submitted to a chronic social defeat stress model (CSDS) based in the resident-intruder paradigm. The main objective of this investigation was to assess its effects on neuroendocrine and neurochemical functions as well as on behavior. Furthermore, it sought to explore if behavior displayed by intruders during resident-intruder encounters (the coping strategies), could be making an individual more susceptible or resilient to the effects of stress. The results indicated that chronic stress precipitated anhedonia and alterations in catecholamine and indolamine pathways across different brain regions, even though mice did not show a different physiological activation pattern regarding the coping strategy adopted. To determine whether the observed trend in males was maintained in females, in the third study, female mice were submitted to chronic social stress. However, the stress protocol applied in females was different that of males, given their inherent differences. Therefore, in order to firstly ensure whether the proposed stress procedure (chronic social instability stress (CSIS)) could indeed elicit physiological and behavioral changes in females, the third study aimed to investigate the effects of this model on the neuroendocrine and immune systems, as well as its potential impact on tumor development and depressive- and anxiety-like behaviors. This

was crucial to establish it as a viable stress model in females. Since alterations were indeed found at the neuroendocrine and immune systems, as well as in the displayed mobility, the same stress procedure was employed in the fourth and last study. The fourth article was aimed to explore the changes in neuroendocrine parameters and cerebral neurochemistry, particularly examining whether the intrinsic social behavior of females could modulate the consequences of social instability stress. Stress did elicit neuroendocrine and neurochemical alterations, with some differences between high and low sociable mice, although sociability should be studied in depth to determine if it indeed plays a key role in the stress response.

These studies provide significant insights into the complex interplay between chronic stress, coping mechanisms, sociability and physiology in mice models. These findings provide an overview of the differences observed between males and females exposed to chronic social stress, as well as differences based on individual behavioral characteristics, laying the groundwork for future research aimed at exploring the relationship between physical and mental states and, consequently, the potential underlying mechanisms of stress-related disorders.

RESUMEN

En el ámbito científico, el estrés crónico hace referencia a un estado sostenido de activación fisiológica y psicológica debido a la continua exposición a estímulos ambientales demandantes, comúnmente conocidos como estresores, que pueden afectar negativamente la salud de los individuos. En la actualidad, representa un problema significativo para la salud pública debido a su potencial impacto en personas de todas las edades, sexos o etnias. Como consecuencia, su prevalencia ha aumentado entre la población en los últimos años. El estudio del estrés, su naturaleza, desarrollo e implicaciones para la salud, es esencial para desarrollar intervenciones destinadas a prevenir o reducir sus efectos negativos en el bienestar físico y mental. El estudio de las diferencias individuales también es fundamental, incorporando no solo diferencias biológicas, como el dimorfismo sexual, sino también conductuales. Por lo tanto, investigar tanto a hombres como a mujeres, junto con sus características comportamentales intrínsecas, es esencial para comprender cómo el estrés afecta a los individuos en función de sus características específicas en lugar de estudiar el estrés como un fenómeno que afecta por igual a toda la población. Los modelos animales son particularmente relevantes para lograr este objetivo, ya que obtener una muestra representativa de machos y hembras y estudiar sus diferencias fisiológicas y comportamentales es comparativamente más factible que en la investigación en humanos. Por ello, esta tesis presenta los resultados obtenidos en cuatro estudios experimentales, dos en machos y dos en hembras, que analizan las diferentes consecuencias fisiológicas y comportamentales del estrés crónico en ratones teniendo en cuenta el sexo y las diferencias comportamentales individuales.

Por lo general, en un laboratorio, los ratones machos se estabulan individualmente entre los procedimientos experimentales. Sin embargo, el aislamiento puede ser una fuente de estrés en animales sociales como los ratones. Por lo tanto, el primer estudio tuvo como objetivo examinar las consecuencias fisiológicas del aislamiento social en ratones machos, para determinar si esta condición podría afectar los resultados de los siguientes estudios experimentales sobre estrés crónico. De hecho, el aislamiento indujo cambios significativos en los niveles de glóbulos blancos, junto con alteraciones en la corticosterona fecal, lo que revela que el aislamiento puede inducir alteraciones en variables fisiológicas y, por lo tanto, debe considerarse al estudiar el estrés en ratones. En el estudio siguiente, los ratones machos fueron sometidos a un modelo de estrés crónico por derrota social (CSDS) basado en el paradigma residente-intruso. El objetivo principal de esta investigación fue evaluar sus efectos en las funciones neuroendocrinas y neuroquímicas, así como en el comportamiento. Además, se buscó explorar si el comportamiento exhibido por los intrusos durante los encuentros entre residentes e intrusos (las estrategias de afrontamiento), podría hacer que un individuo fuera más susceptible o resistente a los efectos del estrés. Los resultados indicaron que el estrés crónico precipitó anhedonia y alteraciones en las vías de las catecolaminas e indolaminas en diferentes regiones cerebrales, aunque los ratones no mostraron un patrón de activación fisiológica diferente en función de la estrategia de afrontamiento adoptada. Para determinar si la tendencia observada en los machos se mantenía en las hembras, en el tercer estudio, las hembras también fueron sometidas a estrés social crónico. Sin embargo, el protocolo de estrés fue diferente al de los machos, dadas sus diferencias

inherentes. Por lo tanto, para determinar en primer lugar si el procedimiento de estrés propuesto (estrés crónico por inestabilidad social (CSIS)) podía inducir cambios fisiológicos y comportamentales en las hembras, el tercer estudio tuvo como objetivo investigar los efectos de este modelo en los sistemas neuroendocrino e inmunológico, así como su impacto potencial en el desarrollo tumoral y en conductas tipo depresivas y ansiosas. Esto fue decisivo para establecerlo como un modelo de estrés viable en hembras. Dado que se encontraron alteraciones en los sistemas neuroendocrino e inmunológico, así como en la movilidad, se empleó el mismo procedimiento de estrés en el cuarto y último estudio. El cuarto artículo tuvo como objetivo explorar los cambios en los parámetros neuroendocrinos y la neuroquímica cerebral, examinando particularmente si el comportamiento social intrínseco de las hembras podría modular las consecuencias del estrés por inestabilidad social. El estrés indujo alteraciones neuroendocrinas y neuroquímicas, con algunas diferencias entre ratones más y menos sociables, aunque la sociabilidad debe estudiarse a fondo para determinar si realmente juega un papel clave en la respuesta al estrés.

Estos estudios proporcionan información significativa sobre la compleja interacción entre el estrés crónico, las estrategias de afrontamiento, la sociabilidad y la fisiología en modelos de ratones. Estos hallazgos ofrecen una visión general de las diferencias observadas entre machos y hembras sometidos a estrés social crónico, así como de las diferencias en función de las características conductuales individuales, sentando las bases para futuras investigaciones dirigidas a explorar la relación entre estados físicos y mentales y, con ello, los posibles mecanismos subyacentes a los trastornos relacionados con el estrés.

SECTION I: OVERVIEW

Introduction and Theoretical Framework

1. INTRODUCTION

In scientific research, a considerable amount of published studies have mostly been centered on men or, alternatively, on male animal models. Females, as experimental subjects, remain noticeably underrepresented in the samples of the experimental studies within the fields of psychology, behavioral neurosciences and social sciences. Although this sex imbalance has decreased over the years and an increasing number of studies are taking into account sexual dimorphism, there continues to be a greater emphasis on studying men or males compared to women or females in the context of psychobiology, and, more specifically, in the psychoneuroimmunology of stress.

This assertion is sustained on the basis of a systematic review search that I undertook during the predoctoral research period. Its primary goal was to gather information on the State of the art in this field with the aim of creating a conceptual framework that encompassed information regarding the effects of stress on the physiology and behavior in both male and female mice. At the beginning, the primary aim of the review was to examine the differences in the behavioral coping strategies of both sexes when subjected to chronic social stress, as well as to explore if there were any differences in their underlying physiological mechanisms. After an initial search and overview, I observed a significant scarcity of studies that included females in their sample. Consequently, the systematic review, which initially embraced both male and female subjects, eventually evolved into a study that exclusively examined male mice. In this sense, my personal experience serves as an example of the research landscape, in which the lack of reliable information on a given subject matter risks perpetuating a cycle of misinformation if not addressed. Thereby, it

becomes important to consider conducting studies that include both male and female subjects, thereby achieving a more ecologically valid and representative viewpoint.

In my particular case, I wanted to contribute to this objective by including both male and female subjects in my Doctoral Thesis and by always considering their idiosyncratic characteristics. Thus, given their inherent differential characteristics, I tried to explore stress response in male and female mice by carrying out different experimental procedures that involved different stress protocols and laboratory techniques and therefore obtaining a wide range of results.

For its part, male mice frequently exhibit territorial and aggressive behaviors when confronted by intruders in their territory. For this reason, one of the most used stress models is referred to as "Chronic Social Defeat Stress" (CSDS). In fact, this model involves a confrontation between two males, one acting as the resident and the other as the intruder, resulting in stress induction in the intruder male. Hence, this was the model employed to examine stress physiological and behavioral consequences in male mice. Precisely, due to their idiosyncrasy, male mice are usually kept in isolated conditions until the start of the social defeat stress procedure. Nevertheless, this period of isolation during which male mice adapt to their new environment in the laboratory facilities prior to the stress procedure may act as a stressor itself. In fact, some studies have implemented large periods of isolation in order to study chronic stress. This prompt me to question whether the isolation in which males are frequently housed before the actual stress procedure could be biasing the physiological outcomes

observed after implementing CSDS. For that reason, I also sought to explore the physiological response of mice following an isolation condition. Thus, alongside the CSDS, I also examined the effects of a 4-week period of social isolation in the physiological response of male mice.

On the other hand, when attempting to extend this research to female subjects, I encountered certain challenges. Not only was there a considerable lack of scientific literature, but the intrinsic behavioral characteristics of females differed significantly from those of males. Although female mice also form hierarchies and can display aggressive behaviors, these behaviors are less frequently observed in comparison to males. Additionally, females do not exhibit the same level of territoriality and generally exhibit fewer observed dominant behaviors, even though it could be possible that female mice engage in other less easily observable dominant behaviors. Consequently, the CSDS proved to be an unsuitable stress model for female mice. Given their heightened sociability and natural inclination to live in groups, I implemented a different model of stress referred to as Chronic Social Instability Stress (CSIS). This model was designed to create unpredictable social conditions by subjecting female mice to alternating periods of isolation and overcrowding over a four-week period. The unexpected and different results obtained in female mice compared to what is usually found in males, make CSIS a more interesting model for studying stress in females. Thus, I aimed to take an additional step and, in addition to exploring the effects of stress on the behavioral and physiological response of females, I aimed to examine whether intrinsic sociability could be mediating the response of female mice to stress. This approach, exploring the role of the inherent sociability in the

stress response, can provide a more extensive understanding of the role of this new variable in the female mice response to stress.

This Doctoral Thesis aims to underline the importance of including both sexes in the preclinical and experimental psychology research in order to attain a more representative sample that reflects the existing variability among sexes. Therefore, through this approach, individual differences within the population are implicitly attended, resulting in a better understanding of the stress response. This is crucial for advancing research in the clinical field, which would eventually lead to treatments for stress-related disorders that consider the physiological and behavioral differences between individuals.

FOREWORD

Nowadays, stress is a very frequently used word in everyday discourse. It has become one of the most commonly used terms by the general population to describe unpleasant and negative events, such as traumatic episodes, thus associating it with a negative connotation. However, stress itself is not an illness and is not necessarily prejudicial. The raison d'être of the stress response to an aversive or threatening stimulus is precisely survival, is the physical preparation of an individual to face such stimuli and to adapt to the new environment. The demanding life and social circumstances that require an extraordinary effort from us and consequently act as sources of stress are unlikely to diminish. Being able to generate an effective response to confront them and thus adapt to the new situation, whether temporary or permanent, has been and still is crucial for our continuity as individuals and as specie.

Stress is adaptive and essential for survival.

Instead of viewing stress as the enemy and attempting to avoid it at all costs, I advocate for understanding it from a multidimensional perspective to assess its actual risks and even uncover its potential benefits.

2. THEORETICAL FRAMEWORK

2.1. INTRODUCTION TO CHRONIC SOCIAL STRESS AND ITS HEALTH IMPLICATIONS

The correlation between psychological and physical health has been a main subject of extensive research throughout history, spanning from ancient philosophical inquiries to contemporary studies in medicine and psychology. In the psychological field, studies on this topic have become increasingly important, although their origins can be traced back to two important figures: Wilhelm Wundt (1832-1920) and William James (1842-1910). Both of them were particularly interested in the study of the mind-body relationship. On the one hand, W. Wundt, acknowledged as the founder of modern psychology and recognized for establishing the first laboratory specialized on experimental psychology, suggested that physical and mental states were interrelated in the human experience, thus, emphasizing their interdependence. Similarly, W. James was interested in this relationship and, more specifically, in the role of mind. This philosopher and psychologist proposed the functionalist role of the mind, underlying its importance in facilitating our adaptation to the environment. Since those early hypotheses and investigations on mental-physical interactions, understanding the complex interrelation between mental states and physical health, together with the influence of environmental and contextual factors on this interaction, has become a significant topic of interest in the psychological area.

In this context, social stress stands out due to its dual nature, as it involves environmental and psychological components, and it has demonstrated to exert an effect at a physiological level. In this regard, in the early years of the

last century, psychologist Walter B. Cannon (1871-1945) conducted different studies precisely in order to explore the physiological reaction to different stimuli perceived as stressful, including hunger, cold or emotional disturbances. After the exposure to some of those stressors, W. B. Cannon observed an automatic physiological response triggered by the activation of the Sympathetic Nervous System (SNS). He observed that physiological functions responsible for maintaining the body's energy reserves during rest were either intensified or temporarily halted in response to a stressor, enabling the mobilization of a significant amount of energy. This energy mobilization allowed the individual to confront the stressful situation by engaging in behaviors such as escape, attack, or defense responses. In this way, W. B. Cannon introduced the *fight or flight* response by which psychological distress or strong emotions affected the physical state by activating the SNS, prompting the release of adrenaline (A), also termed as epinephrine (EPI), from the adrenal medulla and thereby preparing the individual for either fight or fly (Cannon, 1914, 1915). The secretion of A allows to maintain *homeostasis*. This term was also proposed by W. B. Cannon and it constitutes a key concept for the study of the relationship between mind and body and the subsequent studies on the field of stress. *Homeostasis* refers to the balance that the body maintains by activating self-regulating mechanisms in order to preserve the internal stability despite external challenges or highly demanding external requirements (Cannon, 1929). Thus, W. B. Cannon proposed that stressful stimuli elicit adaptive physiological mechanisms that are designed to provide the organism with the energy and physiological needs to cope with the stressor. Once the stressor ceases, the body returns to its original state of homeostasis. Therefore, these physiological alterations occur with the

aim to maintain long-term homeostasis, that is, the stable internal balance, in a temporarily altered environment by a stressor.

Following this line of study of the organism's response to stress, Hans Selye (1907-1982) was a scientist and physician pioneer in this field of research. He is widely recognized as one of the most important figures in this area as he introduced and thoroughly defined the term *stress*, laying the groundwork for the development and subsequent study of the concept. In fact, stress is one of the most studied topics in psychology research domains, in particular in basic and experimental psychology (McEwen et al., 2015). According to him, stress involves a series of physiological responses to a perceived threat, whether real or imagined, to an individual's well-being. Although he started studying the effects of acute stress on physiological functioning, his research evolved towards understanding chronic stress and its implications in health and well-being. Selye was particularly interested in the physiological response to persistent stressors over time and he suggested that a chronic physiological activation due to chronic stress could have adverse effects in psychological and physical health. Hence, during these years, he developed a new concept to which he referred to as *General Adaptation Syndrome* (GAS), with which he precisely described the physiological changes after a prolonged exposure to stressful conditions. From his standpoint, individuals could face the stressful stimuli either by engaging adaptive responses, as described by GAS, or by engaging non-adaptive responses, considered as such due to their inadequacy, repetitiveness, or excessiveness, and referred to by Selye as diseases of adaptation (Selye, 1936, 1946).

Since then, various researchers have focused on studying the effects of chronic stress on health. It has been demonstrated that chronic stress can lead to numerous alterations in the organism, such as weight loss, atrophy in the thymus, hypertrophy in the adrenal gland (Sapolsky, 2002), alterations in brain structures such as the hippocampus (HC) (Magariños et al., 1996; Sapolsky, 1985) or immunosuppressive effects (Dhabhar & McEwen, 1997).

The immunosuppression as a result of chronic stress (CS), has been one of the most extensively studied effects following chronic stress as the impairment of the immune system could make an individual more susceptible to suffer other diseases (Cohen et al., 2001; Irwin & Miller, 2007; Kiecolt-Glaser et al., 1996). Thus, CS has been studied and, in some cases, identified as a key contributing factor in the onset or development of physical and emotional illnesses (Korte et al., 2005; McEwen, 2003; McEwen & Wingfield, 2003) such as cancer (Dai et al., 2020; Parker et al., 2004; Thaker et al., 2006) and depression (Kendler et al., 2003; Slavich et al., 2010a, 2011). Remarkably, the nature of the stressor also plays a crucial role in the progression of these diseases. Among them, stressors of a social, unpredictable and uncontrollable nature have been demonstrated to be a risk factor in the development of these diseases (Cherry et al., 2006; Matheson et al., 2006; Pflanz & Ogle, 2006; Sandin et al., 2004).

This all underlines the importance of broadly understanding the physiological and behavioral response to chronic social stress (CSS) and its negative health issues. This understanding could be useful when approaching different treatments for various illnesses that may be influenced by stress, ensuring that its potential influence is taken into account.

2.2. PHYSIOLOGICAL AND BEHAVIORAL RESPONSE TO STRESS

To comprehend the effects of stress on the immune system and thus on the development of diseases, it is essential to understand the underlying physiological mechanisms in response to stress. It has already been discussed the GAS as part of this physiological response. This syndrome involves a series of changes in the body aimed to cope with the stressor and adapting to it if it remains stable over time. These changes are precisely part of what is called *allostasis* (Sterling & Eyer, 1988). *Allostasis* refers to the process by which an organism aims to maintain the internal balance, or *homeostasis*, through various physiological changes in response to a stressful stimuli (McEwen, 1998b, 2004). In fact, the GAS involves an allostatic process, as the organism tries to adapt to the changes in the environment while maintaining internal balance. This process can vary regarding the type and intensity of the stressor. However, it usually entails the release of two hormones, cortisol (CORT) and noradrenaline (NA), which mediate the stress response and are the final substances released following activation of the hypothalamic-pituitary-adrenal (HPA) and the sympathetic-adreno-medullary (SAM) axes, respectively. Nevertheless, if these hormones are not adequately liberated because they are either over-released or because they are not metabolized correctly persisting in the organism once they are no longer required, they can result in what is known as *allostatic load* (McEwen, 2004, 2007). McEwen and Stellar (1993) introduced this physiological state and defined it as following:

> The strain on the body produced by repeated ups and downs of physiologic response, as well as by the elevated activity of physiologic

> systems under challenge and the changes in metabolism and the impact of wear and tear on a number of organs and tissues, can predispose the organism to disease (p.2094) (McEwen & Stellar, 1993).

Numerous studies have revealed some of the negative health consequences of the *allostatic load*. Among others, Seeman et al., (2001) assessed *allostatic load* using different biological parameters of physiological functioning, including primary mediators (such as CORT, A or NA), metabolic effects of primary mediators (cellular processes involving enzymes or receptors) and secondary mediators (such as blood pressure or heart rate). Higher scores on baseline levels of *allostatic load* were associated with the development of diseases and health impairments, such as cardiovascular diseases or cognitive dysfunction, even a higher mortality risk (Juster et al., 2010). Its close relationship with the development of diseases has led to an increase in the number of studies and it has been found that *allostatic load* has been linked to stressful life experiences emerging from social interactions, such as the position in social hierarchy or psychosocial deprivation (Singer & Ryff, 1999; Weinstein et al., 2003). These data suggest that chronic social stressors can lead to negative health outcomes, and thus it is crucial to amplify the study of stress to social stress and to analyze stress from a holistic perspective considering both physical and psychosocial factors and its role in susceptibility to develop different illnesses.

2.2.1. Physiological response to stress

The physiological response to stress is a fundamental process by which the organism reacts and adapts to stressful stimuli. This process encompass a

wide variety of underlying biological mechanisms including hormonal, neural and metabolic responses that are essential for the survival of the individual. In this context, the GAS proposed by Selye (1946) and defined as “the sum of all non-specific, systemic reactions of the body which ensue upon long continued exposure to stress” (p.119) is crucial to describe the physiological response to a stressful condition. The GAS refers to the non-specific physiological changes that are usually observed regardless of the nature of the threatening stimulus presented. When stress becomes a chronic condition, the GAS is eventually divided into three stages: the “alarm reaction”, the “stage of resistance” and the “stage of exhaustion”. These stages are characterized by the following (Selye, 1946):

- **Alarm stage:** any stimulus that enables the alarm reaction is called *alarming stimulus*. This stimulus leads to the first physiological response of an organism and includes an over activation of the HPA axis and of the SAM axis, which results in neurovegetative signs. In this stage, two phases have been recognized, although they are not always clearly distinguished. In the first phase of *shock*, the individual may exhibit, among other symptoms, tachycardia, decreased body temperature, decreased muscle tone, gastric ulcers, an increase followed by a decrease in glucose, leukopenia followed by leukocytosis. This phase is followed by the *counter-shock* phase, with a reversal of the experimented symptoms in the shock phase to restore *homeostasis*. Additionally, functions such as sexual behaviors and reproductive processes that demand a substantial amount of energy, but are not essential for individual survival are temporarily suppressed. For instance, the

hypothalamic-pituitary-gonadal (HPG) axis is inhibited the with the resulting inhibition of sexual hormones (Matsuwaki et al., 2006; Rivier & Serge, 1991).

- **Resistance stage:** if the exposure to the *alarming stimulus* is continuous over time, the counter-shock phase gives rise to the next phase, the phase of resistance, during which the organism continues trying to adapt to the new prevailing environmental conditions, that is, *allostasis*. The HPA axis and the SAM axis activity are enhanced in order to deal with the stressor. If this phase is prolonged over time, the over-activation give rise to *allostatic load*. As this phase is unsustainable in the long term, thus, it gives rise to the next phase.
- **Exhaustion stage:** if the stressful stimuli persists, the organism cannot maintain the resistance phase, and, therefore, the initial physiological state of the alarm stage returns. This is called the exhaustion stage.

For this syndrome to take place, a series of changes at the physiological level involving both the activation of the HPA axis and the activation of the SNS and the SAM axis occur. These systems are activated during the alarm phase, releasing CORT and catecholamines, primarily A, which are maintained during the resistance stage, but whose response becomes dysfunctional in the exhaustion stage. These systems are further detailed below.

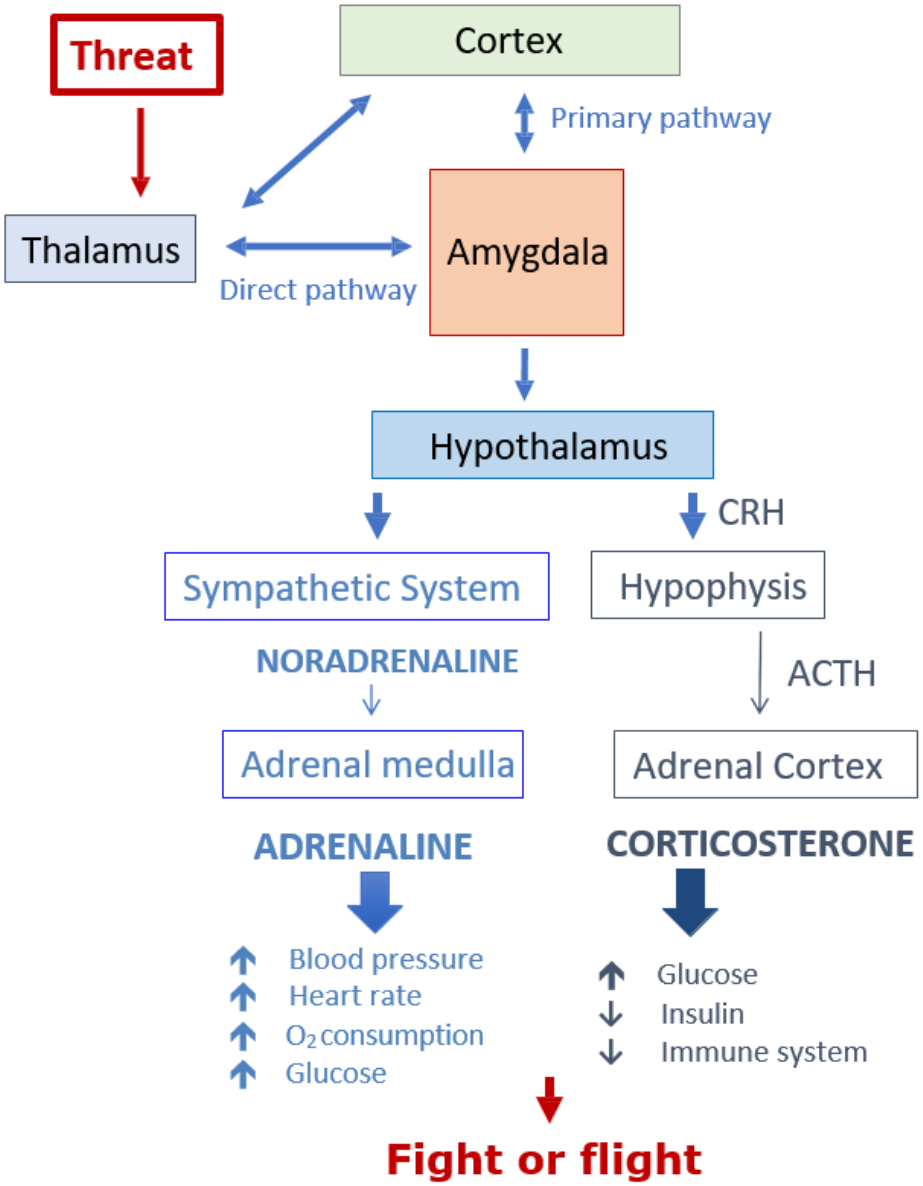

Fig.1. Fight or flight response physiological pathways. The activation of the HPA and SAM axes.

2.2.1.1. Neuroendocrine response: HPA axis

One of the proposed mechanisms underlying the GAS is the activation of the HPA axis. This axis, together with the hypothalamic-neurohypophyseal system, the hypothalamic-pituitary-thyroid (HPT) and the HPG axes are the main systems through which the hypothalamus (HT) and the pituitary gland regulate the relationship between the nervous system and the endocrine system, constituting the neuroendocrine system (Gore, 2013).

The HPA axis includes a complex set of physiological interactions between its three main structures (Gore, 2013):

- The **hypothalamus**. This brain structure lies just above the brainstem, and below the thalamus. It is the central neuroendocrine organ and regulates diverse functions such as growth, basal metabolism or stress response through the release of the growth-hormone releasing hormone (GHRH), the thyrotropin-releasing hormone (TRH) and the corticotropin-releasing hormone (CRH), respectively.
- The **pituitary gland**. It consists of the andenohypophisys (anterior pituitary) and the neurohypohysis (posterior pituitary) and it is the primary target of the HT. When stimulated by the hormones secreted by the HT, it produces and releases new hormones regarding the specific metabolic process.
- The **adrenal cortex**. It is the outer layer of the adrenal glands, situated above each kidney. In the stress response, it is responsible of the glucocorticoids (GCs) release (cortisol in humans and corticosterone in rodents (CORT)) (De Kloet, 2013).

Thus, when an individual faces an aversive stimulus, regardless of whether it is physical or psychological, different brain areas are activated. This cascade of biological reactions starts in the HT, which initiates a series of biochemical reactions that end up in the release of different hormones. The paraventricular nucleus of the HT (PVN) synthesizes and secretes three well-known neuroendocrine peptides: arginine vasopressin (AVP), the CRH and oxytocin (OT) (Sawchenko et al., 1996). While AVP and OT arrive via axonal

transport to the posterior pituitary, the CRH reaches the anterior pituitary through the bloodstream, where it stimulates the secretion of the adrenocorticotropic hormone (ACTH) (Vale et al., 1981). The ACTH reaches the middle layer called *fascicullata*, of the adrenal cortex in the adrenal glands (Herman et al., 2003), in which GC are synthesized from cholesterol (Miller & Auchus, 2011), thereby increasing the amount of steroids in the systemic circulation. As mentioned before, these stress hormones prepare the body to cope with the challenging situation by increasing the amount of energy and eliciting a state of alertness in the individual. When GCs are no longer required, they bind to mineralocortidoid (MR) and glucocorticoid (GR) receptors sequentially, that is, they bind first to MRs due to their higher affinity for them and then to GRs. These receptors are expressed in different peripheral tissues, as well as in different brain structures. However, MRs are significantly more present in the limbic system, specifically in the dentate gyrus and subiculum of the HC, lateral septum, HT and amygdala (AMYG); while GRs are more scattered throughout the brain. Given their lipophilic nature, GCs cross the blood-brain barrier (BBB), enter the brain, and bind to these receptors (McEwen et al., 1968; Reul et al., 1987; Reul & De Kloet, 1985). This binding enable the negative feedback loop of the HPA axis, by which GCs levels decrease because of the signal received by both, the anterior pituitary and the HT. However, when stress becomes chronic, the functioning of this axis and its negative feedback can be altered either due to the dysregulation of the axis itself, resulting in increased GCs secretion, or by a higher resistance of GCs receptors, which would require higher GCs levels to be activated and to trigger the negative feedback (Cohen et al., 2012; McEwen, 1998a).

2.2.1.2. Autonomic Nervous System response: SAM axis

On the other hand, another system involved in the stress response is the ANS. The ANS is part of the peripheral nervous system (PNS) and it is further divided into the SNS and the parasympathetic nervous system (PSNS). The ANS regulates various neurovegetative functions and body processes such as the heart rate, the body temperature, metabolism and digestion, the blood pressure and the breath rate. The physiological response will depend on whether the sympathetic or the parasympathetic system are stimulated and activated. These systems are tonically active, which means that the neurotransmitter release into the synaptic space is continuous, which allows for its constant regulation. The increase or decrease of neurotransmitter release regulates the activation of the tissue to which the system sends the signal. In many of the mentioned physiological processes, SNS and PSNS respond antagonistically in order to allow the body to adapt and to react appropriately to the circumstances. For instance, when the individual is resting, the PSNS is activated so as to maintain the state of relaxation. However, the SNS is essential to induce changes in the relevant organs and tissues to support physical activity or to carry out the *fight-flight* response (Alshak & Das, 2023; McCorry, 2007).

Thus, the SNS activation is fundamental in the stress response. The SNS is constituted by preganglionic (cholinergic fibers) and postganglionic (adrenergic fibers) neurons. The preganglionic neurons originate in the spinal cord and their axons synapse with postganglionic neurons in autonomic ganglions located outside the spinal cord. Within these ganglia, acetylcholine is released and binds to nicotinic receptors on the postganglionic neurons. Postganglionic neurons,

which innervate the effector tissue forming the neuroeffector junction, depolarize and release of A and NA through multiple swellings along the axon, thus stimulating many tissue cells. This stimulation is further enhanced through the gap junctions, which allow the spread of the stimulation to adjacent cells resulting in the alteration of the entire tissue. Both A and NA are part of the adrenergic pathway and can bind to different adrenergic receptors (AR) (alpha-1 (α-1), alpha-2 (α-2), beta-1 (β-1), beta-2 (β-2) and beta-3 (β-3)) which will have different effects depending on the organ or tissue in which they are located (i.e. the activation of alpha-1 receptors contract certain muscles while beta-2 relax them). Notably, SNS activation has different effects including, among others, a higher heart force of contraction and rate of conduction, which results in an increased heart rate; bronchodilation, which allows entering more airflow into the lungs; and decreased intestinal motility which helps to slow down digestion (Alshak & Das, 2023; Boron & Boulpaep, 2009; McCorry, 2007).

In addition to this direct sympathetic effects, preganglionic neurons can directly target adrenal medulla cells, called as chromaffin cells, as they act as modified sympathetic postganglionic neurons. However, instead of releasing neurotransmitters into a synaptic space, these cells synthesize A and NA hormones that are directly released into the bloodstream through which they reach different tissues. The synthesis of these catecholamines is enhanced during stress to prepare the body to cope with the aversive stimulus. This indirect sympathetic pathway involving the adrenal medulla, which is commonly known as the SAM axis (or system), works synergistically with the previously explained direct activation thus enhancing the stress response. In fact, SAM axis reinforces the stress response. The released catecholamines remain in the bloodstream

longer than neurotransmitters in the synaptic space and also reach tissues and organs that neurotransmitters do not. The physiological responses triggered by sympathetic activation ensure increased blood supply to the brain and muscles by decreasing blood supply to non-essential organs for an immediate stress response. Adrenaline release also stimulates glycogenesis in the liver, increasing glucose levels and facilitating a rapid mobilization of stored energy resources (Boron & Boulpaep, 2009; McCorry, 2007).

2.2.1.3. Interaction between the HPA and the SAM axes

The interaction between the HPA axis and the SAM axis plays a crucial role in the stress response. The quick reaction of the SAM axis that has an immediate effect on the organism is complemented by the slightly slower HPA axis activation that ensures an amplified and more prolonged response. These two physiological systems communicate and regulate each other to coordinate the physiological response to stressful stimuli. Nevertheless, the interaction between GCs and catecholamines is complex. Although GCs can exacerbate the effects of catecholamines, they can also exert inhibitory effects on the SAM axis, thereby helping to restore *homeostasis*.

2.2.2. Behavioral response to stress

The activation of the HPA and the SAM axes is part of the adaptive functions of the stress response system (SRS), which is relatively stable across species (Del Giudice et al., 2011; Nesse et al., 2016). As abovementioned, the activation of the HPA axis and the sympathetic pathways is aimed to adapt to new environmental stimuli and it is fundamental in the physiological response to

stress, although their dysregulation or over activation may lead to the onset or progression of certain diseases (Mariotti, 2015; Salleh, 2008; Sapolsky, 2000). However, inconsistent findings have been encountered regarding the health issues after stress exposure. The same events do not necessarily elicit the same effects in all individuals and, in fact, even under highly controlled and standardized laboratory conditions, some individuals have shown to better adapt to stressors. These discrepancies may arise from either the characteristics of the stressor (such as its nature or duration) or from the inherent differences among individuals (Ebner & Singewald, 2017).

Individual differences in stress sensitivity and reactivity may affect individuals' ability to adapt to the given conditions regarding the employed stress coping strategies. This idea was proposed by the American psychologist Richard S. Lazarus (1922-2002), who suggested that the same situation or stimulus could affect people in a different way. According to this author, a stimulus itself is not inherently aversive; rather, it is the perception of an event what determines whether an event is threatening or not. Consequently, its impact on health can vary among individuals based on their evaluation and the effectiveness of their subsequent coping strategies used, that is, the capacity of an individual to cope with it (Lazarus & Folkman, 1984; Ursin & Olff, 1993).

2.2.2.1. Behavioral coping strategies to stress

Coping strategies include a wide range of behavioral responses that individuals develop to manage, minimize, or tolerate stressful situations. These responses vary across individuals, although they remain relatively stable over time and across similar stressors. One of the pioneering works in suggesting

different behavioral responses to stress among individuals was the one of Henry, Ely and Stephens (1974) who proposed two distinct response patterns to stress. They suggested that the behavior typically displayed by an animal within their relatives could be classified as dominant/active or subordinate/avoidant and that each pattern was related to specific physiological correlates. For instance, they revealed that active mice exhibited a more pronounced activation of the SAM axis, whereas avoidant mice displayed a more pronounced HPA response (Henry et al., 1974). Since then, numerous investigations have consistently demonstrated that the behavioral responses to stress are coupled with distinctive physiological and neurobiological responses, which could significantly influence the underlying functioning of the organism, thereby increasing the susceptibility to certain diseases and thus, compromising individuals' health (Rensina F. Benus et al., 1991; J. M. Koolhaas et al., 1999; Marchetti & Drent, 2000).

Understanding the differences between coping strategies is challenging due to substantial variability in nomenclature, its interpretation, and the methodology for its assessment. However, most studies have opted for bicategorical classifications and, of these, many of them have employed the active/proactive and passive/reactive categories (Avitsur et al., 2003; Gómez-Lázaro et al., 2012; Pérez-Tejada et al., 2016; Koolhaas et al., 1999). Active strategies have generally been considered the most effective strategies when facing stress and they have been related to reduced stress levels (Heshmati et al., 2018; Pérez-Tejada et al., 2013) as well as with resiliency (Zhang et al., 2021). On the other hand, passive strategies have frequently been proposed as a model of dysfunctional and maladaptive strategies and have been linked to disease susceptibility and negative health consequences. Despite the relative

consensus that existed on this idea, these statements have been refuted by other studies and theories, such as the match-mismatch theory, which postulates that resilience or vulnerability to disease depends on the adequacy of the coping strategies adopted by an individual for the external demands of the environment (Schmidt, 2011). In this sense, there would be no single optimal coping style, but its appropiateness would depend on the particular circumstances. For example, active or proactive strategies could be more effective if the event that is causing stress can be addressed. However, if this event cannot be controlled, passive strategies may yield further benefits (Cabib et al., 2021). Therefore the suitability of one strategy or the other would depend on the specific circumstances and the involved behaviours.

Active coping, in both animals and humans, is characterized by behaviors aimed at directly engaging with the stressor by using one's resources. For instance, humans can employ various active strategies, such as modifying the stressful event itself, altering their thoughts and feelings regarding it, or seeking for social support. In their case, animals, such as rodents, may exhibit different behaviors, such as territorial defense or flee when confronted with a stressful situation. This coping strategy is what Cannon (1915) originally defined as the *fight or flight* response. Physiologically, these individuals have been characterized by elevated levels of blood NA, heightened SNS reactivity, and diminished HPA reactivity (Dantzer, 1993; Fokkema et al., 1988; Koolhaas et al., 1999; Sgoifo et al., 1996). Engel and Schmale (1972) originally described the passive coping strategies, which are characterized by immobility, low social activity and low levels of aggression. Both animals and humans will resort to strategies such as escaping, avoiding and social withdrawal. This coping style is

related to increased activation of the PSNS as well as heightened reactivity of both the HPA and the SAM axes (De Boer et al., 1990; Korte et al., 1992). Given their different underlying physiological mechanisms, coping styles are also believed to determine individuals' vulnerability or resilience to different stress-related illness. For example, it has been shown that individuals with active or proactive strategies have a higher risk of suffering cardiovascular diseases such as tachyarrythmia or hypertension; gastrointestinal disorders, such as gastric ulcerations; and some behavioral disorders such as violent-like aggressiveness or substance abuse disorder. In contrast, passive individuals have a higher risk of suffering infectious diseases, an increased tumor progression and social phobia and anxiety-like behaviors (Cabib et al., 2021; de Boer et al., 2017; Del Giudice et al., 2011; Sapolsky, 1994; Vegas et al., 2006; Wood, 2014).

Thus, while CS is associated with increased vulnerability to health impairment, not every individual exposed to stress suffers these adverse consequences. The behavioral coping responses that individuals develop have been proposed to mediate the negative effects of stress mitigating or aggravating its impact on health. Hence, it is important to understand the moderating role of individuals' coping strategies and behaviors in the relationship between stress, its neurochemical response and health.

2.3. STRESS AND THE IMMUNE SYSTEM

The Immune System (IS) is a group of different cells and organs that constitute the body's defense system. It protects the organism from the possible invasion of harmful pathogens such as bacteria, viruses or abnormal cells, such as cancer cells, with the ultimate goal of ensuring the survival of the individual.

For the IS to effectively carry out its functions, its components must interact accurately. However, diverse factors can disrupt the balance the IS needs, leading to increased susceptibility to pathogens or to the onset of autoimmune diseases. These factors may stem from physiological, cognitive or emotional sources. Among them, stress stands out as a possible factor, which in fact can be both physical, such as high-intensity exercise, and psychological, such as social stress.

Psychoneuroimmunology is the sub-discipline that studies how these environmental factors can alter the homeostatic balance of the organism and therefore the functioning of the IS. This discipline of study has played a fundamental role in exploring the relationship between these factors, the central nervous system (CNS), the endocrine system, and the IS, as well as the impact these interactions have on health. While investigations exploring the connections between the brain, emotions, and the immune system date back to the early 20th century, the term psychoneuroimmunology was first introduced in 1981 when Robert Ader (1932-2011) published a review presenting findings from studies that revealed intriguing associations among behavior, neural processes, the brain function, and the immune response (Ader, 1981). Some of the most relevant results found for the development of this discipline are the following demonstrated neuroimmune interactions:

Hence, the nervous and the immune systems do not operate independently, but they are involved in a complex network of relationships whereby neurotransmitters, hormones, immune cells and cytokines are interrelated. To better understand these interactions, it is beneficial to have an

accurate overview of the elements of the immune system and their functions in the organism that I are presented below.

2.3.1. Innate immune response

The innate immunity, or non-specific IS, constitutes the first line of defense of the organism. It responds in the same way to all the pathogens and foreign substances that could be harmful to the organism. This system acts immediately and consists of four types of barriers: anatomic (skin and mucosal cells), physiologic (pH or temperature), phagocytic and inflammatory response (immune system cells and proteins).

Myeloid progenitor cells give rise to erythrocytes (red blood cells), platelets, and leukocytes (white blood cells). Leukocytes are fundamental in the immune response and they can be, in turn, divided into phagocytes (dendritic cells (DC), macrophages, and monocytes), granulocytes (basophils, eosinophils, and neutrophils), and lymphocytes (B cells, natural killers (NK) and T cells). The white blood cells are part of the innate immune system except for B and T cells.

When physical and chemical barriers cannot prevent the penetration of microorganisms into the individual, cells damaged by these pathogens secrete histamine. This molecule acts as a vasodilator and thus it dilates the blood vessels and increases their permeability allowing a greater influx of blood flow and, consequently, of leukocytes to the site of cell damage. Here is where they initiate the immune response. Phagocytic cells, such as macrophages, neutrophils and DCs, are the first to act against these pathogens and attempt to eliminate them by phagocytosis. If macrophages and DCs fail to eliminate an

antigen by themselves through phagocytosis, they can act as antigen-presenting cells (APCs), whose function is to detect, capture, and degrade antigens to present them to T cells, which are part of the adaptive immune system and will continue with the immune response (Marshall et al., 2018).

2.3.2. Adaptive immune response

On the other hand, the adaptive immunity occurs when the innate response fail in its purpose. Unlike the innate immunity, the adaptive response targets a specific type of pathogen that has caused the infection, and, thus, it is also known as specific immune response. Therefore, this immune response first involves identifying accurately the antigen that has transgressed the innate immunity. If an antigen had already been previously identified, the response of the adaptive system acts faster and more efficiently due to its ability to "remember" prior encounters with pathogens. This explains why some diseases are only experienced once in a lifetime.

When an antigen invades an organism for the first time and has not been previously presented to the adaptive system, the response typically operates through two main types of cells: T cells and B cells. For these lymphocytes to function, it is necessary an initial detection of the specific antigen. This first detection can be achieved through APCs, which are primarily cells of the innate immune system. In this context, DCs are particularly relevant, although macrophages also function as such. These cells do not inherently recognize the foreign antigen but can capture, phagocytize, and degrade it within their vesicles into smaller fragments, also called antigenic peptides, which then bind to major histocompatibility complex (MHC) proteins found on their own surface

membranes. Similarly, the B cells, can also act as APCs, although they specifically recognize antigens through the specific cell receptor (BCR) they express on their surface, and therefore they do not follow the phagocytosis process, but they directly bind to these antigens and become activated. All these cells migrate to the lymph nodes, where T cells are usually located. These lymphocytes possess a specific receptor (TCR) for each peptide-MHC combination or for the antigen presented by B cells. As a result, the B cells, upon interacting with a T cell, begin to release antibodies against the recognized antigens (Maier & Watkins, 1998).

However, certain pathogens subsist inside the cells they infect, making them unreachable to secreted antibodies. Therefore, an alternative immune strategy is started. T cells come into play as the responsible for this intracellular line of defense. In this sense, T cells are divided into different types of cells with different functions. Among them, one type of T cell, the CD8+ cytotoxic T cells, can directly induce apoptosis in the infected cells by releasing cytotoxic molecules, such as perforin and granzymes. On the other hand, CD4+ T helper cells amplify the immune response. They can bind to the peptide-MHC combination and release cytokines to attract immune cells such as macrophages and other immune cells in order to enhance the inflammatory response. This way they enhance the overall effectiveness of the immune system against elusive foreign pathogens (Abbas & Lichtman, 2001; Roitt et al., 1997)

2.3.3. Cytokines

Cytokines are soluble glycoproteins fundamental in the immune response. Cytokines share pleiotropic (one cytokine exerts many actions) and redundant

(many cytokines have similar effects) characteristics and they usually act synergistically to regulate immune responses (Silverman et al., 2003). They encompass different proteins, including chemokines, interleukins, interferons, the tumor necrosis factor and growth factors. Regarding their function, they have been classified in two groups: pro-inflammatory cytokines and anti-inflammatory cytokines. Pro-inflammatory cytokines, such as interleukin-1 (IL-1), 1β (IL-1β) and 6 (IL-6), or tumor necrosis factor-alpha (TNF-α), are associated with immune activation and are responsible for recruiting immune cells in response to a pathogenic invasion and therefore promote inflammation. For its part, anti-inflammatory cytokines, such as IL-10, repress the expression of IL-6 and TNF-α, thus they regulate the intensity and duration of the inflammatory response, reducing the activity of pro-inflammatory cytokines and therefore maintaining the internal balance. Nevertheless, it is worth mentioning that some interleukins, such as IL-1β and IL-6 can play both roles, pro- and anti-inflamatory, depending on the specific circumstances (Abbas et al., 2021; Kronfol & Remick, 2000; Lydyard et al., 2011; Zhang & An, 2007).

Cytokines can be secreted both centrally (by astrocytes, microglial cells, and, in some cases, some neurons) and peripherally (by the IS cells such as macrophages, monocytes, and lymphocytes during immune activation). However, even when secreted peripherally, they can reach the CNS, by crossing the BBB, where they bind to specific receptors and cause what is known as neuroinflammation (Szelényi, 2001). Their reported functions remain ambiguous. Some findings illustrate their beneficial effects, for example, on neuroplasticity, neurogenesis, and tissue repair (Kohman & Rhodes, 2013; Vezzani & Viviani, 2015), while others reveal adverse effects and their role in the development and

progression of different diseases (Vial & Descotes, 1995). When synthesized and released over a short period, such as after exposure to acute stress, they act as regulators of the immune function, controlling the differentiation and survival of immune cells. However, prolonged release of cytokines can lead to persistent inflammation and tissue damage.

Cytokines can be released by both innate and adaptive immune cells. Many of them are released by monocytes in response to a foreign pathogen that has activated the phagocytic response. These cytokines enhance the immune response against infections and viral invasions and thus they play a crucial role in regulating the immune response. They can act in different ways. For instance, these molecules can directly kill pathogens, mark them for enabling their recognition and elimination by immune cells, or facilitate antigen presentation and activation of T cells of the adaptive immune system by stimulating APCs (Marshall et al., 2018). Remarkably, the CD4+ T cells can also secrete cytokines, so they are also known as helper T (Th) cells. According to their cytokine expression profiles and immune function, these cells are divided into different functional subsets, i.e. Th1, Th2, and Th17. Th1 cells are fundamental in cellular immunity enhancing the macrophages' function against bacteria. On the other hand, Th2 cells also secrete cytokines, although they have been associated with mediating humoral immunity, which consists of regulating defense against extracellular pathogens (Dong, 2021; J.-M. Zhang & An, 2007).

2.3.3.1. The immune response in the CNS: microglial activation

When cytokines are found in the CNS, they can bind to specific receptors on microglia cells, regulating the immune function in the CNS (Augusto-Oliveira et al., 2019).

Microglial cells are non-neuronal glial cells of the CNS. They are produced from myeloid cells and their functions include the maintenance of the homeostasis and the protection of the CNS though ensuring the proper functioning of the brain (Augusto-Oliveira et al., 2019). Microglia cells are central in the physiological process of neuroinflammation, to which they contribute by initiating the immune response in the CNS. Normally, microglia can be found in a quiescent state, referred to as M0, in which it expresses membrane receptors that will detect signals in order to initiate the necessary response. However, when a signal is received, the microglia acquire different activation states. In fact, when activated, they can enhance the secretion of cytokines, in particular chemokines, in order to facilitate the immune response in the CNS.

Chemokines are signaling cytokines that play a fundamental role in the inflammation and immune response in the CNS due to their chemoattractant properties, meaning they have the ability to direct specific immune cells to a specific site. For example, they can attract leukocytes to the focus of an infection. A chemokine of particular relevance in recent years has been the Fractalkine, also known as chemokine C-X3-C-motif ligand 1 (CX3CL1). This chemokine belongs to the CX3C chemokine family and, like other cytokines, can be released by endothelial, immune and neuronal cells. Additionally, it shares the same chemoattractant properties. However, unlike other chemokines, CX3CL1 is a

unique chemokine functioning in two distinct forms: the membrane form (mCX3CL1), which is attached to the surfaces of endothelial and epithelial cells, and the soluble form (sCX3CL1). The soluble form originates as membrane-bound CX3CL1, but through a series of enzymatic processes involving different metalloproteases, it is cleaved from the membrane and released into the bloodstream. While the membrane-bound form facilitates cell adhesion and possesses chemoattractant properties, the soluble form acts as a conventional chemokine, attracting immune cells from the innate (NK cells, monocytes and macrophages) and the adaptive (T cells) IS. The chemo-attraction starts when sCX3CL1 binds to its receptor, the CX3CR1. This receptor is the only high-affinity receptor of Fractalkine and it is expressed in the mentioned immune cells, including DCs. This binding promotes the migration of these cells to infected or transformed cells, where CX3CR1 may also be expressed, indicating its role in, for example, tumor processes (Bazan et al., 1997; Garton et al., 2001). Nevertheless, it is a chemokine with complex functions, as it has proven to play opposite roles such as anti-tumor functions through apoptosis (Tang et al., 2015; H. Wang et al., 2017) or tumorigenesis through apoptosis resistance and cell survival (Ohta et al., 2005; Park et al., 2012).

Notably, the CX3CL1 is the only chemokine that is more centrally expressed than in the periphery. This is primarily because, unlike other chemokines, CX3CL1 is highly expressed in different brain structures, being the neurons the primary CNS cell type that synthesize it. Furthermore, its receptor, CX3CR1, is also highly expressed centrally, on microglial cells. Thus, the CX3CL1-CX3CR1 axis establish a critical link between the IS and the CNS. As CX3CL1 acts as a potent chemoattractant for immune cells expressing CX3CR1,

which includes microglia, this reflect their role as mediators between the immune and nervous systems, meaning a cross-talk between both systems. Through this binding, microglia can be activated and mobilized to sites of injury or inflammation within the CNS facilitating immune response within the CNS (Hughes et al., 2002; Nishiyori et al., 1998; Sheridan & Murphy, 2013).

2.3.3.2. Interaction between HPA axis and the immune system

The immune response, and subsequent release of cytokines, represent the organism's attempt to maintain homeostasis following pathogen invasion. While primarily involved in immune functions, pro-inflammatory cytokines can also directly activate the HPA axis, which, as above explained, is one of the physiological mechanisms by which the organism responds to stressful demands in order to maintain internal balance.

Cytokines can cross the BBB via active or passive mechanisms and reach different structures of the HPA axis, including the HT, the pituitary and the adrenal glands. Cytokines can impact the HPA axis at the hypothalamic level, either exerting a direct effect on the median eminence (ME) or acting on endothelial and glial cells to stimulate the release of secondary messengers and activate hypothalamic neurons, both pathways ultimately leading to CRH release. Both IL-1 and TNFα have been implicated in this process, with IL-6 being predominantly associated with chronic inflammation. These molecules have also been shown to exert a direct action on the pituitary, thereby stimulating the secretion of ACTH. Moreover, the pituitary not only possesses receptors for pro-inflammatory cytokines but can also contribute to the process through its own production of these cytokines, thus enhancing the inflammatory response. At the

adrenal glands, receptors for IL-6 and TNF-α have also been identified across various species, with IL-6 exhibiting excitatory effects and TNF-α displaying inhibitory effects on CORT synthesis and release. Once the GCs are released, they can bind to receptors on immune cells, halting further pro-inflammatory cytokine secretion and thus preventing an exacerbated immune response, thus contributing to the negative feedback (Silverman et al., 2003). Additionally, GCs not only inhibit the production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, but also can stimulate the secretion of anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 (Elenkov & Chrousos, 2002; G. E. Miller et al., 2002). Therefore, cytokines can modulate the stress response by acting on the HPA axis, and in turn, the HPA axis can modulate the immune response by regulating cytokine secretion.

However, chronic stress can exert varied effects on the immune system regarding its nature or duration. For instance, Tian et al. (2014) proposed three stages of CS with different effects in the immune system. During the early stage, in which stress has already begun, the activation of the HPA axis and the subsequent release of GCs would inhibit the release of pro-inflammatory cytokines (Miller et al., 2002), thus regulating the inflammatory state. In the second stage, CS would lead to HPA axis “fatigue” and GC resistance, resulting in a potential inflammatory state due to the maintenance of pro-inflammatory cytokine levels in the organism (Cohen et al., 2012; Webster et al., 2002). Finally, in the third stage, the pro-inflammatory cytokines levels would further increase, inducing an inflammatory state and heightening the individual's susceptibility to disease (Tian et al., 2014).

2.3.3.3. Interaction between SAM axis and the immune system

As well as GCs, catecholamines can also influence the release of cytokines. Catecholamines, including both A and NA, play a crucial role in the secretion of stress-induced pro-inflammatory cytokines, with both α-AR and β-AR contributing to cytokine release into the bloodstream. β-ARs are particularly important for cytokine release at the peripheral level, while α-ARs also influence cytokine production centrally, as evidenced by the increase of cytokine levels in specific brain regions (Johnson et al., 2005).

Adrenergic pathways are fundamental in the interaction between the nervous and the immune systems, especially in the adaptive immune response. Immune cells, such as monocytes, NKs and DCs, exhibit ARs in their surface allowing the catecholamines to bind to them. The activation of the β-AR has been primarily associated to anti-inflammatory effects in human monocytes (Scanzano & Cosentino, 2015); although, under certain conditions, its activation can lead to inflammatory effects on monocytes, leading to an increase of pro-inflammatory cytokines (Takahashi et al., 2003). Regarding the DCs, the effect of the activation of the AR vary depending on the specific context and stimuli. For instance, in human cells, the activation of β2-AR can inhibit the release of pro-inflammatory cytokines, thus inhibiting the inflammatory response (Goyarts et al., 2008; Panina-Bordignon et al., 1997). In this line, in murine DCs, NE binding also inhibits the secretion of IL-12 and increases IL-10 levels (Maestroni, 2004), which would suggest its anti-inflammatory effects. On the other hand, α-AR activation can modulate the immune response of monocytes and macrophages, promoting either a pro-inflammatory or an anti-inflammatory response; influence cytokine

production in DCs; and increase the cytotoxicity of NK cells. Nevertheless, its functionality requires further investigation (Scanzano & Cosentino, 2015).

Taken together, these data reveal the close relationship between catecholamines and the IS through the secretion of cytokines.

2.4. HEALTH IMPLICATIONS OF CHRONIC STRESS

Chronic stress and the associated *allostatic load*, resulting from persistent physiological activation, can report negative health consequences and can promote the onset and development of certain diseases. These pathologies can range from minor health disorders to physical illnesses such as cardiovascular and autoimmune diseases or cancer and psychiatric illnesses such as anxiety disorders or depression. Given the scientific evidence regarding these relationships, several countries have expressed their concern about the increase in mortality from stress-related illnesses as well as about the increase in stress-related complaints in medical practices. The study of stress and its effects on health has therefore increased considerably in recent years. However, the study of stress in humans has certain limitations due to the effects of stress on various physiological variables of the immune, endocrine and nervous systems. For this reason, the study of stress in rodents has become increasingly important. Chronic stress applied in rodents allows us to study multiple parameters at the biological and behavioral level, which is promising as it has been ratified to imitate the chronic stress condition in humans and the related development of diseases (Belzung & Lemoine, 2011; Willner, 1997). Despite the inherent limitations when it comes to extrapolation from studies in animals to humans, studying rodents can provide us with valuable data to better understand the

underlying mechanisms and pathways of stress-related illnesses in humans. This comprehension can lead to more effective therapeutic strategies and interventions when trying to control or reduce the impact of chronic stress on human health and well-being.

2.4.1. Stress, immune system and tumor development

Numerous studies have demonstrated the effects of stress on the body. As it has been explained, stress triggers the activation of various systems in the organism to prepare it for the *fight or flight* response. Therefore, it could be expected that stress would also enhance the immune response with the aim of coping with a stimulus that could be threatening and cause damage in the individual (Dhabhar, 2009). Indeed, acute stress has been shown to have these immune-protective effects on the organism making it carry out a more effective and adaptive immune response. However, these effects are highly dependent on the characteristics of the stressor itself, being duration one of these key differentiating characteristics. In fact, chronic stress, unlike the acute one, can induce suppression of the immune system and thus make an individual more susceptible to certain diseases such as cancer (Ader, 1981; Andersen et al., 1994).

The immunosuppression caused by CS primarily affects cellular immunity. For instance, CORT has been shown to have immunosuppressive effects promoting humoral over cell-mediated immunity (Daynes & Araneo, 1989). In addition, lymphocytes proliferation and NKs immune activity, which are essential in the cellular immunity against cancer cells, can also be compromised (Andersen et al., 1998). It is worth mentioning that high concentrations of GCs

and catecholamines may also affect humoral immunity by altering the production of antibodies by B cells (Rabin, 1999). The impact on cellular immunity is particularly relevant in cancers such as malignant melanoma as it can be immunogenic. This means that melanoma cells are capable of inducing a cell-mediated immune response, which is essential in the fight against cancer. Thus, an altered cell-mediated immune response could facilitate cancer development (Dunn et al., 2002).

Skin cancer, which can be divided into basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and melanoma, is one of the most common cancers within white populations and affects both men and women. Although mortality rates appear to be declining, their incidence continues to exhibit an upward trend and it is particularly high in countries with high sun exposure and non-native white populations, such as Australia, the United States and Europe. Melanoma has a lower incidence compared to the other types of skin cancer, however, it is considered the most dangerous of the skin cancers as it is responsible for the majority of skin cancer deaths due to its metastatic potential. Its incidence is subject to geographic (i.e. latitude) and ethnic variations, and has followed the same trend as other types of cancer increasing in both sexes in recent decades. However, melanoma mortality rates are increasing to a lesser extent due to early interventions and early detection, which leads to a better prognosis and underlies the importance of an early diagnosis (Garbe & Eigentler, 2007; Leiter et al., 2014).

Many melanoma models have been developed in the scientific research field in order to understand its etiology and progression. Different B16 melanoma

sublines, originally developed in C57BL/6 mice, have been regularly used, being the B16-F10 one of the most used ones in experimental research due to its capacity to produce metastases, mainly in the lung, and to generate very aggressive tumors in mice when injected via the tail vein. These tumor cells elicit an immune response by the host and thereby they can be interesting in psychoneuroimmunology field of research. For instance, when implanted locally in the ear of experimental mice, the immune system responds to the tumor through cell-mediated immunity. It has been observed an increased vascularity, and a higher presence of macrophages and T cells in the tumor microenvironment. These immune cells infiltrate the tumor as part of the body's response against cancer cells (Hart, 1979; Potez et al., 2018). However, despite its immunogenic response, B16-F10 has demonstrated resistance to various immunotherapeutic interventions (Overwijk & Restifo, 2001). Hence, its study continues to be of special relevance in order to understand the immunogenic behavior of this tumor.

Given its resistance to immunotherapeutic therapies, various treatments are being studied for B16-F10 melanoma developed in the lungs, some of them showing promising results in slowing down tumor progression. The presence of CX3CL1 in a tumor niche has been related to a better prognosis in different types of cancer, and thus, the potential therapeutic effects of CX3CL1 against these tumor cells have been revealed (Hyakudomi et al., 2008; Park et al., 2012; Ren et al., 2019). For instance, regarding B16-F10 tumor cells, the intratumoral administration of an adenoviral vector expressing CX3CL1 activates T cells and NKs against tumor (Xin et al., 2005), and the injection of mesenchymal stem cells (MSCs) modified to express CX3CL1 have shown to significantly inhibit the

development of lung metastases in mouse models (Xin et al., 2007). Nevertheless, it is worth mentioning that the opposite effects have been also observed, as it has been shown that CX3CL1 could play an important role in tumor angiogenesis due to its properties as an adhesion cell (T. Ren et al., 2007).

The CX3CL1/CX3CR1 axis has shown to be expressed in different tumor cells (Liu et al., 2018; Wada et al., 2015) and hence, it should have effects on tumor development. Indeed, the anti-cancer response is characterized by an increase in the release of pro-inflammatory cytokines such as IL-1β and TNF-α, which can increase the expression of the membrane form of CX3CL1 (Garcia et al., 2000; Sidibe et al., 2018). Here, mCX3CL1 has two main functions: it acts as a quemoattractant protein that attracts DCs (Guo et al., 2003); and it acts as an adhesion protein for immune cells that express CX3CR1 in their surface, such as the DCs, NKs and CD8+ T cells (Dichmann et al., 2001; Imai et al., 1997), retaining them in the site of cell injury. The expression of CX3CL1 on DCs is upregulated upon cellular maturation (Papadopoulos et al., 1999), thus, once matured, these cells expressing CX3CL1, enable the NK cells (that express CX3CR1 on their surface membrane) migration to the tumor niche (Pallandre et al., 2008). When NK cells bind to mCX3CL1, they begin their trans-endothelial migration to areas of high cellular activity such as tumor sites. Upon arrival at the focus of infection, they can act directly against tumor cells, further releasing cytokines to initiate a process known as cytotoxicity (Hertwig et al., 2016; Hess et al., 2009). Thus, the CX3CL1-CX3CR1 pathway could have important therapeutic implications against tumors.

Therefore, CS is indeed related to the progression of cancer. Specifically, CSS is one of the most associated stressors with the cancer onset and development. This association is particularly significant as it highlights the interaction between social and biological factors. In addition, the diagnosis of cancer itself causes in many of the patients a continuous state of stress, which can predispose them to psychological disorders such as depression (Smith, 2015; Vignjević Petrinović et al., 2023). In this manner, stress, cancer, and depression frequently co-occur simultaneously and thus it is especially relevant to understand this association.

2.4.2. Stress, immune system and depression

The relationship between stress and mental illness is very complex as it involves a large number of physiological, emotional and social factors. Initially, research was primarily focused on the effects of stress on physical illnesses, with mental disorders pushed into the background. In recent decades, however, the study of psychological and emotional disorders has increased considerably, and thereby its possible causes, including stress, have also been studied. In fact, the influence of psychosocial stress on the appearance of different psychological or mood disorders, has been widely demonstrated (Calabrese et al., 2009; Post, 1992). Studies in both, animals and humans, have reported the link between stressful experiences and the onset or aggravation of depressive-like behaviors (Mazure, 1998; Mineur et al., 2006), with chronic social stress being one of the most strongly associated with depression (Slavich et al., 2010b, 2011). Several studies have shown that this association can be mediated by physiological mechanisms by which stress could alter the effectiveness of the immune system,

leading to an increased risk of developing depression. The exploration of these mechanisms is fundamental for the development of novel therapeutic approaches aimed at mitigating the impact of stress on mental health.

Although the multifactorial model of the relationship between stress and mental illnesses remains to be fully established, several biological mechanisms have been proposed as mediators of this bidirectional relationship. For instance, a dysregulation of the HPA axis during chronic stress can result in an increase in GCs levels that can lead to functional and structural alterations in some brain regions, including the HT, the HC or the AMYG, which are also observed in depressed subjects (aan het Rot et al., 2009; Sapolsky, 2003; Sheline et al., 2003). Moreover, as abovementioned, HPA activation also has an influence in the immune response. Typically, GCs have been related to immunosuppressive effects and anti-inflammatory effects (Coutinho & Chapman, 2011; Marx, 1995; Strehl et al., 2019) and thus, GCs have been widely used as anti-inflammatory agents in clinical practice (Schleimer, Claman & Oronsky, 1989). In fact, acute stress and the release of GCs in a short period of time have been associated with anti-inflammatory effects, however, paradoxically GCs released after a chronic stress have been linked to a pro-inflammatory state. It has been shown that GCs can induce this inflammatory state by increasing the expression of different genes and proteins that promote inflammation as well as pro-inflammatory cytokines and chemokines (Busillo et al., 2011; Lannan et al., 2012; Sukkar et al., 2004). Although immunosuppressive and inflammatory responses may appear to be contradictory or mutually exclusive, both states can coexist in the same individual, leading to the appearance of diseases with different etiologies concerning the immune system. Indeed, the hypothesis of immunosuppression as a result of

chronic stress would explain the susceptibility to diseases such as cancer discussed in the previous section. However, it would not explain why stress has been shown to be associated with diseases whose main feature is inflammation, such as autoimmune or infectious diseases (Cohen et al., 1998; Zautra et al., 1994; Whitacre et al.,1995).

One of the main hypotheses to explain the effect of stress on inflammation is the GC resistance model. This hypothesis proposes that chronic activation of the HPA axis and the SAM axis could downregulate the expression or function of GRs and MRs through the action of white blood cells (WBCs), which would reduce the ability of the IS to respond to the anti-inflammatory effects of GCs and thus result in an inflammatory state (Miller et al., 2002). The inflammatory state would be characterized by the release of pro-inflammatory cytokines, including IL-6, TNF-α or IL-1β. The presence of these inflammatory agents can lead to what is known as sickness behavior that includes nonspecific symptoms such as fatigue, weakness, listlessness, apathy, loss of appetite or avoidance of social relationships (Kent et al., 1992). These symptoms had been frequently overlooked or undervalued, as they were considered as not significant in the illness process. However, the concept of sickness behavior is fundamental since, when it becomes chronic, the underlying mechanisms that contribute to its development could play a very important role in the onset of depression (Dantzer, 2001).

The study of sickness behavior did not begin until 1995 (Aubert et al., 1995), although some studies had already suggested that the characteristic symptomatology of a disease was not only due to the direct action of the invading

pathogen, but that the own response of the host played an important role in the onset of disease symptoms (Winther et al., 1984). In fact, sickness behavior in response to inflammation represents a well-organized response of the organism aimed at preserving the energy needed to cope and fight against the stressful stimulus that would represent a threat to the organism's survival. Therefore, it is an adaptive response of the organism itself in order to survive. Nevertheless, if the sickness behavior becomes chronic due to persistent inflammation, this may affect brain neurochemistry, which in turn would affect the cognitive functions and have effects on mental health, facilitating the development of diseases such as depression (Dantzer & Kelley, 2007; Hart, 1988).

Consequently, the prolonged presence of pro-inflammatory cytokines may underlie multiple medical or psychiatric conditions with a neurochemical basis, as they can disrupt different metabolic and neuronal pathways that can alter brain neurochemistry. Among these pathways, tryptophan (Tryp) and phenylalanine (Phe) metabolism pathways are particularly significant due to their role in the synthesis and regulation of neurotransmitters such as serotonin (5-HT), dopamine (DA) and NA, which play a key role in depression etiology (Dantzer et al., 2008; Ruhé et al., 2007). The impact of inflammation on the Tryp pathway may be highly relevant to the onset of mood disorder symptomatology (Correia & Vale, 2022), while its effect on the dopaminergic pathway may have implications in activity-related symptomatology, including fatigue, anergia, anhedonia, and decreased motivation (Capuron et al., 2012; R. Dantzer et al., 2014).

Cytokines such as TNF-α can alter these metabolic pathways. For instance, they can activate indoleamine 2,3-dioxygenase (IDO), which is a

fundamental enzyme in the Tryptophan metabolism pathway as it catalyzes Tryp into Kynurenine (Kyn) (O'Connor et al., 2009). Tryptophan is the precursor of 5-HT, and thereby the synthesis of this monoamine, which is then metabolized into 5-hydroxyindoleacetic Acid (5-HIAA), is limited by the amount of Tryp available. A prolonged IDO enzyme activation diverts the metabolization of Tryp, and instead of being metabolized to 5-HT it is metabolized into Kyn, thus reducing the amount of 5-HT that can be released, which has been widely related to depressive disorders. Kynurenine is also the precursor of two neuroactive glutamatergic compounds, the Kynurenic acid (Kyna) and 3-hydroxyKynurenine (3-HK). These metabolites have very different health consequences. While Kyna has been shown to have neuroprotective properties; 3-HK is neurotoxic, as it has been proven to stimulate NMDA receptors promoting oxidative stress, and it has been linked to neurodegenerative and psychiatric disorders (Campbell et al., 2014).

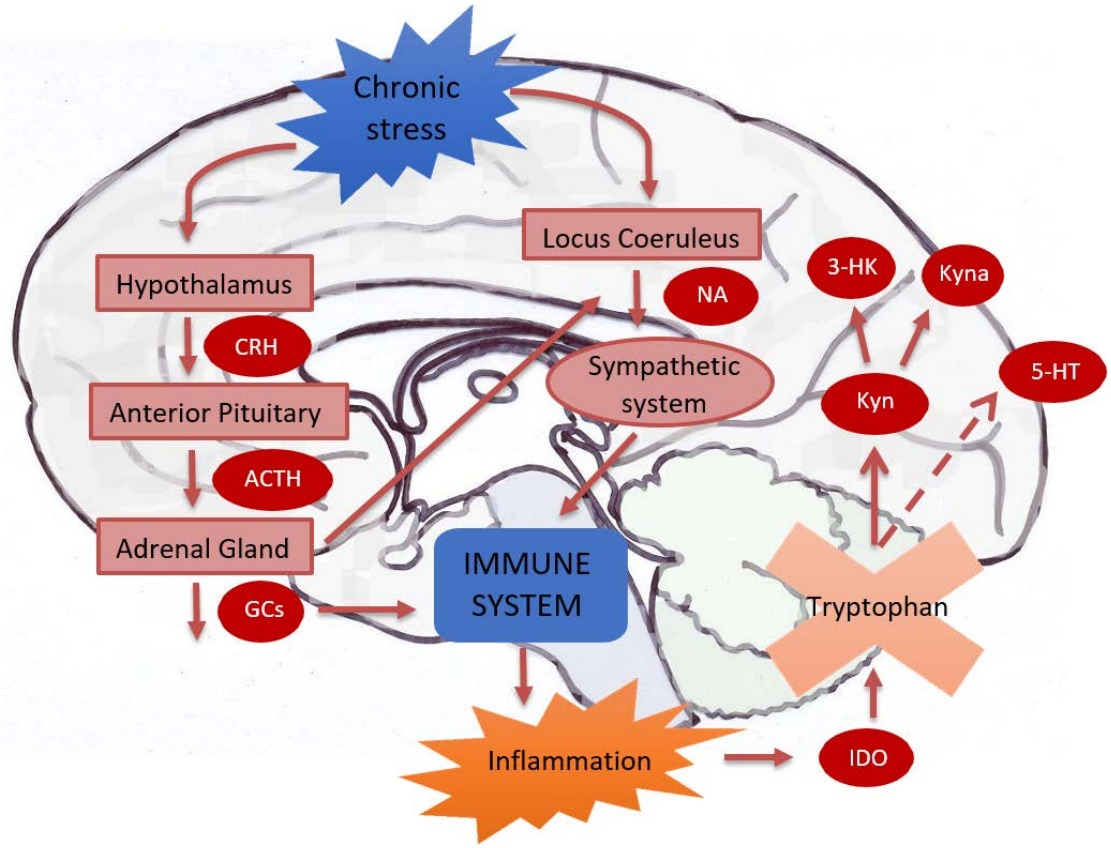

Fig. 2. The Kynurenine pathway in stress-related depression. The conversion of Tryp into Kyn rather than 5-HT requires induction of IDO enzyme. IDO may be induced in response to stress through the activation of the SAM axis, which can result in the release of pro-inflammatory cytokines, such as IL-1β and IL-6 (Elenkov et al., 2000).

Additionally, pro-inflammatory cytokines, such as TNF-α and IL-1β, can also induce oxidative stress by stimulating the production of reactive oxygen species (ROS) (Angeloni et al., 2015). ROS can cause oxidative damage to cells and biomolecules such as tetrahydrobipterin (BH4), which is a key cofactor for the synthesis of inducible nitric oxide synthase (iNOS), which, in turn, is essential in the secretion of nitric oxide (NO), and in the DA metabolism, as it converts Phe into Tyr, thus allowing the synthesis of DA. By inducing ROS, cytokines can reduce the availability of BH4, which limits the possible DA synthesis (Fanet et al., 2021). Moreover, the pro-inflammatory cytokines released during the Th1 immune response, such as IL-1β or TNF-α, can increase iNOS production over DA synthesis, again contributing to less DA synthesis (Suschek et al., 2005). All

these neurochemical changes may affect neuronal function and neurotransmission, thereby altering the neurochemical balance and contributing to neuropsychiatric disorders such as depression.

2.5. INDIVIDUAL DIFFERENCES: MALES AND FEMALES

Interestingly, although chronic stress can have an effect on the development of certain diseases, not all individuals develop them, and if they do, they do not even develop them in the same way. Numerous research studies distinguish between those individuals who are more susceptible to suffer from the physical and psychological effects of CS, while others, the so-called resilient individuals, do not develop these disorders and may even benefit from chronic stress. In fact, these groups of individuals can be distinguished by both physiological and psychological differences (Del Giudice et al., 2011; Sapolsky, 1994; Wood, 2014). Inter-individual differences in personality when referring to humans, or behavioral differences in animals, may be making an individual more or less susceptible to developing these diseases.

Using animal models provides valuable insight into the physiological factors associated with individual differences in response to social stress. Male mice (Mus musculus) serve as a frequently utilized model for investigating how behavioral coping strategies to social stress influence health outcomes. For this purpose, contrary to the popular recommendation to use inbred strains, the use of outbred OF-1 or CD-1 mice is particularly relevant precisely due to their genetic diversity (Aldinger et al., 2009; Festing, 1976), which facilitates a better and more comprehensive examination of individual differences. Employing outbred mice

allows for a more accurate simulation of the heterogeneity characteristic among humans.

2.5.1. Coping strategies in male mice

As mentioned, the inter-individual variability means that not all individuals submitted to the same stressor develop a stress-related disease, and when they do, they do not always develop them at the same time or with the same intensity. Because susceptibility to stress-related diseases varies, various mechanisms have been postulated to mediate or modulate this association. Among the different factors that could be influencing the vulnerability to suffer from any of these stress-related diseases, the individual differences including the genetic background and the behavioral coping styles have been some of the most studied ones (Armario & Nadal, 2013).

Coping is one of the most prominent mechanisms that has been studied in recent year, although is a complex concept lacking a single definition. One of the most extensively used is the one proposed by Lazarus & Folkman in 1984 by which they stated coping as “thoughts and behaviors that people use to manage the internal and external demands of situations that are appraised as stressful strategies”. However, much research has gone into arriving at this definition, since Richard Lazarus published his book "Psychological Stress and the Coping Process" in the 1960s, which introduced this new concept and orientation in the study of stress by focusing on the cognitive and behavioral strategies that individuals use to cope with stress (Lazarus, 1966). From that moment on, studies dealing with coping strategies increased in different research fields, such as medicine, nursing or psychology. As a result, it has been shown that coping

is a multidimensional concept that encompasses different biological, cognitive and behavioral aspects (de Boer et al., 2017).

Coping strategies are usually divided into active and passive ones, with active strategies being more directed towards coping or problem solving. There are, however, other dimensions, besides activity, which may characterize these subjects. Aggressive behaviors may be one of them. Although levels of aggression in male mice may change over time, increasing, maintaining or decreasing (Weber et al., 2023), it seems to be a personality trait that correlates directly with active coping strategies. Individuals exhibiting more aggressive behaviors have also been found to engage in more active coping behaviors in response to a threatening event. For instance, they display more climbing and swimming in the Forced Swimming Test (FST) (Veenema et al., 2005), more active behaviors trying to escape from an inescapable shock (Benus et al., 1990) and more defensive burying (De Boer et al., 2003); whereas low aggressive mice show more freezing behaviors (Benus et al., 1991), which is more characteristic of the passive coping strategies.

Coping strategies also differ in different biological parameters. For example, differences have been found at the neuroendocrine level, SAM axis and IS. After exposure to chronic social stress, passive mice present a higher reactivity of the HPA axis (De Miguel et al., 2011; Gómez-Lázaro et al., 2012; Pérez-Tejada et al., 2013), less adrenergic activity (De Miguel et al., 2011) and a pro-inflammatory profile (Ballestín et al., 2021; Gómez-Lázaro et al., 2012; Pérez-Tejada et al., 2016). These differences in coping style, both behavioural and physiological, have often been attributed to variability in corticolimbic neural

activation. This circuit plays a fundamental role in decision-making and behavioural regulation and includes regions such as the prefrontal cortex (PFC), HT, HC and striatum (ST). The interactive functioning of this circuit is largely dependent on the release of monoamines such as 5-HT, DA and NA (de Boer et al., 2015), which have been shown to play a critical role in depressive symptomatology.

Although a general understanding of coping has been achieved, the majority of its neurobiological bases remain unknown. Furthermore, despite the progress made in their exploration, the great part of experimental studies have been conducted on males, resulting in behavioral ethograms based on the behaviors usually displayed by males. Since males and females present sexually dimorphic social behaviors with distinct neural circuits (Bayless & Shah, 2016), it is highly recommendable to the behaviors that females display toward conspecifics to better understand their stress-related coping strategies in a disruptive social environment.

2.5.2. Intrinsic sociability in female mice

Experimental studies in animal models have a long tradition in scientific research. However, until recent years, experimental subjects employed in investigation have predominantly been males. This fact has entailed numerous issues, as findings from male subjects have been extrapolated to females, leading to significant challenges as both sexes exhibit behavioral and biological differences. Despite the higher prevalence of many illnesses, such as autoimmune diseases and depression, in women, female subjects have remained in the background in experimental research. The exclusion of women

in clinical studies, as well as the omission of female subjects in preclinical research, has resulted in a negative impact on women's health. This research bias concerning sex has led to treatment failures, as treatments working in men did not do so in women, as well as in more and more pronounced drugs side effects in women (Carey et al., 2017; Plevkova et al., 2020). Regarding research in mice, females have only been introduced into experimental designs much more recently, and given the existing sex differences in behavior and their underlying hormonal, molecular and genetic dissimilarities, as well as neurobiological differences in brain structure (Viveros et al., 2012), results obtained in studies which only use male subjects should be interpreted with caution when making generalizations.

As previously observed, the chronic stress induction in outbred male mice has commonly been achieved employing a model of CSDS. However, this model is difficult to transfer to female mice due to the sexual dimorphism in behavior, which is a well-documented phenomenon (Kelley, 1988). Some studies have implemented CSDS in female studies, albeit with modifications, as the standard model can only be effectively implemented in males confronted to males due to their intrinsic characteristics, including the presence of marked hierarchies, territoriality and aggressive behaviors. However, given the more social nature and the less pronounced dominance hierarchies of females and considering that male mice are expected to display aggression only towards same-sex conspecifics rather than exhibiting aggression towards a female (Dadomo et al., 2018; Palanza & Parmigiani, 2017; Shansky, 2019; Williamson et al., 2019), some manipulations of the model are required when aiming to study females. Some of the modifications involve either changes on the "aggressor side", such

as replacing the resident male with a lactating female (Jacobson-Pick et al., 2013) or keep using females but from another more aggressive strain (Trainor et al., 2011). Other modifications have to do with changes on the “intruder female side”, such as coating her with male urine to make the resident aggressor male perceive her as a male and not as a female (Harris et al., 2018).

Remarkably, other studies prefer to employ stressors involving disruption of their social environment, thus considering the inherent characteristics of females and, ultimately, making the study more ecologically valid for this population. For instance, prolonged social deprivation as a consequence of isolation (Martin & Brown, 2010), or Chronic Social Instability Stress (CSIS) achieved through unpredictable changes in their social environment (Goñi-Balentziaga et al., 2018) represent a significant source of stress for females more in line with their intrinsic characteristics.

Chronic Social instability Stress model has been used as a valuable paradigm to induce depressive-like behaviors in rodents (Haller et al., 1999; Herzog et al., 2009a; Jarcho et al., 2016). Nevertheless, not all the studies have encountered this same relationship (Saavedra-Rodríguez & Feig, 2013). This discrepancy could be indicating that there are inherent factors in the nature of females that could be mediating the relationship between stress and the development of depressive-like behaviors. For instance, sociability could be playing a key role in this association. Although the greater sociability of females compared to males has been discussed, it is a complex and multidimensional concept that involves numerous biological and behavioral traits. Sociability could be defined as the propensity for conspecifics to interact with each other in a

nonaggressive way. Sociability encompasses some of the most intricate mechanisms of the CNS, as it involves detecting, processing and producing specific responses within the specific context in which the individual finds himself or herself. Additionally, sociability exists on a spectrum, meaning that individuals can be more or less sociable (López-Tobón et al., 2020), what could make their perception of social instability different, with some subjects perceiving it as much more stressful, thus eliciting a more pronounced physiological and behavioral response. The perception and evaluation of a socially changing environment will differ depending on sociability, i.e. my predisposition and need for companionship with peers. Sociability could thus be a key factor in the perception and coping with stress, and, additionally, the evaluation of social interactions has been suggested as a relevant behavioral trait when studying preclinical depression (Berger et al., 2019). Thereby, considering this factor could shed light on the relationship between stress and depression, and implicitly, its impact on resilience or susceptibility to diseases.

SECTION II: PUBLISHED STUDIES

Introduction, primary objective, specific objectives,

hypothesis and published studies

2. CHAPTER 1. Study #1: How does Chronic Isolation Stress affect physiological response in male mice?

2.1. Introduction

Many of the scientific laboratories working with experimental mice individualize them for housing. However, social isolation can be a significant source of stress as it entails social deprivation, and thereby it can have effects on physiological and behavioral parameters, which could interfere with the main aim of the study.

2.2. Primary Objective

The main objective of this study was to examine the possible effects of a 4-week procedure of social isolation in CD-1 male mice in certain physiological variables, specifically in hematological and neuroendocrine parameters.

2.3. Specific objectives

In pursuit of the aforementioned primary objective, specific objectives were stated:

- To assess HPA axis activation by quantifying fecal CORT metabolites levels.
- To examine white and red blood cell changes by conducting blood cells count (WBC, RBC, HGB, HCT, MCV, MCH, MCHC, RDW, PLT, MPV, PDW, PCT). Blood samples were collected at the beginning (week 1) and at the end (week 4) of the stress procedure.

- To assess body weight alterations by assessing body weight before (week 0), during (weeks 1, 2 and 3) and after (week 4) isolation procedure.

2.4. Hypothesis

The hypothesis of this study suggests that the housing conditions of male mice during an experimental procedure, specifically the social isolation to which they are subjected in a substantial number of laboratory facilities, may constitute a stressor itself capable of altering their physiological responses and potentially introducing bias into research outcomes.

2.5. Publication: Individualized Housing Modifies the Immune-Endocrine System in CD1 Adult Males

animals

MDPI

Article

Individualized Housing Modifies the Immune–Endocrine System in CD1 Adult Male Mice

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Simple Summary: In recent years, awareness of laboratory animals' wellbeing and the refinement of their house conditions have increased considerably. Mice (*Mus musculus*) are the most widely used animal species in research in the European Union and are sociable and hierarchical creatures. It is important to determine whether experimental conditions may affect research results and whether housing conditions (isolated or grouped) may be one such condition. The aim of this study was, therefore, to determine whether 4 weeks of social isolation (usual practice in our animal facility and some laboratory procedures) could induce changes in different physiological parameters (body weight, number of blood cells, and stress hormones) in adult mice. Although we did not observe changes in body weight, red blood cells, and platelets, mice that were socially isolated for 4 weeks did have a decreased count of some white blood cells. Moreover, levels of the main stress hormone were higher in single-housed mice after 1 week, although they decreased after 4 weeks to the same levels as those recorded for grouped mice. We can, therefore, conclude that social isolation affects some physiological parameters, and that this should be taken into account in the interpretation of research data.

Abstract: In the last years, different research groups have made considerable efforts to improve the care and use of animals in research. Mice (*Mus musculus*) are the most widely used animal species in research in the European Union and are sociable and hierarchical creatures. During experiments, researchers tend to individualize males, but no consideration is given to whether this social isolation causes them stress. The aim of this study was, therefore, to explore whether 4 weeks of social isolation could induce changes in different physiological parameters in adult Crl:CD1(ICR) (CD1) males, which may interfere with experimental results. Body weight, blood cells, and fecal corticosterone metabolites levels were the analyzed parameters. Blood and fecal samples were collected at weeks 1 and 4 of the experimental procedure. Four weeks of single housing produced a significant time-dependent decrease in monocytes and granulocytes. Fecal corticosterone metabolite levels were higher in single-housed mice after 1 week and then normalized after 4 weeks of isolation. Body weight, red blood cells, and platelets remained unchanged in both groups during this period. We can, therefore, conclude that social isolation affects some immune and endocrine parameters, and that this should be taken into account in the interpretation of research data.

Keywords: CD1 male; single-housed; stress; white blood cells; fecal corticosterone metabolites

Citation: Ortega-Saez, I.; Díez-Solinska, A.; Grífols, R.; Martí, C.; Zamora, C.; Muñoz-Culla, M.; Vegas, O.; Azkona, G. Individualized Housing Modifies the Immune–Endocrine System in CD1 Adult Male Mice. *Animals* **2023**, *13*, 1026. https://doi.org/10.3390/ani13061026

Academic Editor: Vera Baumans

Received: 21 February 2023
Revised: 8 March 2023
Accepted: 9 March 2023
Published: 10 March 2023

1. Introduction

People working with laboratory animals display a high level of awareness of and sensitivity to their wellbeing [1]. Indeed, perceived animal stress/pain has been found

to negatively affect their professional quality of life [2]. In the last few years, different research groups have made considerable efforts to improve the care and use of animals in research, regardless of receiving specific funding for that purpose [3]. In the near future, this new scientific knowledge will provide new evidence to improve the welfare and housing conditions of animals used in scientific procedures. Current European legislation on the protection of animals used for scientific purposes (Directive 210/63/EU) establishes suitable environmental conditions and minimum enclosure measures by age and animal species. It likewise indicates that social laboratory animals must be socially housed in stable groups of compatible individuals. Moreover, procedures in which social animals (e.g., dogs and monkeys) are completely isolated for prolonged periods are classified as "severe" [4]. However, the legislation does not specify what exactly is considered to be a "prolonged period", and it does not mention other social species.

Despite the current debate about their predictive value in basic and regulatory studies [5–10], mice (*Mus musculus*) continue to be the most widely used animal species in research in the European Union [11]. Mice are sociable and hierarchical animals that, in nature, live in small groups. These groups are usually composed of a dominant male, along with various females with their offspring, both young and juvenile. The size of the territory occupied by a mouse family varies according to different factors. These include the availability of different resources such as water and food, as well as the density of the group. Occasionally, depending on the aggressiveness of the dominant male and the density of the group, young males are found in the aforementioned family groups. Generally, however, males are usually rejected from the group when they reach sexual maturity and can be found in the wild alone or in groups of young males. As for the females, they usually become part of the family group once they reach sexual maturity [12].

Unfortunately, in animal facilities, mice are not housed as in their natural environment, thus interfering with their natural ethogram. Standard laboratory protocols stipulate that mice's weaning and maternal separation should occur 21 days after birth. Thereafter, it is recommended that animals should be housed separately by sex and strain in stable groups of 2–5 members, a step that fosters the formation of affiliate relationships between individuals in the same group [13] and reduces aggression between males [14]. The main reason for housing male mice individually is aggression between cage mates [15,16]. Recently, a series of recommendations were published to minimize aggression between males [17].

Keeping newly weaned animals in the company of other animals is important for the correct development of their brains. It has been shown that post-weaning social deprivation by isolating mice induces neurochemical and morphological alterations, which have a behavioral impact in adulthood [13,18–23]. Indeed, the lack of social experiences before adulthood has been used in mice as a model to study some impaired behavioral phenotypes, such as depression and anxiety-like behavior types [21–23], as well as social and cognitive deficits [19,22]. In light of the above, in our animal facility, we implemented two different strategies in order to minimize the number of single-housed newly weaned male mice [24,25].

There is still an ongoing debate about whether adult male mice should be housed individually [15,26]. Years ago, "isolation syndrome" was described, with authors arguing that the inability to interact socially is likely to have a harmful effect on the animal's emotional state [27]. Indeed, it has been proven that adult male mice prefer the proximity of another male over individual housing [28], which is considered a stressor. The gold standard to measure the immediate physiological responses to stress is the activation of the hypothalamic–pituitary–adrenal (HPA) axis, which induces the secretion of corticosterone from the adrenal gland [29]. The effect of solitary versus social housing on corticosterone levels has been explored with varying results. Some studies observed that single-housed male mice had increased corticosterone levels after 14 days [30] and 15 months [31], whereas others found that corticosterone levels remained stable up to 42 days of individual housing [32–36], and two studies reported that single housing caused less stress for mice than

group housing [37,38]. Other indications of stress include changes in body weight and a decrease in circulating leukocytes. A meta-analysis of the effects of individual housing on body weight found considerable heterogeneity in different mice strains, with higher, unchanged, or lower body weights being reported after social isolation [39]. Although it is well documented that chronic stress results in immunosuppression [40], differences in the total number of white blood cells have also been observed [36,41]. Among other factors, these discrepancies may be due to differing isolation periods.

In our animal facility, researchers tend to individualize males during experiments for a maximum period of 4 weeks, mainly for reasons of convenience and habit. However, no consideration is given to whether individually housing animals may cause them stress. The aim of the present study was, therefore, to determine if 4 weeks of social isolation could induce changes in body weight, blood cells, or fecal corticosterone metabolite levels in adult Crl:CD1(ICR) (CD1) males, which may interfere with experimental results.

2. Materials and Methods

2.1. Animals

Mice born in our specific pathogen-free (SPF) breeding zone were housed in pressurized and individually ventilated 1145T (403 × 165 × 174 mm; 435 cm^2 floor area; Tecniplast) (PIV) cages (70 air changes/h). We used black poplar/aspen shavings (Lignocel Selectfine; Rettenmaier Ibérica S.L.) as litter bedding, two sheets of tissue (Tork®; Essity Spain S.L) irradiated by Ionisos Iberica as nesting material, and an in-house autoclaved cardboard cylinder (12.5 × 9 × 0.5 cm; Sodispan Research S.L.) as enrichment. Once a week, socially housed mice (four mice per cage), together with their nesting material, were transferred to clean cages by picking them up at the base of their tails. This same procedure was carried out with individually housed mice every other week. New irradiated tissue was added if the nest was dirty or did not have enough material. Similarly, if the cardboard was broken, a new cylinder was provided. Mice had ad libitum access to water and diet (irradiated Special Diet Services RM1). Rooms were maintained under standard environmental conditions (humidity: 55 ± 10%; temperature: 20–24 °C) with a 12 h light/dark cycle (lights on at 8:00 a.m.). Animals were monitored every day. The animal care and use program was accredited by AAALAC International. The Catalan Government and the PRBB Ethics Committees approved the experimental protocol (DAAM 10576).

2.2. General Procedure

Eight-week-old CD1 mice were randomly assigned to two groups (grouped or single; n = 8 per group, 16 in total) and housed in the same room in which they were born. We selected CD1 adult male mice because they are outbred, are the most commonly used strain in toxicology studies [42], and have a high propensity to fight, resulting in suggestions that they may benefit from individual housing [15]. This does not apply to females, since chronic social isolation is used to model separation-induced depression [43].

Animals were weighed on the same day of the week for 5 weeks (weeks 0–4; 9:00–11:00 a.m.). Sampling was carried out in a laboratory adjacent to the room where they were housed, and the animals were transferred there 1 h before sampling, around 8:00 a.m., because the technician started their working day at this time. Sampling was carried out at two different time points to minimize the influence of handling as much as possible. Thus, on weeks 1 and 4 (9:00–11:00 a.m.), whole blood and fecal samples were obtained from each animal (Figure 1). No signs of fighting were observed during the experimental period. None of the animals had adverse events, and all completed the procedure. Animals became part of our colony once the experiment was completed.

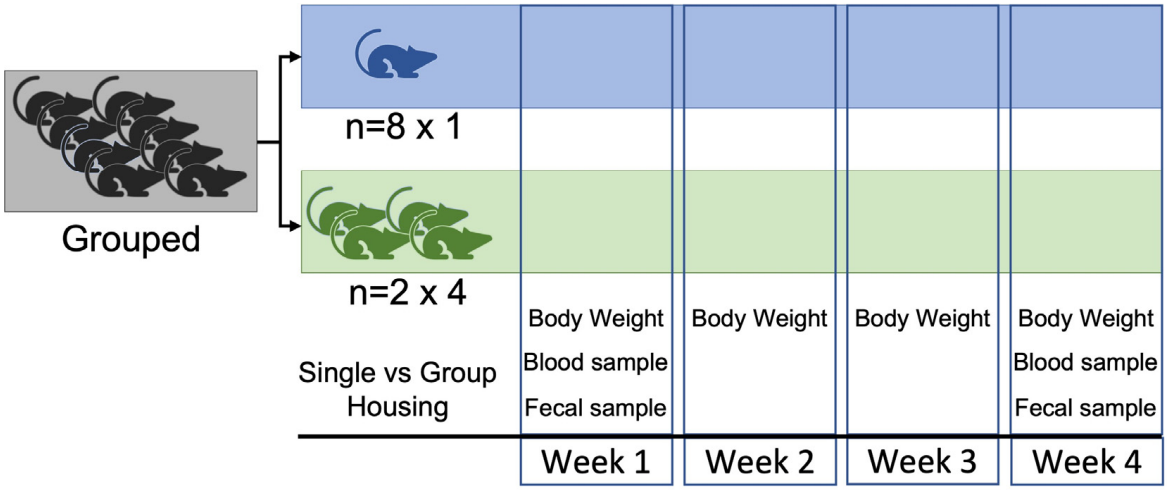

Figure 1. Experimental procedure.

2.3. Hematological Parameters

Blood samples were obtained by facial vein puncture with a 21 G sterile hypodermic needle. We collected blood from the facial vein because this procedure has been found to have the least adverse effects on welfare parameters in mice [44,45]. Samples (15 µL) were collected using a Microvette® 200K3E with potassium salt of ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. After sampling, mice were returned to their home cage. No residual bleeding was noted in any of the animals. The blood was immediately analyzed for complete blood count: white blood cells (WBC), lymphocytes, monocytes, granulocytes, red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), hemoglobin (MCH) and hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets (PLT), mean platelet volume (MPV), platelet distribution width (PDW), and platelet crit (PCT), using the fully automated CVM-Procell analyzer (CVM Diagnóstico Veterinario SL). Since the provider could not give us information about the exact mouse strain, age, or sex where the values were obtained, we first determined if the blood value range of male and female adult mice of different commonly used strains were within the normal range indicated by the analyzer. Our results indicated that the normal range provided for mice by the CVM-Procell analyzer can be used for adult male and female inbred C57BL/6J, outbred CD1, and immunodeficient CB17.Cg-*Prkdc*^{scid}*Lyst*$^{bg\text{-}J}$/Crl (SCID Beige) mice (see Supplementary Materials).

2.4. Fecal Corticosterone Metabolites

Fecal samples were obtained by placing each animal on a grid. The fecal boluses were obtained directly, without possible contamination, placed in an Eppendorf, and stored at −80 °C to determine corticosterone metabolite levels. After sampling, mice were returned to their home cage. This sampling method may allow a more accurate interpretation of chronic stress [46]. Moreover, since there is no need to restrain the animals when collecting the samples, this is a good method for enabling repeated sampling without affecting the animal, meaning that fecal samples are less affected by hormone secretion fluctuation or pulsatility. Each fecal sample was homogenized, and an aliquot of 0.05 g was shaken with 1 mL of 80% methanol in Tris/HCl 20 mM, pH 7.5, for 30 min on a multi-vortex. After centrifugation, each aliquot was frozen at −80 °C until analysis. Fecal corticosterone metabolite levels were quantified in duplicate using an enzyme immunoassay (Corticosterone Elisa Kit, Enzo Life Sciences; ADI-900-097), 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 four-parameter logistic curve fit using MyAssays (Data Analysis Tools and Services for Bioassays; available at https://www.myassays.com/ accessed on 10

March 2023). The sensitivity of the assay was 27.0 pg/mL, and the intra- and inter-assay variation coefficients were between 7% and 8%.

2.5. Statistical Analyses

Experimental data were analyzed using GraphPad Prism software (6.01, GraphPad Software, Inc, San Diego, CA, USA). Group comparisons were performed using a two-way repeated-measures ANOVA, followed by Bonferroni's post hoc test. Values of $p < 0.05$ were considered statistically significant (95% confidence). Data are expressed as the mean ± standard deviation (SD). The results are described in accordance with the ARRIVE guidelines [47].

3. Results

3.1. Body Weight

Both groups of animals gained weight over the duration of the experiment ($F_{(4,56)}$ = 34,78, $p < 0.0001$). Grouped mice weighed 36.27 ± 2.46 g at week 0 and 39.46 ± 2.99 g at week 4. Single-housed mice weighed 38.20 ± 3.55 g at week 0 and 41.19 ± 4.29 g at week 4 (Figure 2). No significant differences were observed between grouped or single-housed mice.

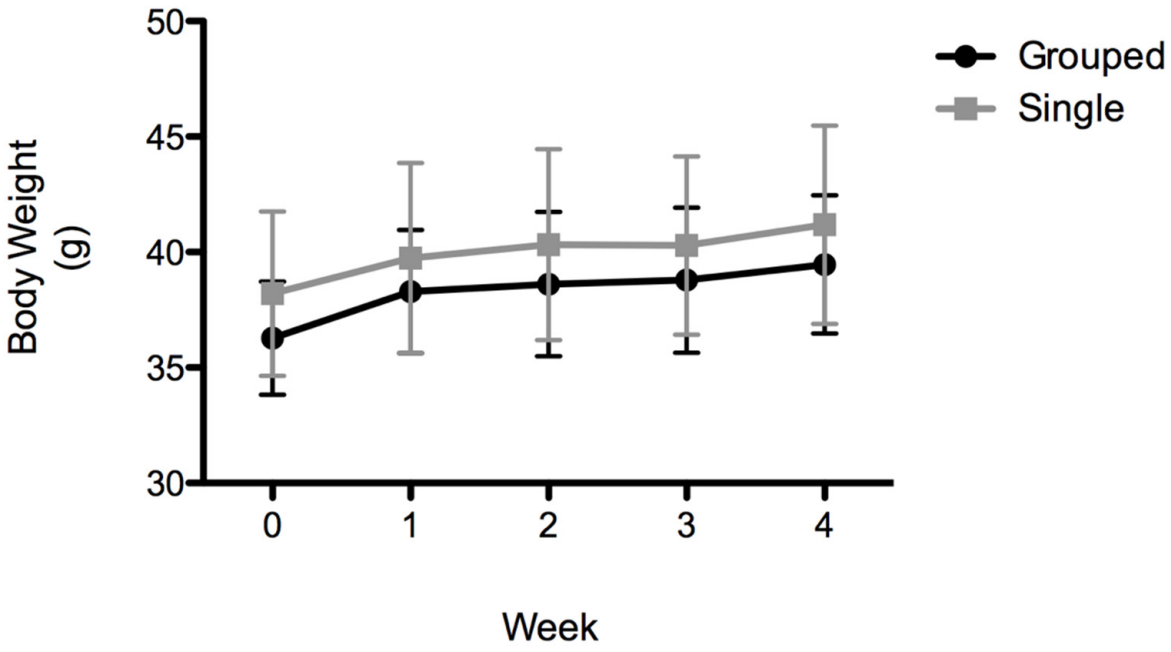

Figure 2. Body weight (g). Data are expressed as the mean ± SD; n = 8 per group.

3.2. Hematological Parameters

The results indicated no significant differences between grouped and single mice in the number of cells in the white series at either week 1 or week 4. However, significant differences were observed as a function of time ($F_{(1,14)}$ = 5.52; $p < 0.05$; Table 1). The post hoc analysis indicated a significant decrease in WBC after 4 weeks of single housing (t = 2.21; $p < 0.05$). When white cell type was analyzed in more detail, significant time-dependent differences were observed in monocytes ($F_{(1,14)}$ = 10.45; $p < 0.01$), and the post hoc analysis indicated a significant drop in monocytes in single-housed mice after 4 weeks (t = 2.714 $p < 0.05$). Similarly, significant time-dependent differences were observed in granulocytes ($F_{(1,14)}$ = 7.63; $p < 0.05$), which dropped in single-housed mice after 4 weeks (t = 2.46, $p < 0.05$).

Table 1. White blood cell population values. Data are expressed as the mean ± SD; n = 8 per group; * $p < 0.05$ (week 1 single vs. week 4 single).

	Week 1		Week 4		Normal Range	Unit
	Grouped	Single	Grouped	Single		
WBC	8.45 ± 3.33	8.15 ± 4.59	7.18 ± 2.59	4.50 ± 1.83 *	0.8–6.8	10^9/L
Lymph	5.81 ± 2.42	5.73 ± 3.08	4.76 ± 0.81	4.13 ± 0.52	0.7–5.7	10^9/L
Mon	0.33 ± 0.14	0.34 ± 0.31	0.26 ± 0.11	0.10 ± 0.08 *	0.0–0.3	10^9/L
Gran	2.33 ± 0.93	1.96 ± 1.50	1.70 ± 0.48	1.07 ± 0.56 *	0.1–1.8	10^9/L

* White blood cell (WBC), lymphocyte (Lymph), monocyte (Mon), and granulocyte (Gran).

The results indicated no significant differences between groups or timepoints in terms of the number of red blood cells and platelets (Table 2).

Table 2. Red blood cell and platelet values.

	Week 1		Week 4		Normal Range	Unit
	Grouped	Single	Grouped	Single		
RBC	8.79 ± 1.18	8.32 ± 1.42	8.76 ± 0.95	8.53 ± 0.93	6.36–9.42	10^{12}/L
HGB	14.59 ± 1.85	13.81 ± 2.79	14.73 ± 1.67	13.71 ± 1.42	11–14.3	g/dL
HCT	43.20 ± 5.24	42.15 ± 6.79	44.08 ± 4.64	42.09 ± 4.65	34.6–44.6	%
MCV	49.30 ± 1.09	50.83 ± 0.98	50.20 ± 1.69	49.41 ± 1.88	48.2–58.3	fL
MCH	16.58 ± 1.42	16.38 ± 0.42	16.46 ± 0.36	14.79 ± 0.78	15.8–19	pg
MCHC	337.13 ± 4.78	325.63 ± 5.06	328.86 ± 18.8	325.63 ± 11.4	302–353	g/L
RDW	13.30 ± 0.77	14.74 ± 0.33	13.55 ± 1.13	12.86 ± 1.27	13–17	%
PLT	1021.8 ± 582.3	762.4 ± 498.0	1140.0 ± 226.6	892.4 ± 273.7	450–1590	10^9/L
MPV	4.91 ± 0.35	5.39 ± 0.15	5.31 ± 0.430	5.13 ± 0.53	3.8–6	fL
PDW	16.88 ± 1.10	17.31 ± 1.47	16.67 ± 1.14	17.21 ± 1.28	-	-
PCT	0.41 ± 0.20	0.30 ± 0.27	0.44 ± 0.25	0.31 ± 0.23	-	%

Hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), hemoglobin (MCH) and hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets (PLT), mean platelet volume (MPV), platelet distribution width (PDW), and platelet crit (PCT).

3.3. Fecal Corticosterone Metabolites

The statistical study of fecal corticosterone metabolite levels revealed a significant interaction between variables ($F_{(1,14)}$ = 11,40, $p < 0.01$). The post hoc analysis indicated significantly higher corticosterone metabolite levels in single-housed (0.225 ± 0.05 ng/mg) than in grouped animals (0.132 ± 0.02 ng/mg) after 1 week (t = 4.523; $p < 0.001$). At 4 weeks, no differences were observed between groups (grouped: 0.165 ± 0.06 ng/mg vs. single: 0.168 ± 0.04 ng/mg; t = 0.488, $p > 0.05$), and single-housed corticosterone metabolite levels were normalized (Figure 3).

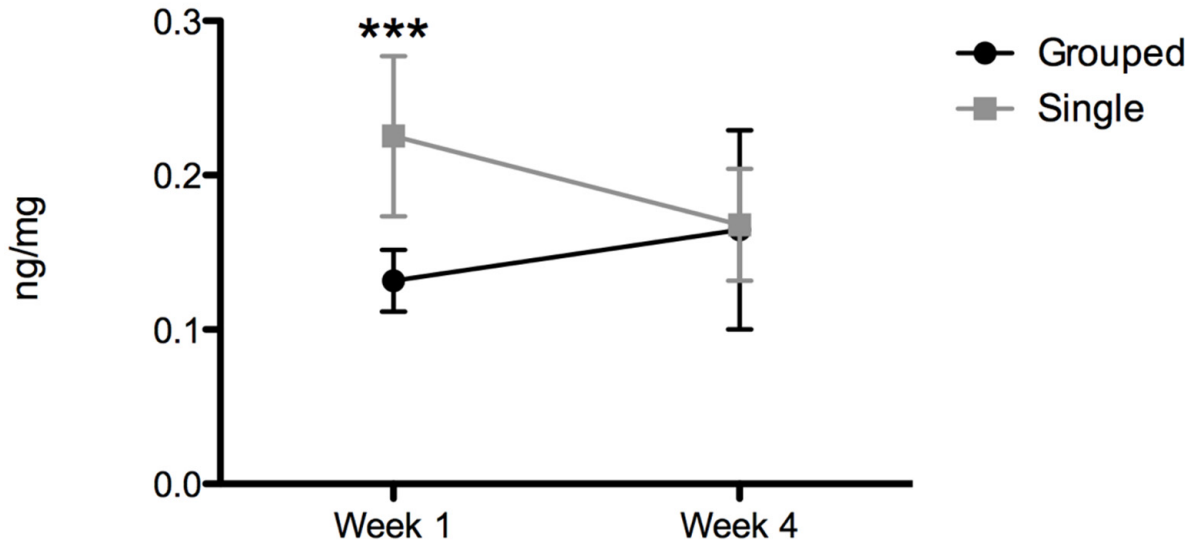

Figure 3. Fecal corticosterone metabolites (ng/mg feces). Data are expressed as the mean ± SD; n = 8 per group; *** $p < 0.001$.

4. Discussion

It is well known that animal welfare has an effect on the outcome of experiments. We must, therefore, always consider this factor when designing and carrying out experimental procedures. However, many researchers systematically tend to individualize animals in their experiments. Thus, the question we aimed to answer in this study was whether a lack of social interaction may modify physiological parameters, which may in turn interfere with experimental results. Our findings indicate that social isolation modifies some physiological parameters.

As previously reported for CD1 male mice [48–50], social isolation for 4 weeks did not affect body weight gain. Similarly, our results revealed that social isolation did not modify RBC parameters. As far as we are aware, this is the first study in mice to analyze RBC parameters; thus, we cannot compare our results with previous findings.

Mice that were changed from sharing a cage with littermates to living alone showed higher fecal corticosterone metabolites than those maintained in the group after the first week, although levels normalized after 1 month. These same results were recently observed in adult CD1 mice housed in the same conditions as our animals, in a ventilated rack with environmental enrichment [50], which may indicate habituation to the new situation. Due to the nature of our experimental design, we were unable to determine when exactly corticosterone metabolite levels normalized, and this is one of our study's limitations. However, data from a previous study [33] indicated that fecal corticosterone metabolite levels start to decrease and remain stable from the second week onward. These data are consistent with those described previously in relation to the return of plasma glucocorticoids to baseline values during the first week after transport or translocation [51–54]. Among the grouped animals, no significant changes were observed across individuals, and the standard deviation within groups was very small. Our data, therefore, seem to suggest that, in contrast to observations by some authors [37,38], remaining grouped together does not appear to cause the animals any stress. We believe the main reason for this is that, as has indeed been pointed out previously [32], our mice were littermates and were grouped together from weaning.

It is well known that increased glucocorticoid levels suppress cellular immunity [55]. No changes in monocytes and granulocytes were observed in single-housed animals after 7 days, although changes were found after 4 weeks. A previous study found no significant differences in the overall number of blood-circulating leukocytes between CD1 male mice that were socially isolated for 2 weeks and their socially housed counterparts [36]. However,

C57BL6/J adult mice separated into individual cages for 2 h every day for 25 days were found to have a decrease in T cells, B cells, monocytes, and neutrophils [41]. Unfortunately, our system is not able to distinguish between the different types of lymphocytes and granulocytes; however, overall, our results are consistent with these findings and highlight the fact that isolation time is a factor to be considered. Another limitation of the study is that we did not study humoral immunity; previous studies found that fecal immunoglobulin A (IgA) excretion (a marker of long-term stress) takes at least 4 weeks to normalize [53]. It is important to note that CD1 adult males isolated for 21 days and subjected to mild psychological stress had lower splenocyte proliferation and lower IL-2 and IL-4 cytokine plasma levels than their grouped counterparts [32]. The same results were reported using shock as a stressor [55].

In addition to the limitations outlined above, our study had some further limitations. When designing the experiment, we wanted it to be as realistic as possible in terms of the day-to-day management of our animal facility technicians and researchers. Therefore, the animals were moved from dirty to clean cages by picking them up by the tail. In recent years, less aversive handling methods (e.g., tunnel or cup handling) have been shown to mitigate anxiety and depressive-like behaviors [56–58]. However, a recent study showed that picking mice up by their tail may not be a significant source of chronic husbandry stress [59]. In view of the results of this study and our daily practice, we decided to change the location of animals in this experiment by picking them up by their tail. We are all aware that efforts have to be made to implement less aversive methods of handling in daily practice in animal facilities. Nevertheless, it should also be kept in mind that this procedure takes more time; hence, the amount of work assigned to each technician when changing cages should also be reviewed. In our work, we did not study whether social isolation induced behavioral changes in our animals, because we were more interested in peripheral biomarkers than behavioral parameters. In a recent study performed on C57BL/6JRj mice housed singly for 10 weeks, no behavioral changes were observed in exploratory activity, anxiety, working memory, and fear memory [60]. However, a previous study using C57BL/6J and DBA/2 kept in individual housing for 7 weeks revealed that individual housing has strong strain- and test-specific effects on emotional behavior and impaired memory in certain tasks. Single-housed mice were hyperactive and displayed reduced habituation to novel environments. Reduced anxiety was established in the elevated plus-maze, but not in the dark/light test. Immobility in the forced swimming test was reduced by social isolation. Novel object recognition and fear conditioning were impaired in the single-housed mice, whereas water-maze learning was not affected [61]. In the same way, 2 weeks of single housing plus acute injection stress induced anxiety-like behavior in C57BL6/J mice [30]. Mouse strain and social environment also influence depression-like behavior caused by an immune challenge. In this sense, group-housed CD1 mice exhibited depression-like behavior 1 day after bacterial lipopolysaccharide (LPS) injection, while the behavior of single-housed CD1 mice was little affected during the 4 weeks of the experiment. In contrast, both grouped and single-housed C57BL/6 mice responded to LPS with an increase in depression-like behavior [62]. It would be interesting to conduct future behavioral studies to determine if, under our conditions, single-housed CD1 male mice show any behavioral changes. Another parameter we did not measure was body temperature. In recent years, it has been observed that laboratory mice suffer from thermal stress, and that this affects their immune system, among other physiological parameters [63,64]. In this sense, huddling, a form of social thermoregulation, is a major contributor to mice's thermal physiology. Thus, single-housed mice are usually more affected by cold temperatures than grouped mice [65]. In order to mitigate this effect, two sheets of tissue were added to their home cage, and we ensured that they made a proper nest.

In light of all these data, we recommend keeping males in stable groups from weaning onward. Researchers should be aware that the change from grouping to living alone

induces stress and mild immunosuppression in CD1 male mice; hence, if the mice need to be separated for experimental reasons, these factors should be taken into consideration.

5. Conclusions

We conclude that social isolation has an effect on the immune–endocrine system. Consequently, the stress associated with the new social situation should be taken into consideration in the interpretation of research data.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ani13061026/s1: Determination of CVM-Procell analyzer reference values for each strain. References [66–71] are cited in the supplementary materials

Author Contributions: Conceptualization, G.A.; methodology, I.O.-S., A.D.-S., M.M.-C. and G.A.; acquisition and analysis of the data, I.O.-S., A.D.-S., R.G., C.M. and C.Z.; analysis and interpretation of the data, I.O.-S., A.D.-S., O.V. and G.A.; interpretation of the data and writing—original draft preparation, I.O.-S., A.D.-S., M.M.-C., O.V. and G.A.; writing—review and editing, G.A. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the University of the Basque Country (UPV/EHU) GIU18/103 grant.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Catalan Government and the PRBB Ethics Committees (DAAM 10576).

Informed Consent Statement: Not applicable.

Data Availability Statement: All the data of the study can be made available upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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4. CHAPTER 2. Study #2: How do intrinsic coping strategies in response to Chronic Social Defeat Stress influence physiological and behavioral parameters in male mice?

4.1. Introduction

Chronic Social Stress is one of the most related stressors to health issues, particularly to depression. However, the underlying physiological and behavioral mechanisms have not been fully elucidated. The coping strategies employed during stress and/or the differences on brain neurochemistry or neuroendocrine parameters could be playing a key role.

4.2. Primary objective

The aim of this study was to determine the behavioral, neuroendocrine, and neurochemical effects of stress in OF-1 male mice regarding the coping strategies that stressed mice use during the first social defeat encounter. The purpose was to explore the effects of CSDS in male mice and to examine whether the use of Active-Aggressive (AA) or Passive-Reactive (PR) strategies could be related to a differential behavioral and physiological response.

4.3. Specific objectives

With the aim of achieving the aforementioned primary objective, the following specific objectives were proposed:

- To assess the behavior and to identify coping strategies used by the subjects during the first confrontation of CSDS.

- To assess the activation of HPA axis by assessing CORT plasma levels before, during and after CSDS.
- To assess brain neurochemistry by determining monoamines and their metabolites (Phe, Tyr, DA, DOPAC, NA, MHPG, Tryp, Kyn, Kyna, 3-HK, 5-HT and 5-HIAA) in the HC and the PFC after CSDS exposure and in relation to the coping strategy.
- To examine the behavioral profile of stressed and non-stressed subjects, and to study whether there were differences within the stressed group regarding the coping strategy used after CSDS exposure.
- To analyze alterations in body weight by assessing body weight both before and after CSDS procedure.

4.4. Hypothesis

This study hypothesis posits that CSDS will induce changes at both the behavioral and physiological levels in male mice, and that the coping style employed by them will mediate this relationship, resulting in a differential response depending on the coping strategies used.

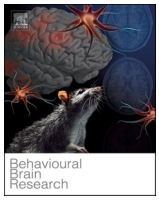
4.5. Publication: Chronic defeat stress induces monoamine level dysregulation in the prefrontal cortex but not in the hippocampus of OF1 male mice

Behavioural Brain Research 467 (2024) 115023

Contents lists available at ScienceDirect

Behavioural Brain Research

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



Chronic defeat stress induces monoamine level dysregulation in the prefrontal cortex but not in the hippocampus of OF1 male mice

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ARTICLE INFO

Keywords:
Chronic defeat stress
Coping strategies
Depressive like-behavior
Monoamines
Prefrontal cortex

ABSTRACT

Chronic social stress can increase susceptibility to chronic diseases such as depression. One of the most used models to study the physiological mechanisms and behavioral outcomes of this type of stress is chronic defeat stress (CDS) in male mice. OF1 male mice were subjected to a stress period lasting 18 days. During that time, non-stressed animals were housed in groups. The cluster analysis of the behavioral profile displayed during the first social interaction divided subjects into two groups: active/aggressive (AA) and passive/reactive (PR). The day after the end of the stress period, the following behavioral analyses were performed: the sucrose preference test (SPT) on day 19, the open field test (OFT) on day 20, and the forced swim test (FST) on day 21. Immediately after completing the last test, animals were weighed, and blood samples were obtained. Then, they were sacrificed, and their prefrontal cortices and hippocampi were removed and stored to analyze monoamine levels. Stressed animals displayed anhedonia, and solely the PR mice continued to show higher levels of immobility in the OFT and FST. All stressed animals, regardless of the coping strategy, presented higher plasma corticosterone levels. In addition, stressed mice showed lower levels of tyrosine, dopamine, DOPAC, MHPG, kynurenine, kynurenic acid, and 5-HIAA levels but higher serotonin levels in the prefrontal cortex, not in the hippocampus. In conclusion, our results show that CSD induces differences in monoamine levels between brain areas, and these differences did not respond to the coping strategy adopted.

1. Introduction

Chronic stress, particularly chronic psychosocial stress, can have negative health consequences and increase susceptibility to chronic diseases such as depression [40]. One of the most common chronic stressors in humans and other social animals is stress arising from social interactions [10]. For example, loss of social rank, status and/or control are examples of chronic stressors that are increasingly recognized as risk factors for depression [36]. Animal models using the resident-intruder paradigm, applied in different ways, have been very valuable in studying individual differences in patterns of behavioral and physiological responses to chronic social stress [66]. Several studies using these models in male mice have focused on the consequences of chronic defeat stress (CDS), sorting subjects into different coping strategy groups based on their social behavior. Coping is the behavior engaged in by an individual in response to an aversive or stressful situation [65].

Many studies classify subjects as either resilient or susceptible based on their performance in the social avoidance test following the stress procedure [32,47]. However, some assess subjects' behavioral coping response during the social defeat encounters themselves. These studies frequently classify subjects into categories that generally involve some degree of behavioral activity (i.e., mobility/immobility, social/non-social exploration, escape or avoidance behaviors) [33,37,53]. Other studies focusing on coping strategies in animals distinguish between two major strategies: active and passive coping [8,26,46]; and others still employ different classifications and terminology, such as proactive/reactive strategies [7,25,46,52], or active escape behavior/passive submissive responses [3], or emphasize aggressiveness

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https://doi.org/10.1016/j.bbr.2024.115023
Received 28 February 2024; Received in revised form 19 April 2024; Accepted 26 April 2024
Available online 28 April 2024

levels [35]. Despite the differences in nomenclature, it has been observed that coping responses vary across individuals, and the strategies an individual uses to cope with stress have significant effects on the characteristic activation profile of the neuroendocrine axes [4,19].

The physiological response of the organism to a stressful situation involves the co-activation of the sympathetic-adrenal-medullary (SAM) and hypothalamic-pituitary-adrenal (HPA) axes. The activation of these axes begins with the perception of the stimulus as a threat and the activation, among others, of the hypothalamus (HT), the hippocampus (HPC) and the prefrontal cortex (PFC). During stressful events, rapid physiological adaptation is mediated by adrenaline (A), noradrenaline (NA) and glucocorticoids (CORT) [31,41]. Chronic stress-induced HPA axis hyperactivity has been linked to HPC and PFC degeneration, HPC–PFC pathway dysfunction and depression [16,57,58,64].

The monoamine hypothesis proposes that diminished dopamine (DA), NA and serotonin (5-hydroxytryptpamine, 5-HT) signal pathways may cause depression [20]. The ventral tegmental area (VTA) of the midbrain is the main source of DA for limbic and cortical structures, and this transmission is involved in numerous cognitive and emotional functions. The locus coeruleus (LC) is the main source of NA, and modulates arousal and attention [11,60]. The raphe nuclei (RN) is the main 5-HT nuclei, has widespread innervations to the HPC and the PFC, and is involved in cognition, mood and impulse control [15]. Catecholamine, DA and NA, biosynthesis starts with phenylalanine (Phe), which is converted to tyrosine (Tyr). Dopamine is metabolized to 3, 4-Dihydroxyphenylacetic Acid (DOPAC), and NA to 3-methoxy-4-hydroxyphenylglycol (MHPG) [22]. 5-HT is synthesized from tryptophan (Tryp) in serotonergic neurons and metabolized to 5-Hydroxyindoleacetic Acid (5-HIAA). However, the vast majority of Tryp enters the kynurenic pathway. Kynurenine (Kyn), produced from Tryp, can be further converted to either kynurenic acid (Kyna) or 3-Hydroxykynurenine (3-HK), the second of which is converted to quinolinic acid that affects the function of both monoaminergic and glutamatergic neurons [39]. Chronic stress can alter this pathway and redirect the metabolization away from 5-HT production towards the kynurenine pathway [51].

To the best of our knowledge, there are no studies that have analysed monoamine levels in the PFC and HPC after CSD as a function of coping in mice. We hypothesise differences in monoamine levels in the aforementioned brain areas regarding coping strategy adopted. Thus, the objectives of the present study were, firstly, to identify coping strategies based on the behavior manifested by subjects during the first social interaction, and to analyze their involvement in depression-like behavior following CDS. And secondly, to analyze prefrontal cortex and hippocampal monoamine levels in relation to CDS and coping strategies.

2. Methods

2.1. Subjects and husbandry

Six-week-old OF1 outbred male mice (Charles River, France) were housed 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 22 ± 2 °C with a relative humidity level of 70 % and a reverse 12-h light/dark cycle (white lights on from 19:00–07:00 h) to enable the testing of these nocturnal animals during their active phase (1 h after the beginning of the dark cycle). All experimental procedures were conducted under dim red lighting in a room adjacent to the holding facility. All procedures involving mice were performed in accordance with the European Directive (2010/63/EU) and were approved by the Animal Welfare Ethics Committee of the University of the Basque Country (CEEA-UPV/EHU; M20/2018/090) and the Gipuzkoa Provincial Council (PRO-AE-SS-062).

2.2. Experimental procedure

Animals were randomly separated into three groups; (1) animals that were subjected to the stress procedure and performed the behavioral tests (S, n = 28); (2) animals that were not subjected to stress and performed the behavioral tests (NS, n = 15); and (3) animals that were not subjected to stress and did not perform the behavioral tests (C, n = 12). The experiment began after a 7-day adaptation period. Basal blood samples and body weight were obtained from NS and S mice on the 4th day of acclimation. After the adaptation period, S mice were submitted to a stress period lasting 18 days (see below). During that time, NS and C animals were housed in groups. On the first day of the stress protocol, the social behavior profile of S animals was analyzed (see below). On day 9, NS and S animals were weighed and blood samples were drawn. The day after the end of the stress period, behavioral analyses were performed on NS and S animals: the sucrose preference test (SPT) on day 19, the open field test (OFT) on day 20, and the forced swim test (FST) on day 21. Immediately after completing the last test, all animals (C, NS and S) were weighed and blood samples were obtained. The C and S groups were sacrificed, and their PFCs and HPCs were immediately removed and placed on ice (Fig. 1). The NS group was not sacrificed and its members were incorporated into our colony as sentinels. We expected the animals to suffer from acute non-social stress following the FST, a hypothesis that was subsequently confirmed by the detection of high plasma corticosterone levels (see results).

2.3. Stress procedure

Animals in the stressed group were exposed to the sensory contact social stress model based on our resident-intruder paradigm (Vegas et al., 2004). Each experimental subject was exposed to daily interaction with different previously-selected and trained highly-aggressive resident mice during 18 days. The mice were subjected to direct physical interaction (DPI) for 5 min, followed by non-physical interaction (NPI) for other 5 min. During NPI, the resident was covered with a wire mesh container that prevented it from attacking the experimental subject, thereby allowing said subject to explore the environment while being protected from attacks. During subsequent encounters, physical interactions were halted after the first attack to avoid physical injuries. After the interactions, the intruders were separated from the residents by perforated methacrylate barriers, which bisected the cage and allowed sensory (non-physical) contact outside the direct confrontation periods. On day 1, interactions were recorded to analyze the animals' behaviors.

2.4. Behavioral assessment

2.4.1. Analysis of the behavioral profile during social interactions

The interactions conducted on day 1 were filmed with a video camera (Panasonic RX66, Osaka, Japan). Behavioral evaluations were performed using Observer XT 14 software (Noldus, ITC, Wageningen, the Netherlands), with a specific configuration based on the ethogram for the mouse developed by Brain, McAllister and Walmsley (1989) and modified by Vegas et al. [62]. This ethogram covers 51 behavioral elements grouped into 12 broad categories: *attack* (chasing, rushing towards or biting the opponent), *threat* (aggressive cleaning, vertical or lateral offence or hitting with tail), *non-social exploration* (exploration of the physical environment), *social investigation* (social exploration of the opponent by following or establishing physical contact, sniffing or cleaning), *exploration from a distance* (paying attention to the opponent from a distance), *digging* (moving the sawdust with front or back legs), *body care* (self-cleaning), *avoidance* (remaining at a prudential distance from the opponent), *flee* (running away when the opponent approaches), *defense/submission* (passive avoidance of an attack by making signs of submission), *sexual behavior* and *immobility* (remaining frozen). Immobility and explorations during NPI were also included in the behavioral assessment.

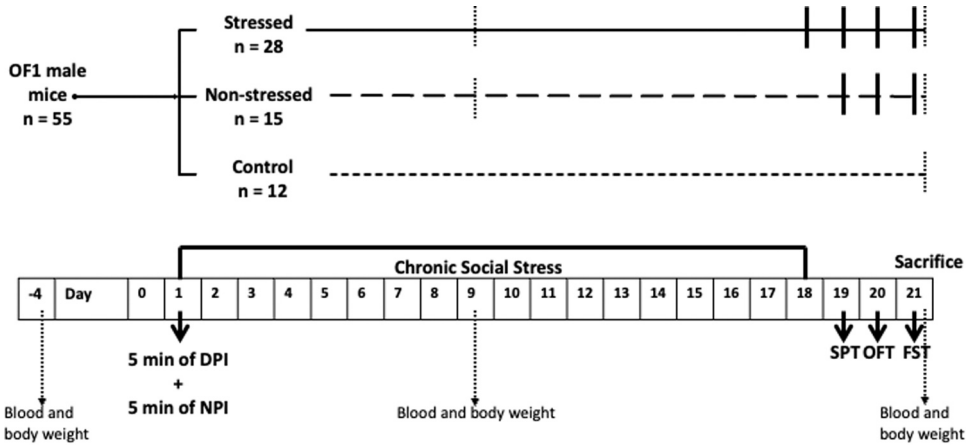

Fig. 1. Experimental procedure. Notes: DPI = direct physical interaction NDI = non-physical interaction; SPT = sucrose preference test; OFT = open field test; FST = forced swim test.

2.4.2. Sucrose Preference Test (SPT)

All mice were offered a free choice between two bottles for 24 h; one bottle contained a 0.8 % sucrose solution and the other bottle contained water. The position of the bottles was counterbalanced to avoid possible effects of a side preference when drinking. The animals were not deprived of food or water before the test. Consumption of both the sucrose solution and water was measured by weighing the bottles at the beginning and end of the test, and linking the result to the animals' body weight.

2.4.3. Open Field Test (OFT)

Mice were placed in a black Plexiglas box (40 × 40 × 30 cm) and allowed to explore for 5 min. The time spent in each zone of the box (center or periphery), the average distance covered and the time spent immobile were analyzed using the ANY-maze© 4.96 software package (Stoelting Europe, Dublin, Ireland). The apparatus was cleaned with a solution of 0.5 % acetic acid between tests to hide animal clues.

2.4.4. Forced Swim Test (FST)

Individual mice were placed in glass cylinders (height 18.5 cm and diameter 12.5 cm) containing 13.5 cm of water at 25 ± 1 °C for 5 min. The following behaviors were assessed: immobility, swimming and climbing. The time spent engaged in each behavior was recorded manually using Observer XT 14 by an experimenter blinded to the stress condition.

2.5. Physiological assessments

2.5.1. Blood collection and plasma isolation

Blood was collected from the submandibular vein between 8:45 and 9:45 a.m. to measure corticosterone levels. Blood samples were collected on day −4, 40–45 min after the direct interactions (day 9), and 5–10 min after the FST (day 21). The blood was stored in a heparinized container and then centrifuged at 1800×*g* for 15 min at 4°C. The resulting plasma was collected and stored at −70 °C until further analysis.

2.5.2. Determination of plasma corticosterone concentrations

Plasma corticosterone concentrations (ng/ml) were determined using a commercially-available enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA) and a Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The sensitivity of the assay was 5 pg/ml, and the intra-assay and inter-assay coefficients of variation were 7 % and 8 %, respectively.

2.5.3. Determination of monoamines and their metabolites by high-performance liquid chromatography (HPLC)

The prefrontal cortexes and hippocampi were weighed and homogenized in a 60 µl solution (1 % formic acid in acetonitrile). Zirconia Ceramic Balls (0.5 and 1.0 mm) were inserted into the sample tubes and then placed in the Bullet Blender for 3 min to bust the brain tissue. Immediately afterwards, the tubes were vortexed for 5 min (Vortex Genie-2; Scientific Industries, Bohemia, NY, USA) and the samples were then centrifuged for 15 min at 15,000×*g* and 4 °C. The supernatants were dried for 30 min with compressed air to concentrate the samples and were then reconstituted with 30 µl of 0.05 % trifluoroacetic acid. Next, the samples were again centrifuged for 20 min at 15,000×*g* and 4 °C and placed in the autosampler unit for analysis.

L-Phenylalanine (Phe), L-Tyrosine (Tyr), dopamine (DA), 3,4-Dihydroxyphenylacetic Acid (DOPAC), noradrenaline (NA), 3-methoxy-4-hydroxyphenylglycol (MHPG), Tryptophan (Tryp), kynurenine (Kyn), Kynurenic Acid (Kyna), 3-Hydroxykynurenine (3-HK), serotonin (5-HT), and 5-Hydroxyindoleacetic Acid (5-HIAA) were determined using 20 µL of each sample injected into the HPLC (Hewlett Packard 1100 System). The samples were separated on a Poroshell 120 EC-C18 column (100 × 4.6 mm, 2.7 µm), with an Analytical Guard Column (12.5 ×4.6 mm, 5 µm) being used for protection (Agilent Technologies). The mobile phase for this study comprised 0.05–2 % trifluoroacetic acid (solvent A) and 98–99.9 % acetonitrile (solvent B). The flow was maintained at a constant rate of 0.5 ml/min. The column was maintained at 25 °C during the analysis, and the samples at 4 °C in an autosampler unit. The samples were analyzed and monitored either by a fluorescence detector (FLD) at an emission wavelength of 320 nm or by a variable wavelength detector (VWD) set at 230 nm. Phe (Excitation wavelength (Ex) 212 nm), and NA, Tyr, 5-HT, DA, MHPG and 5-HIAA (Excitation wavelength (Ex) 229 nm), effluents were monitored with the fluorescence detector. The 3-HK, Kyn, DOPAC, Kyna, and Tryp effluent was monitored with a variable wavelength detector. The total sample analysis time was 27 min. The final data were expressed as ng/mg.

2.6. Statistical analysis

A cluster analysis was performed using SPSS 28.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical analyses of the behavioral and physiological variables and graphic visualization and design were performed using the GraphPad Prism software package (9.0, GraphPad Software, Inc). Kolmogorov-Smirnov test was use for data distribution, and outlier values were identified using the ROUT method and removed from the analysis. Variables were analyzed using Student's t-tests and one-way or two-way ANOVAs, and specific comparisons were analyzed using the post hoc Bonferroni test. Values of $p < 0.05$ were considered statistically

significant (95 % confidence). Data are expressed as mean ± standard error (SEM).

3. Results

3.1. Strategies for coping with social stress

Using the mean percentage of time allocated to each assessed behavioral element during the first interaction, a cluster analysis was performed on all stressed subjects in terms of the behavioral characteristics they displayed in the social stress situation. The analysis resulted in two clusters: active/aggressive (AA, n = 20) and passive/reactive (PR, n = 8) (Fig. 2). A multivariate discriminant analysis was performed to explore the integrity of the groups derived from the cluster analysis and to determine which behavioral variables most efficiently discriminated between the clusters. The discriminant model applied accounted for 97.2 % of the groups obtained from the cluster analysis, thereby confirming the statistical validity of these groups and their behavioral descriptions. Immobility during direct physical interaction (DPI) was the variable that best discriminated between the two clusters, followed by threat, non-social exploration, and exploration from a distance ($F_{(6,182)} = 150.5$, $p < 0.0001$). Both behavioral variables measured during non-physical interaction discriminated between the two clusters ($F_{(1,48)} = 538.6$, $p < 0.0001$).

3.2. Behavioral characterization

In the SPT, chronically-stressed mice consumed less sucrose solution in relation to body weight than non-stressed mice ($t_{(41)} = 3.681$, $p < 0.001$; Fig. 3a). In the OFT, no significant differences were observed between non-stressed and stressed mice in relation to distance travelled (Fig. 3b) or time spent immobile (Fig. 3c). No significant differences were observed either in time spent at the periphery or center of the OF (data not shown). No significant differences were observed in the FST (Fig. 3d).

In relation to coping strategy, although no differences were observed between coping strategies, compared to the NS group, both AA and PR mice consumed less sucrose solution ($F_{(2,40)} = 8.028$, $p < 0.001$; Fig. 3e). In the OFT, PR mice travelled less distance ($F_{(2,40)} = 5.066$, $p < 0.01$; Fig. 3f) and remained immobile for longer than NS and AA animals ($F_{(2,40)} = 4.849$, $p < 0.01$; Fig. 3g). No significant differences were observed between groups in time spent at the periphery or center of the OF (data not shown). Similarly, in the FST, PR mice spent a higher percentage of time immobile and less swimming than NS and AA mice ($F_{(2,120)} = 83.23$, $p < 0.0001$; Fig. 3h).

3.3. Body weight

A two-way ANOVA (group and time) with repeated measures revealed significant differences in weight gained during the experiment for the group factor ($F_{(1,82)} = 4.3$, $p < 0.05$). Stressed mice had a lower weight gain at day 9 but had recovered by day 21 (Fig. 4a). At that time (day 21), no significant differences were found in the final weight of the animals, although a statistical trend was observed ($F_{(2,50)} = 2.478$, $p = 0.094$; Fig. 4b). Moreover, when analyzing these differences in terms of coping strategy, we observed that AA animals had the lowest weight gain at day 9 ($F_{(1,80)} = 4.676$, $p < 0.01$; Fig. 4c), although by the end of the experiment there were no differences in body weight (Fig. 4d).

3.4. Plasma corticosterone levels

No differences were observed between NS and S mice in basal plasma corticosterone, although at day 9, the S group had higher levels of plasma corticosterone than the NS group. The two-way ANOVA (group and time) with repeated measures revealed significant differences for the group factor ($F_{(2,80)} = 22.38$, $p < 0.0001$), the time factor ($F_{(1,80)} = 25.42$, $p < 0.0001$) and their interaction ($F_{(2,80)} = 13.61$, $p < 0.0001$; Fig. 5a). At day 21, the NS and S groups had higher corticosterone levels than the C group. Note that the NS and S groups performed the FST and that blood was collected 5–10 later ($F_{(2,50)} = 27.65$, $p < 0.0001$; Fig. 5b).

In terms of coping strategy, no differences were observed between the AA and PR groups, although both had higher corticosterone levels than the NS group at day 9. The two-way ANOVA (group and time) with repeated measures revealed significant differences for the group factor ($F_{(2,115)} = 40.15$, $p < 0.0001$), the time factor ($F_{(2,115)} = 15.44$, $p < 0.0001$) and their interaction ($F_{(4,115)} = 8.32$, $p < 0.0001$; Fig. 5c). At day 21, the one-way ANOVA revealed that the NS, AA and PR groups all had higher corticosterone levels than the C group ($F_{(3,49)} = 18.49$, $p < 0.0001$; Fig. 5d).

3.5. Effect of the chronic social defeat (CSD) on prefrontal cortex monoamine levels

Stressed mice had lower levels of Tyr ($t_{(35)} = 2.291$, $p < 0.05$), DA ($t_{(31)} = 3.826$, $p < 0.001$), and DOPAC ($t_{(21)} = 2.161$, $p < 0.05$) than their non-stressed counterparts (Fig. 6b–d). They also had higher NA levels, although this difference was only a statistical trend ($t_{(33)} = 1.761$, $p = 0.087$), and lower MHPG ($t_{(35)} = 4.136$, $p < 0.001$) levels (Fig. 6e,f). The S group had a higher NA/Tyr ratio than the C group (0.008 ± 0.001 vs 0.003 ± 0.0007; $t_{(29)} = 2.08$, $p < 0.05$), although no changes were observed in the DA/Tyr, DOPAC/DA or MHPG/NA ratios.

In relation to coping strategies, we observed that PR animals had higher NA levels than the C group ($F_{(2,32)} = 3.45$, $p < 0.05$; Fig. 6k), and a higher NA/Try ratio than both the C and AA groups (C: 0.003 ± 0.0007; AA: 0.006 ± 0.001 and PR: 0.01 ± 0.003; $F_{(2,28)} = 8.23$, $p < 0.001$). We observed marginally significant differences in the DOPAC/DA ($F_{(2,13)} = 3.17$, $p = 0.07$) and MHPG/NA ratios ($F_{(2,19)} = 2.584$, $p = 0.08$).

In terms of the indolamine pathway, we found that stressed mice had lower Kyn levels ($t_{(22)} = 2.773$, $p < 0.05$; Fig. 7b), lower Kyna levels (t

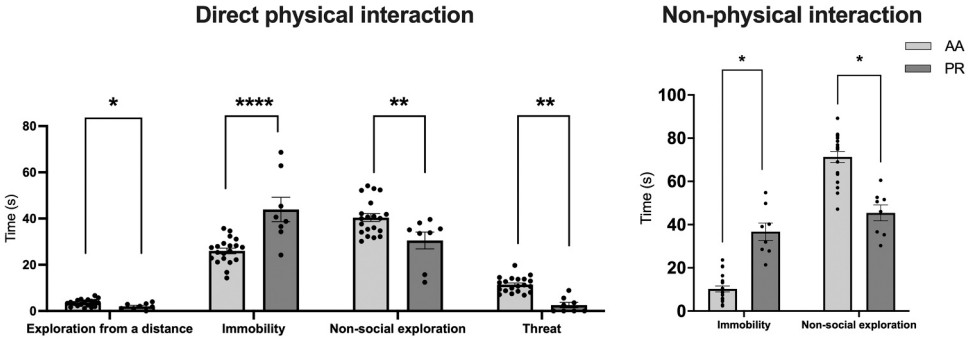

Fig. 2. The mean percentage of time dedicated to each of the behaviors evaluated during the interaction on day 1, analyzed in terms of group membership: active/aggressive (AA) or passive/reactive (PR). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, and ****p < 0.0001.

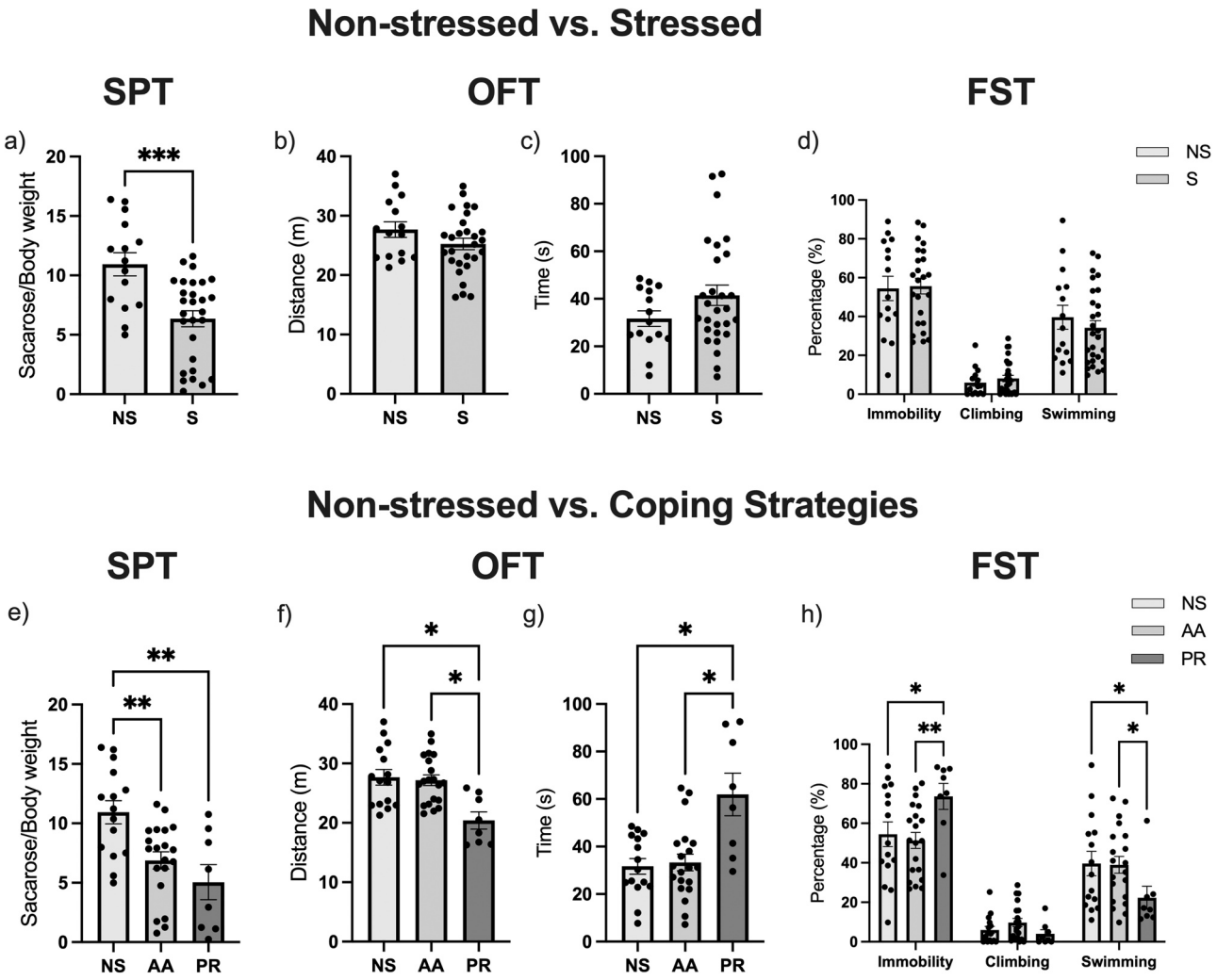

Fig. 3. Non-stressed vs. Stressed: a) Sucrose consumption in the SPT. b) Distance travelled and c) time immobile in the OFT. d) Percentage of immobility, climbing and swimming in the FST. **Non-Stressed vs. Coping Strategies:** e) Sucrose consumption in the SPT. g) Distance travelled and g) time immobile in the OFT. h) Percentage of immobility, climbing and swimming in the FST. Groups: non-stressed (NS), stressed (S), active/aggressive (AA) and passive/reactive (PR). Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.

$_{(25)} = 2.00$, $p < 0.05$; Fig. 7c), higher 5-HT levels ($t_{(34)} = 1.78$, $p = 0.084$; Fig. 7e), and lower 5-HIAA levels ($t_{(26)} = 2.978$, $p < 0.01$; Fig. 7f) than controls. Also, stressed mice had a lower Kyn/Tryp ratio (0.004 ± 0.0009 vs 0.11 ± 0.04; $t_{(14)} = 3.27$, $p < 0.01$), a higher 5-HT/Tryp ratio (0.11 ± 0.01 vs 0.04 ± 0.008; $t_{(22)} = 2.33$, $p < 0.05$), and a lower 5-HIAA/5-HT ratio (0.42 ± 0.10 vs 1.15 ± 0.44; $t_{(17)} = 2.17$, $p < 0.05$) than C mice.

When the analyses were carried out in accordance with coping strategy, we observed that AA animals had lower Kyn levels ($F_{(2,24)} = 3.504$, $p < 0.05$; Fig. 7h) and a lower Kyn/Tryp ratio (C: 0.11 ± 0.04; AA: 0.005 ± 0.001 and PR: 0.08 ± 0.03; $F_{(2,17)} = 4.43$, $p < 0.05$) than C mice.

3.6. Effect of chronic social defeat (CSD) on hippocampal monoamine levels

No significant differences were observed between control and stressed mice, or between controls and coping strategy groups, in hippocampal levels of catecholamines and their precursors (Fig. 8).

Looking at the indolamine pathway, we observed that stressed mice had a lower Kyn/Try ratio (0.001 ± 0.0001 vs 0.002 ± 0.0001; $t_{(26)} = 2.58$, $p < 0.05$), and tended to have lower Kyn levels ($t_{(27)} = 1.803.58$, $p = 0.083$) than C mice, with no change in Tryp levels (Fig. 9a,b). We also observed that stressed mice tended to have lower levels of Kyna ($t_{(21)} = 1.993$, $p = 0.067$; Fig. 9c), lower 3-HK levels ($t_{(28)} = 2.23$, $p < 0.05$; Fig. 9d), and a higher Kyna/3-HK ratio (0.02 ± 0.02 vs 0.01 ± 0.003; $t_{(14)} = 2.39$, $p < 0.05$) than Cs. No significant differences were found in the levels of 5-HT and its metabolite, 5-HIAA (Fig. 9e,f), although a statistical trend was observed, with stressed mice having a lower 5-HIAA/5-HT ratio ($t_{(26)} = 1.71$, $p = 0.098$).

Regarding indolamine levels and coping strategies, no significant differences were observed in either Tryp or Kyn levels (Fig. 9g,h), although the ratio between the two was significantly lower among AA mice than among their C counterparts (C: 0.002 ± 0.0001; AA: 0.001 ± 0.00014 and PR: 0.0015 ± 0.0002; $F_{(2,25)} = 3.41$, $p < 0.05$). AA mice also tended to have lower 3-HK levels ($F_{(2,27)} = 2.60$, $p = 0.093$; Fig. 9j). No significant differences were found in 5-HT and 5-HIAA levels or their ratio (Fig. 9k,l).

4. Discussion

In male mice exposed to CSD, behavioral coping strategies can often be categorized as responding either to the active fight-flight response, which describes proactive individuals exhibiting high levels of aggression and territorial control [14], or to the passive conservation-withdrawal response [23], which describes reactive

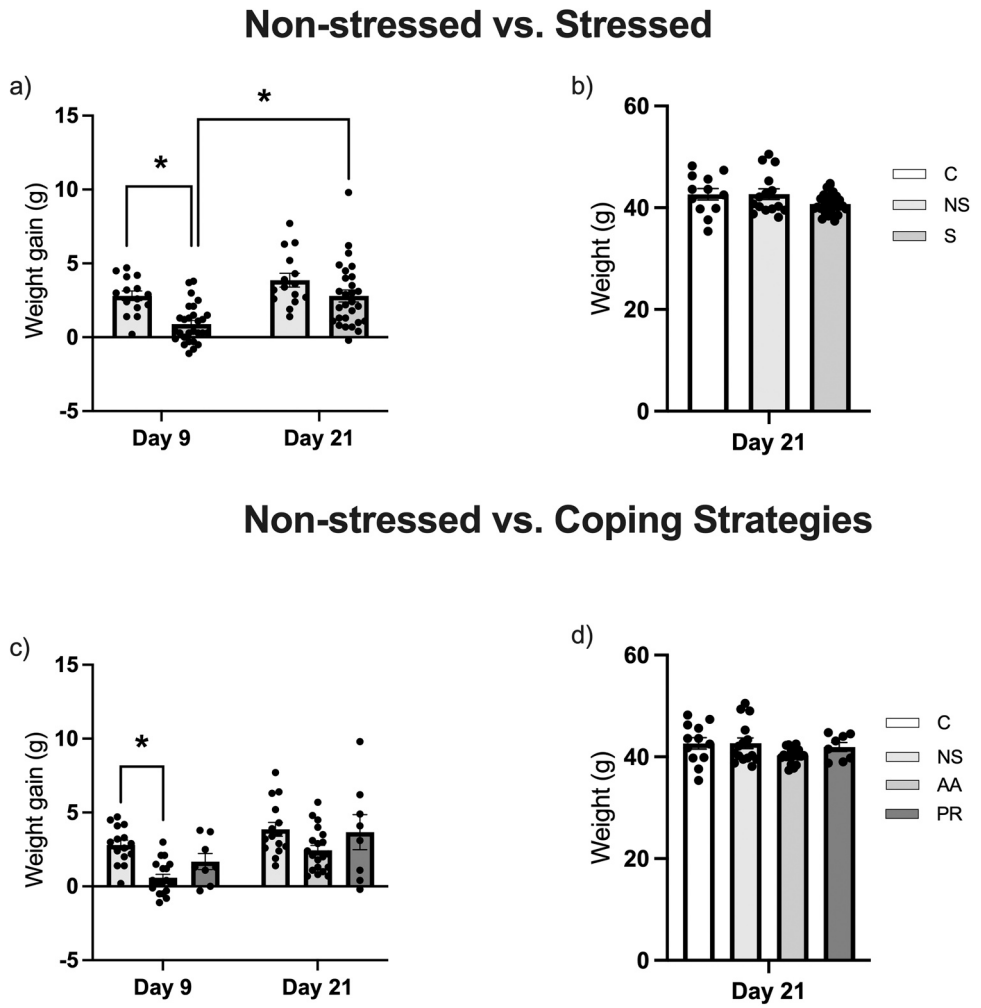

Fig. 4. Non-stressed vs. Stressed: a) Body weight gain at day 9 and day 21. b) Body weight at day 21. **Non-Stressed vs. Coping Strategies:** c) Body weight gain at day 9 and day 21. d) Body weight at day 21. Groups: control (C), non-stressed (NS), stressed (S), active/aggressive (AA) and passive/reactive (PR). Data are expressed as mean ± SEM. *$p < 0.05$.

individuals exhibiting low levels of aggression and high levels of immobility. The passive or reactive coping style has frequently been proposed as a model of dysfunctional and maladaptive behavioral coping [13,66] and has also been linked to negative health consequences [9,12,17,35,62]. However, this assertion has been challenged by other findings collected in both clinical [30] and preclinical [18] studies. According to these studies, the functionality and effectiveness of either coping strategy, as well as the individual's susceptibility or resilience to health problems, vary in accordance with specific conditions [21]. Being able to detect molecular differences between the two coping strategies is therefore of scientific interest.

The cluster analysis of the behavioral profile displayed during the first social interaction divided subjects into two groups. One group, which we called the active/aggressive (AA) group, exhibited a clear pattern of proactive strategies; in direct interaction, this group spent the most time engaged in threatening and exploratory behaviors. The other group engaged in little non-social exploration behavior and spent more time immobile. Since the mice in this group employed a reactive strategy, it was termed the passive/reactive (PR) group. This finding is consistent with that reported in our previous studies [33,34,53,54], and allows us to analyze not only the neurobiological effects of chronic stress, but also the effects of the coping strategies adopted by each individual.

Our results are consistent with those reported in other studies using CSD models in male mice that found that stressed animals displayed anhedonia [27,28,34,54]. When coping strategies were compared, two studies reported that only susceptible mice displayed anhedonia [27,28], although we found no such differences, either here or in our previous work [35]. Thus, the decrease in sucrose consumption induced by social stress showed no differences as a function of coping strategy. However, PR subjects continued to display higher levels of immobility in the OFT and FST after exposure to chronic stress, suggesting, as other authors point out, that coping strategies remain stable over time [19]. In this regard, although some authors report that passive or susceptible mice display higher levels of immobility in the FST [35,50,54], others do not [28,34,48]. The immobile float response in the FST is perhaps the most commonly-used behavioral test in preclinical studies of depressive-like phenotypes [59] and is thought to reflect an inability to sustain escape behavior following stress (i.e., behavioral despair). A variety of antidepressant treatments have been shown to reduce immobility time by increasing active escape behavior during testing [56]. However, some authors argue that immobility in the FST is not indicative of depressive-like behavior, but rather is a learned adaptive behavior. According to this approach, the driving force behind the observed shift to immobility, i.e., from active to passive coping, in the FST is not depression or despair, but cognition that promotes behavioral adaptation and survival [49]. Thus, immobility behavior in the FST may not represent the characteristic behavioral phenotype of an animal, but rather the subject's response to the specific test.

Various studies on the effect of CSD on body weight in male mice

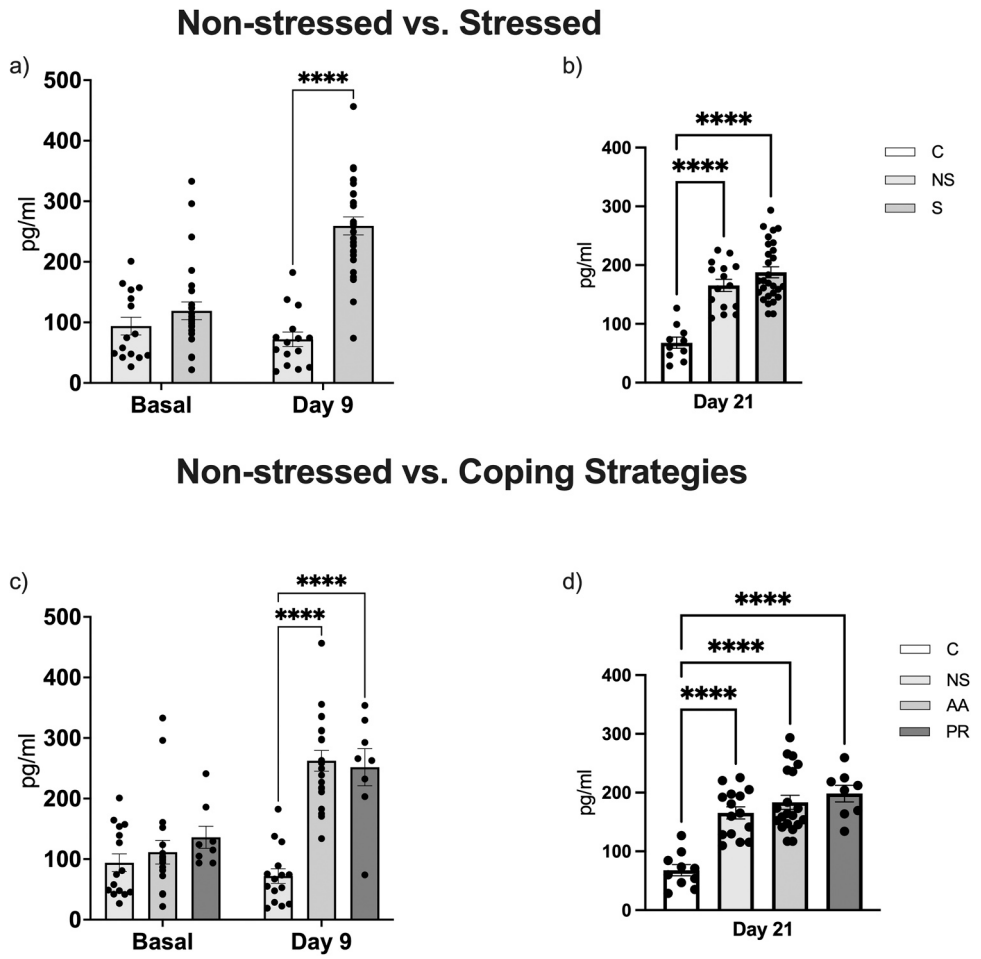

Fig. 5. Non-stressed vs. Stressed: a) Basal, day 9 and b) day 21 plasma corticosterone levels. **Non-Stressed vs. Coping Strategies:** c) Basal, day 9 and d) day 21 plasma corticosterone levels. Groups: control (C), non-stressed (NS), stressed (S), active/aggressive (AA) and passive/reactive (PR). Data are expressed as mean ± SEM. ****$p < 0.0001$.

have observed an increase or decrease in body weight [1,6,35,43]. In this sense, we observed that stressed animals (specifically AA mice) gained less body weight in the middle of the stress process (day 9), although they had recovered by the end of the CSD period (day 21), at which point there were no body weight differences among different stress coping strategies. Our results could indicate some kind of stress habituation that resulted in weight gain in the second half of the experiment. The results reported in the literature regarding coping strategies vary widely. For example, resilient adolescent mice have been to observed to have a lower body weight gain [1], as have susceptible [50], and submissive mice [61].

Increased plasma CORT levels in stressed mice have been repeatedly observed following CSD [5,34,54]. However, the overall picture of the relationship between coping style and HPA axis activity is rather complicated. In male animals, an active coping strategy has been associated with a lower-level response of the HPA axis [44,45]. According to this hypothesis, different studies have observed that animals displaying a passive coping style have even higher CORT levels than those displaying an active coping strategy [34,54]. However, other authors failed to find any differences linked to coping strategy [2,35,38,42,50]. Our results are consistent with the latter findings, as although we observed an increase in plasma CORT after CSD, we did not observe any differences between coping strategy groups.

In our previous studies, we observed higher levels of plasmatic adrenaline in active mice than in passive ones following acute social stress [19] and chronic social stress [54]. In this study, our aim was to analyze catecholamine levels in two brain areas affected by chronic stress and involved in depressive-like symptomatology: the PFC and the HPC. We found lower levels of Tyr, DA and DOPAC in the PFC of stressed subjects, with a tendency towards higher levels of NA and lower levels of MHPG, which may indicate a lower NA depletion. The low levels of DA in the PFC may have two possible causes, one being low levels of Tyr, and the other being the conversion of DA to NA, as low levels of DOPAC may indicate that DA has not been released, as do higher levels of NA and the NA/Tyr ratio in this area. These changes appear to affect only the PFC, as we did not observe any changes in the HPC, which is consistent with that published previously [24,63], although one study did report higher levels of DA in the HPC of C57BL/6 J stressed mice [29]. It has been argued that CSD decreases the density of dopaminergic axonal projections in the deep layer of the PFC in susceptible (but not resilient) male mice [55]. However, we did not find any differences in either DA or DOPAC levels in accordance with coping strategy. What we did find was that PR mice had higher levels of NA than C mice and a higher NA/Tyr ratio than both C and AA mice. These results may indicate that animals adopting a PR strategy have a greater tendency to synthesize NA after stress than their AA counterparts. Nevertheless, the lack of difference in MHPG levels may indicate that there is no difference in the release of NA, which is an indirect indicator of NA activity. These results are not consistent with the findings of our previous studies, in which we observed that active mice had lower levels of alpha2 receptor expression in the PFC than passive mice, which may indicate a down-regulation as a consequence of greater noradrenergic activity after CDS [53].

With regard to indolamines, stressed animals had lower PFC levels of

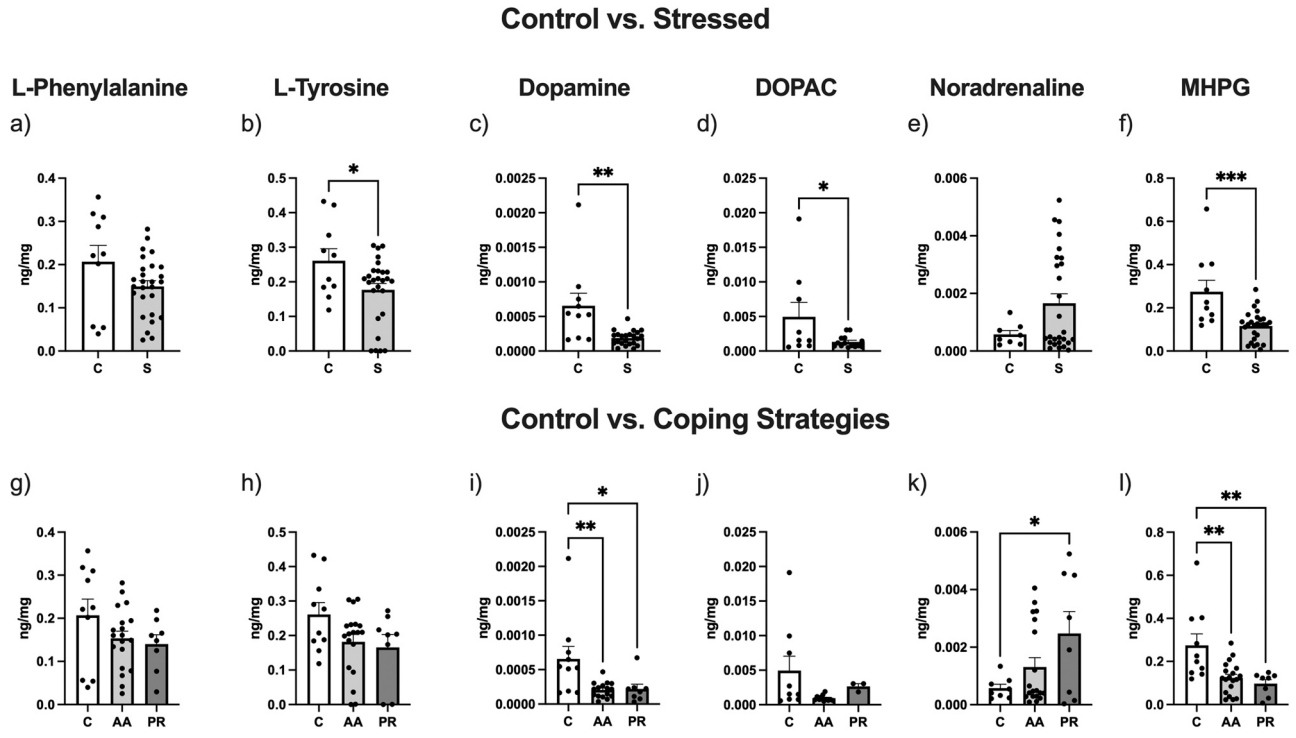

Fig. 6. Control vs. Stressed: Prefrontal cortex levels of a) L-phenylalanine, b) L-tyrosine, c) Dopamine, d) DOPAC, e) 5-Noradrenaline, and f) MHPG. **Control vs. Coping Strategies:** Prefrontal cortex levels of g) L-phenylalanine, h) L-tyrosine, i) Dopamine, j) DOPAC, k) Noradrenaline, and l) MHPG. Data are expressed as mean (ng/mg) ± SEM. Groups: control (C), stressed (S), active/aggressive (AA) and passive/reactive (PR). *p < 0.05, **p < 0.01, ***p < 0.001.

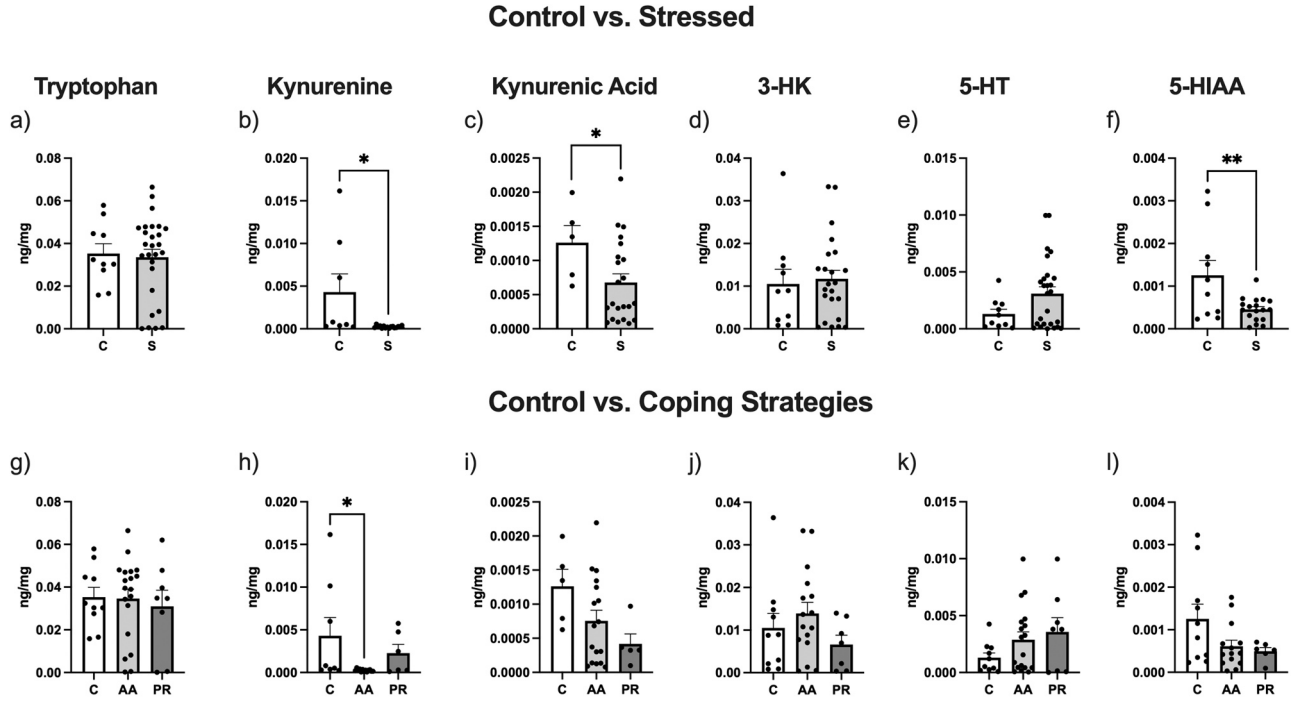

Fig. 7. Control vs. Stressed: Prefrontal cortex levels of a) tryptophan, b) kynurenine, c) kynurenic acid, d) 3-HK, e) 5-HT, and f) 5-HIAA. **Control vs. Coping Strategies:** Prefrontal cortex levels of g) tryptophan, h) kynurenine, i) kynurenic acid, j) 3-HK, k) 5-HT, and l) 5-HIAA. Data are expressed as mean (ng/mg) ± SEM. Groups: control (C), stressed (S), active/aggressive (AA) and passive/reactive (PR). *p < 0.05, **p < 0.01.

Kyn and Kyna, with no change in 3-HK. They also had higher levels of 5-HT and a higher 5-HT/Tryp ratio, although these differences were not significant. However, the fact that they had lower levels of 5-HIAA and a lower 5-HIAA/5-HT ratio may indicate that the synthesized 5-HT was not released. Again, we observed different patterns in the two areas, since in the HPC, we only found lower levels of 3-HK in stressed animals, with no change in levels pertaining to the serotonergic pathway. A previous study reported a significant increase in Kyn levels in both the PFC and the HPC, observing also in this latter area higher levels of 3-HK in stressed C57BL/6 J mice [29]. Our results for 5-HT levels are

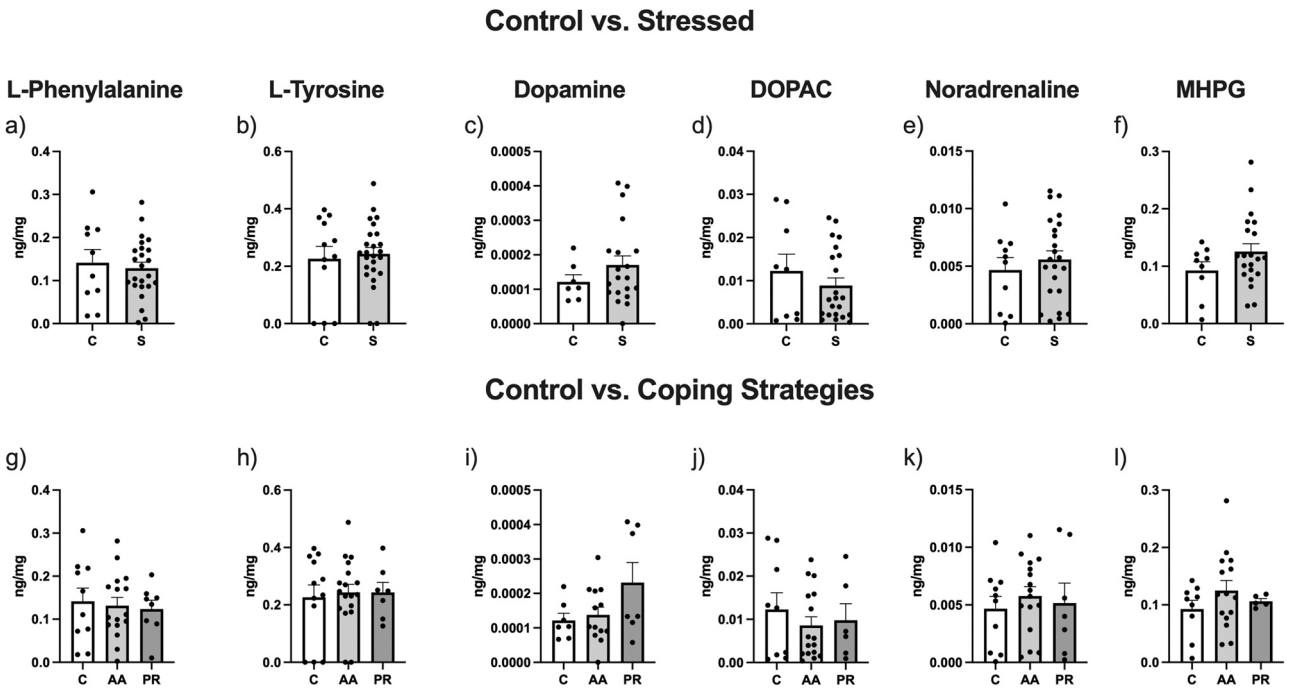

Fig. 8. Control vs. Stressed: Hippocampal levels of a) L-phenylalanine, b) L-tyrosine, c) Dopamine, d) DOPAC, e) 5-Noradrenaline, and f) MHPG. **Control vs. Coping Strategies:** Hippocampal levels of g) L-phenylalanine, h) L-tyrosine, i) Dopamine, j) DOPAC, k) Noradrenaline, and l) MHPG. Data are expressed as mean (ng/mg) ± SEM. Groups: control (C), stressed (S), active/aggressive (AA) and passive/reactive (PR). *p < 0.05, **p < 0.01, ***p < 0.001.

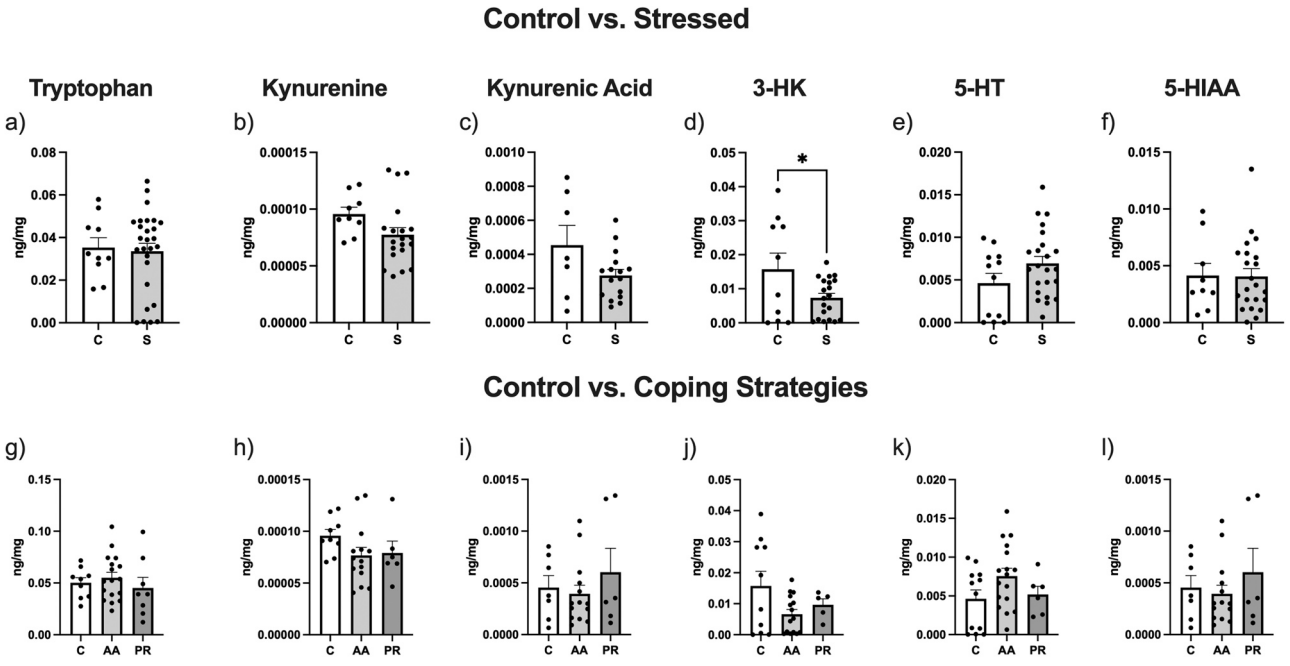

Fig. 9. Control vs. Stressed: Hippocampal levels of a) tryptophan, b) kynurenine, c) kynurenic acid, d) 3-HK, e) 5-HT, and f) 5-HIAA. **Control vs. Coping Strategies:** Hippocampal levels of g) tryptophan, h) kynurenine, i) kynurenic acid, j) 3-HK, k) 5-HT, and l) 5-HIAA. Data are expressed as mean (ng/mg) ± SEM. Groups: control (C), stressed (S), active/aggressive (AA) and passive/reactive (PR). *p < 0.05.

inconsistent with that reported by previous studies, which observed either a decrease [24] or no change [29,63] in the PFC; however, our findings are consistent with the HPC 5-HT levels reported in these same studies. In our study, we were unable to determine whether changes in the Tryp metabolism resulted in altered serotoninergic system activity, although we can state that no changes occurred as a function of the adopted coping strategy.

5. Conclusion

As in previous research, in the present study we classified the mice into AA and PR groups, based on the behavior manifested in the first social interaction with a resident male. Although all stressed mice exhibited anhedonia, we found no differences in accordance with the coping strategy adopted. Interestingly, PR mice maintained an immobile phenotype in the behavioral tests performed after stress. At the

molecular level, CSD was found to alter the HPA axis and induce differences in monoamine levels between the PFC and HPC. However, these differences did not respond to the coping strategy adopted.

Author statement

All procedures involving mice were performed in accordance with the European Directive (2010/63/EU) and were approved by the Animal Welfare Ethics Committee of the University of the Basque Country (CEEA-UPV/EHU; M20/2018/090) and the Gipuzkoa Provincial Council (PRO-AE-SS-062).

CRediT authorship contribution statement

Maider Muñoz-Culla: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Oscar Vegas:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Olatz Goñi-Balentziaga:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Garikoitz Beitia-Oyarzabal:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Alina Diez-Solinska:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Garikoitz Azkona:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability

Data will be made available on request.

Acknowledgments

This study was supported by Basque Government Predoctoral Grant (PRE_2015_1_0085), Basque University Predoctoral Grant (PIF22/192), Basque Government IT757–13 Project Grant, and a Spanish Ministry of Economy and Competitiveness Project Grant (PSI2015-63658-R, MINECO/FEDER, UE). The authors would like to thank SGIker from the UPV/EHU for the technical and human support provided.

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5. CHAPTER 3. Study #3: How does Chronic Social Instability Stress affect the physical and behavioral well-being in female mice?

5.1. Introduction

Stress has been linked to the development of physical and psychological illnesses such as cancer and depression. However, most of the studies and procedures have been carried out in males, ignoring the potential differences between both sexes and leaving females in the background.

5.2. Primary objective

The aim of the present study was to analyze the impact of CSIS on B16F10 tumor development and depressive-like and anxiety-like behaviors, as well as to assess the underlying neuroendocrine and neuroinflammatory mechanisms in female mice. Through this research, the purpose was to clarify the interplay between a chronic social stressor and tumor presence, and the implicit neurobiological processes in OF-1 female mice.

5.3. Specific objectives

In order to achieve the previously stated primary goal, the following specific objectives were proposed:

- To evaluate the activation of the HPA axis by assessing CORT plasma levels before and after CSIS procedure, and GR and MR mRNA relative expression levels in the HT and the HC after the CSIS.

- To examine immune activity by assessing pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and the anti- inflammatory cytokine (IL-10) mRNA relative gene expression levels in the HC and the ST after the CSIS.
- To assess neuron-microglia interaction (CX3CL1 and CX3CR1 mRNA relative gene expression levels) in the HC, the ST and the PFC once the CSIS procedure was completed.
- To assess tumor development in female mice inoculated with B16F10 tumor after CSIS.
- To determine the presence of depressive-like and anxiety-like behaviors after CSIS exposure.
- To analyze alterations in body weight by assessing body weight both before and after CSIS procedure.

5.4. Hypothesis

In this study, the hypothesis posited that the CSIS would lead to behavioral and physiological alterations in female mice. Specifically, CSIS was expected to activate the HPA axis and the IS and to alter the interaction between the IS and the CNS, ultimately contributing to a more pronounced tumor growth and to the emergence of depressive-like and anxiety-like symptomatology.

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

Behavioural Brain Research 435 (2022) 114063

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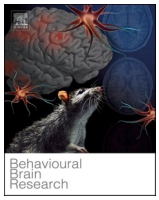

Research report

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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ARTICLE INFO

Keywords:
Female mice
Social stress
Tumor development
Behavior
Cytokines
CX3CL1/CX3CR1

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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https://doi.org/10.1016/j.bbr.2022.114063
Received 26 May 2022; Received in revised form 1 August 2022; Accepted 16 August 2022
Available online 18 August 2022

(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.

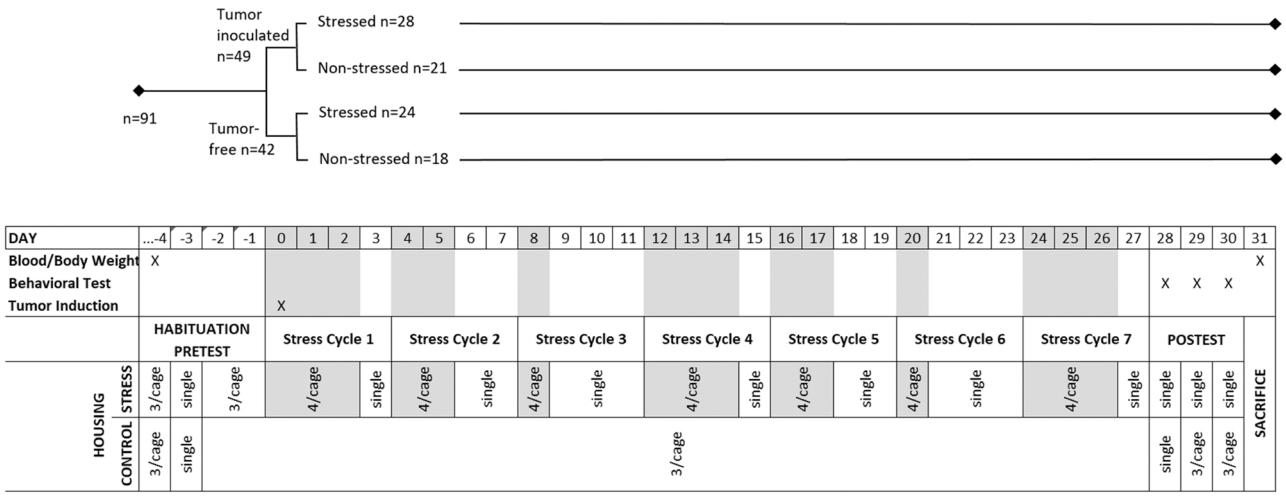

Fig. 1. Experimental procedure.

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

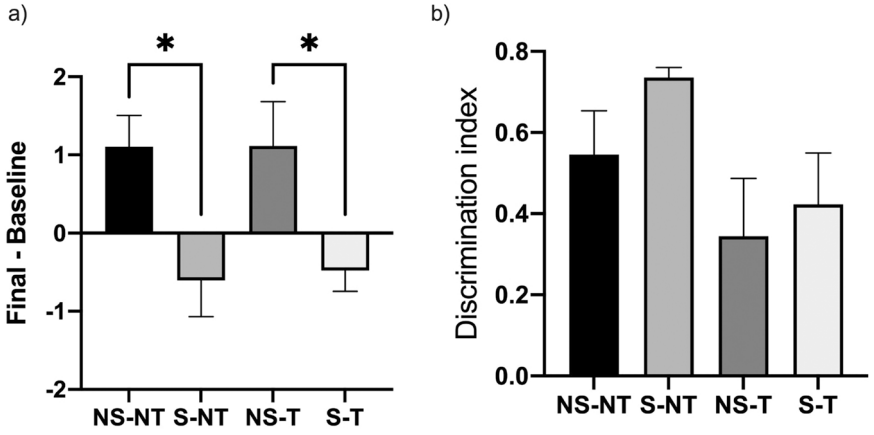

Fig. 2. a) Final-Baseline BW index in grams and b) SPT discrimination index. Data are expressed as mean ± SEM. *p < 0.05.

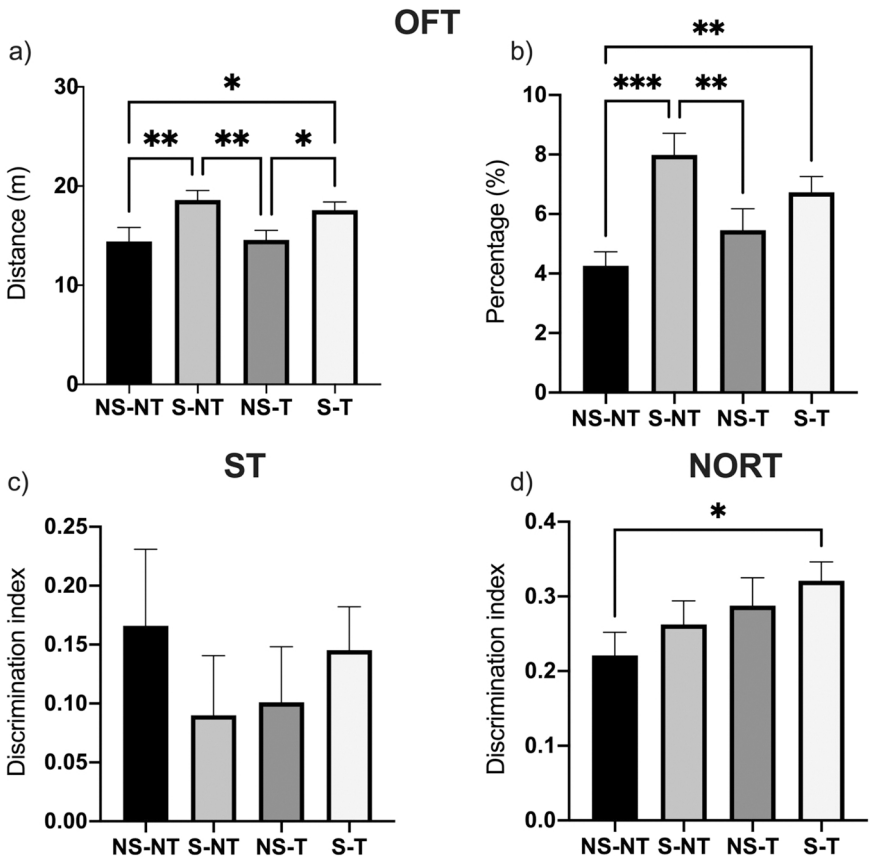

Fig. 3. a) Distance traveled and b) percentage of time spent in the center in the OFT, c) ST and d) NORT discrimination indexes. Data are expressed as mean ± SEM. *p < 0.05 **p < 0.01 and ***p < 0.001.

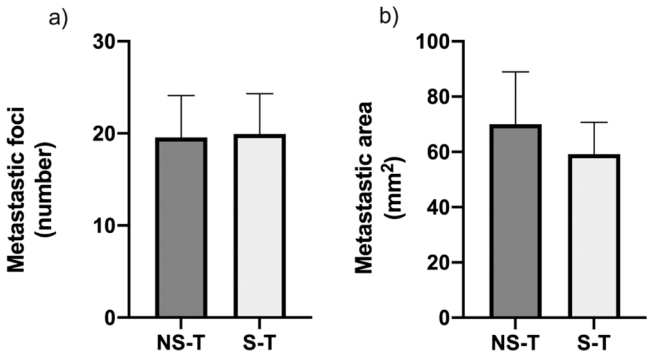

Fig. 4. a) Mean of the pulmonary metastatic foci, and b) total area of the metastatic foci observed. Data are expressed as the mean ± SEM.

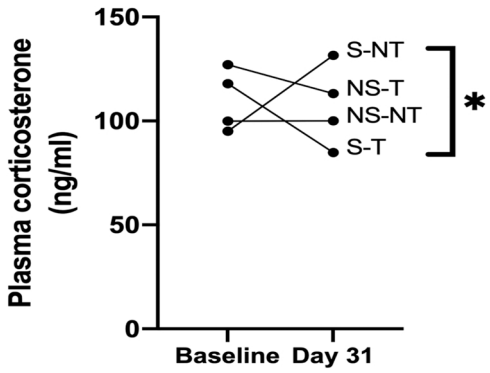

Fig. 5. Plasma corticosterone levels (ng/ml) at days − 4 (baseline) and 31.

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-

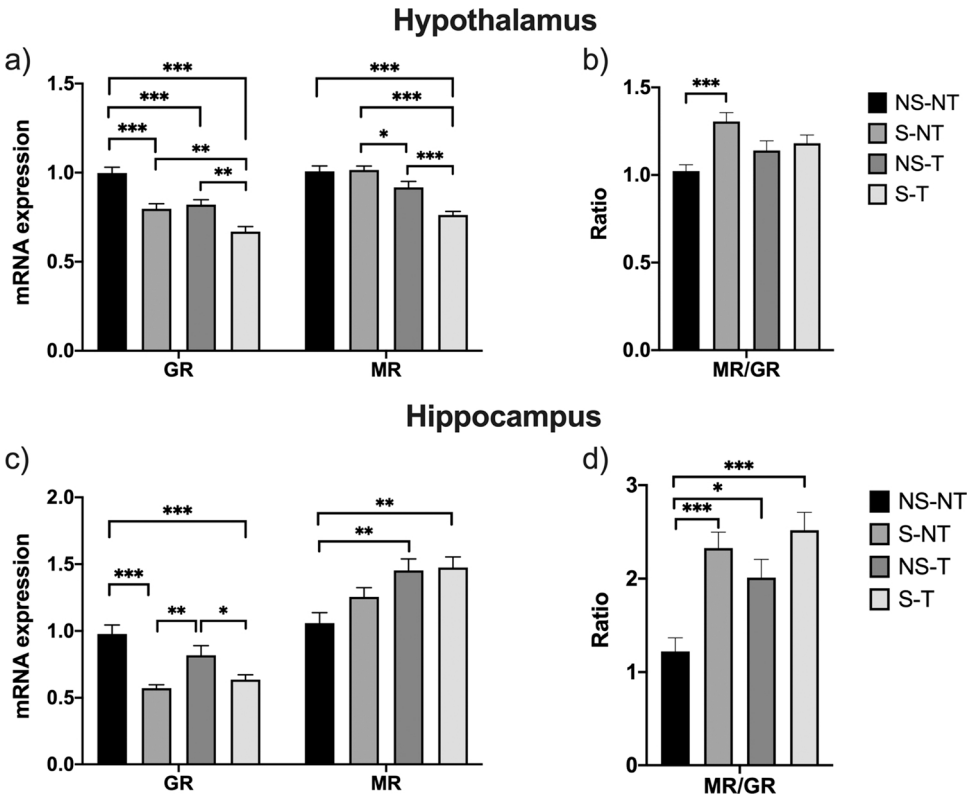

Fig. 6. a) Hypothalamic GR and MR mRNA expression levels, b) MR/GR ratio in the hypothalamus, c) Hippocampal GR and MR mRNA expression levels, and d) MR/GR ratio in the hippocampus. Data are expressed as the mean ± SEM. *p < 0.05, * *p < 0.01 and * **p < 0.001.

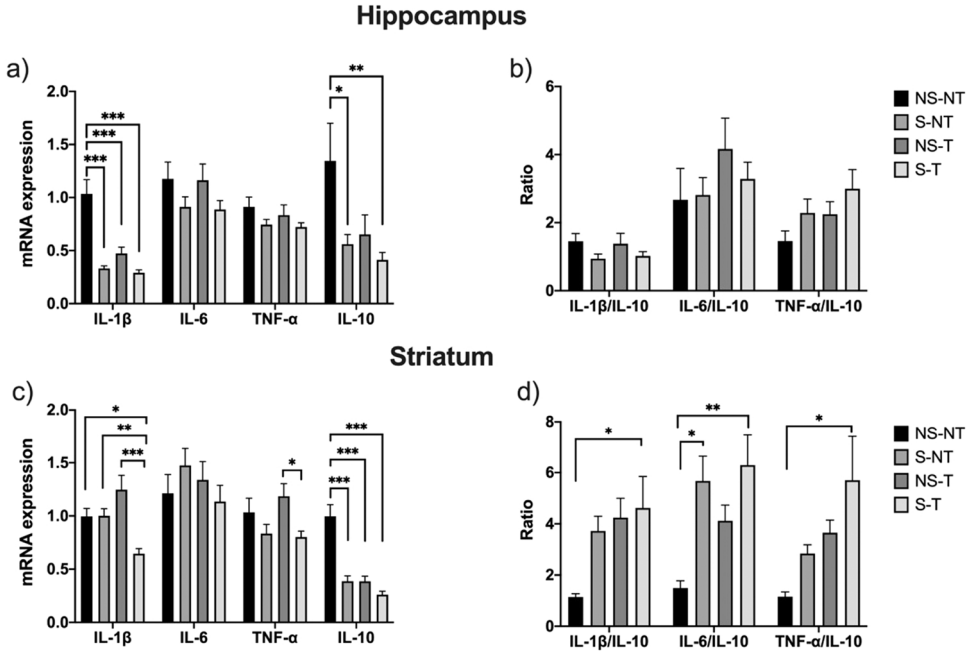

Fig. 7. a) IL-1β, IL-6, TNF-α and IL-10 mRNA expression levels in the hippocampus, b) the pro-inflammatory versus anti-inflammatory cytokine ratios in the hippocampus, c) IL-1β, IL-6, TNF-α and IL-10 mRNA expression levels in the striatum, and d) the pro-inflammatory versus anti-inflammatory cytokine 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

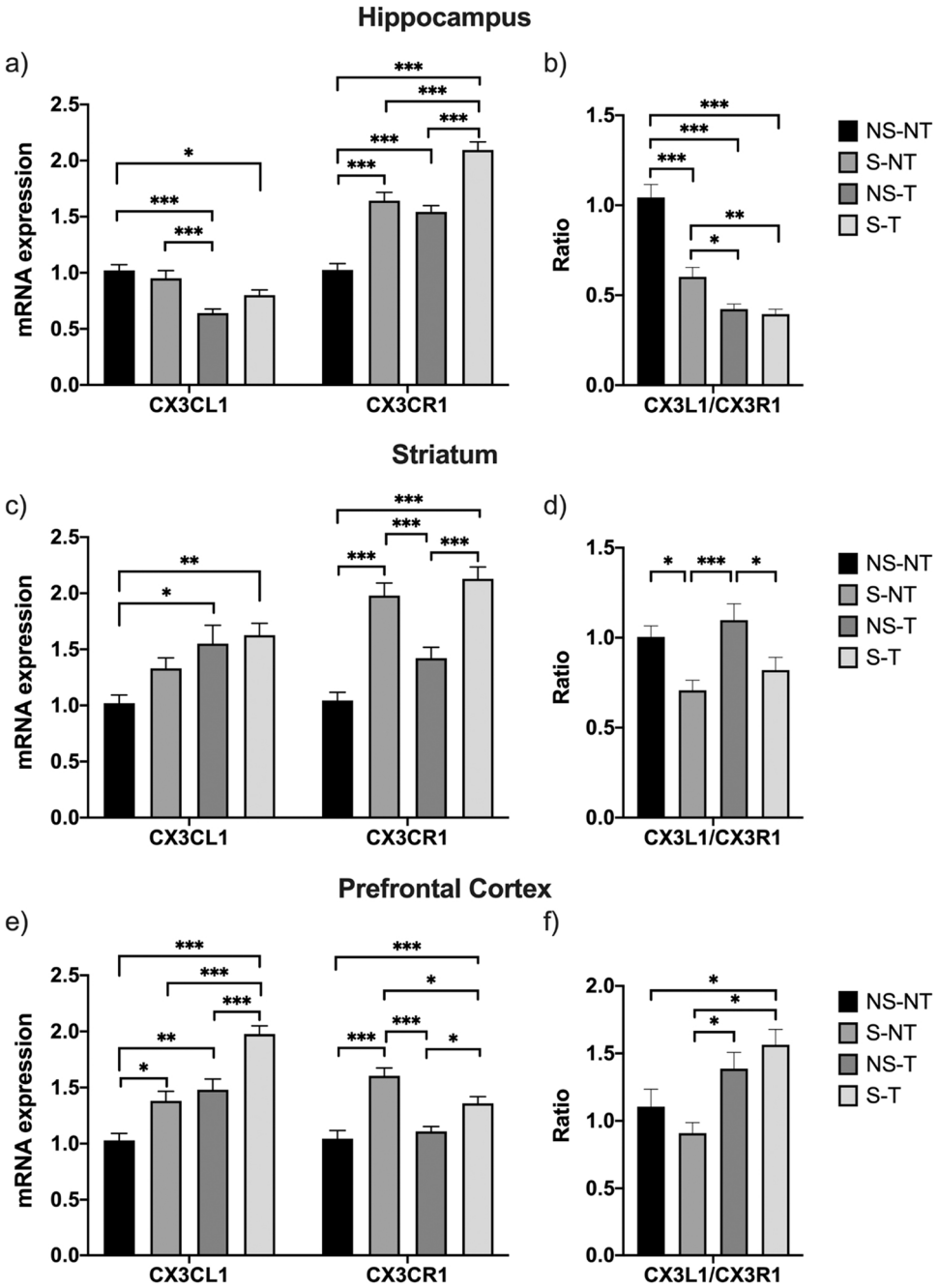

Fig. 8. a) CX3CL1 and CX3CR1 mRNA expression levels and b) chemokine ratio in the hippocampus; c) CX3CL1 and CX3CR1 mRNA expression levels and d) chemokine ratio in the striatum; e) CX3CL1 and CX3CR1 mRNA expression levels and f) chemokine ratio in the PFC. The data are expressed as the mean (± SEM).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

Acknowledgments

This study was supported by the Spanish Ministry of Science, Innovation RTI2018–098264-B-I00 (MCIU/AEI/FEDER, UE), the UPV/EHU GIU18/103 and the PIBA 2019–22 Project Grants.

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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6. CHAPTER 4. Study #4: How does intrinsic sociability modulate behavioral and physiological outcomes of Chronic Social Instability Stress in female mice?

6.1. Introduction

Chronic Social Instability Stress has been shown to affect the physiology and behavior of female mice, as observed in Study #3. However, as in males, where coping strategies may modulate the stress response, individual variability among females could lead to different effects of stress. In females, physiological and behavioral consequences of CSIS could be different given their more social nature.

6.2. Primary objective

The aim of this work was to examine the behavioral, neuroendocrine, and neurochemical effects of stress in OF-1 female mice, with a particular focus on intrinsic social connectedness. The purpose was to explore whether high sociability could be a protective factor when coping with a stressful situation.

6.3. Specific objectives

To address the main goal, the following specific objectives were specified:

- To study animals' inherent sociability before stress procedure in order to establish two different groups of mice (high vs. low sociable) with respect to their social connectedness with their counterparts.

- To assess the activation of HPA axis by assessing neuroendocrine parameters (CORT plasma levels and GR and MR mRNA relative expression levels in the HT and the HC) after CSIS procedure and considering the sociability.
- To assess changes in neurochemical activity by determining monoamines and their metabolites (Phe, Tyr, DA, DOPAC, NA, MHPG, Tryp, Kyn, Kyna, 3-HK, 5-HT and 5-HIAA) in the HC and the ST after CSIS exposure and in relation to the sociability.
- To evaluate immune activity by analyzing IDO and iNOS mRNA relative expression levels in the HC and the ST after CSIS procedure and considering the sociability.
- To examine behavioral differences between the four experimental groups (NS/LS, NS/HS, S/LS and S/HS) after CSIS exposure.

6.4. Hypothesis

The hypothesis of this study suggested that inherent sociability could be exerting a key role when coping with CSIS. We expected to find that female mice with high sociable traits would exhibit enhanced resilience to a stressful event evident through their attenuated physiological (activation of the HPA axis, neurochemical changes, and immune system activation) and behavioral (depressive-like and anxiety-like) responses when compared to low-sociable mice.

6.5. Publication: The role of sociability in social instability stress: Behavioral, neuroendocrine and monoaminergic effects

Physiology & Behavior 270 (2023) 114306

Contents lists available at ScienceDirect

Physiology & Behavior

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

The role of sociability in social instability stress: Behavioral, neuroendocrine and monoaminergic effects

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ARTICLE INFO

Keywords:
Female mice
Social stress
Sociability
Monoamines
Behavior
HPA

ABSTRACT

Extensive literature has reported a link between social stress and mental health. In this complex relationship, individual strategies for coping with social stress are thought to have a possible modulating effect, with sociability being a key factor. Despite the higher incidence of affective disorders in females and sex-related neurochemical differences, female populations have been understudied. The aim of the present study was, therefore, to analyze the behavioral, neuroendocrine, and neurochemical effects of stress in female OF1 mice, paying special attention to social connectedness (female mice with high vs low sociability). To this end, subjects were exposed to the Chronic Social Instability Stress (CSIS) model for four weeks. Although female mice exposed to CSIS had increased arousal, there was no evidence of depressive-like behavior. Neither did exposure to CSIS affect corticosterone levels, although it did increase the MR/GR ratio by decreasing GR expression. Female mice exposed to CSIS had higher noradrenaline and dopamine levels in the hippocampus and striatum respectively, with a lower monoaminergic turnover, resulting in an increased arousal. CSIS increased serotonin levels in both the hippocampus and striatum. Similarly, CSIS was found to reduce kynurenic acid, 3-HK, and IDO and iNOS enzyme levels in the hippocampus. Interestingly, the observed decrease in IDO synthesis and the increased serotonin and dopamine levels in the striatum were only found in subjects with high sociability. These highly sociable female mice also had significantly lower levels of noradrenaline in the striatum after CSIS application. Overall, our model has produced neuroendocrine and neurochemical but not behavioral changes, so it has not allowed us to study sociability in depth. Therefore, a model that induces both molecular and behavioral phenotypes should be applied to determine the role of sociability.

1. Introduction

Epidemiological studies have shown that chronic stress is a risk factor for physical and mental health, both in early developmental stages and in adulthood [1]. The most common type of chronic stress in humans and other social animals is generated by socially demanding situations [2,3]. In addition to stress, several studies have also highlighted the influence of coping strategies and personality on physical and mental health [4].

Two main systems have been found to mediate most of the stress response mechanisms: the sympathetic-adrenomedullary (SAM) axis and the hypothalamic-pituitary-adrenal (HPA) axis. In a situation identified as dangerous, the sympathetic nervous system (SNS) releases noradrenaline (NA), which stimulates the adrenal glands to release adrenaline into the bloodstream. For its part, the activation of the HPA axis causes glucocorticoids (cortisol or corticosterone) to be secreted into the blood, an action that modifies the gene expression of virtually every cell and prepares the body for fight or flight. Following activation of the HPA axis, hypothalamic glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) are critical in the negative feedback

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https://doi.org/10.1016/j.physbeh.2023.114306
Received 18 May 2023; Received in revised form 10 July 2023; Accepted 26 July 2023
Available online 27 July 2023

process that inhibits further glucocorticoid release. Several studies in humans have demonstrated the association between chronic stress and high levels of NA and glucocorticoids [5,6]. These alterations in the activity of the SAM and HPA axes are associated with numerous deleterious psychological and physical health outcomes, including mood disorders [7,8].

Depression is a mood disorder characterized by an intrusive and persistent mood accompanied by low self-esteem and a loss of interest or pleasure (anhedonia). It is one of the most prevalent illnesses in the world, with a consistent gender pattern: women are more likely to suffer from depression, have more severe symptoms, and a poorer clinical outcome [9,10]. Of all stressors, chronic social stress is the one most strongly associated with depression [11–13]. In the same way, depression is associated with social risk factors, social impairment, and poor social functioning [14]. The clinical and etiological heterogeneity of major depressive disorder has made it difficult to elucidate its pathophysiology. Nevertheless, current neurobiological theories point out HPA axis and monoamine alterations, among others [15]. Increasing cortisol secretion and the activity of the sympathetic system induces changes in the immune response [16], which in turn stimulates the release of the enzyme indoleamine-2,3-dioxygenase (IDO) from macrophages. This enzyme degrades tryptophan, the amino acid precursor of serotonin (5-HT), and increases kynurenine levels and/or the kynurenine to tryptophan ratio, which could explain low 5-HT levels in the brain during depressive stages [17]. On the other hand, monoaminergic synthesis may also be compromised by the sequestration of its essential synthesis cofactor, tetrahydrobiopterin (BH4) by the inducible NOS (iNOS), which is also activated by proinflammatory cytokines. In this regard, chronic stress associated with depression is not only associated with altered 5HT neurotransmission [18], but with activation of the kynurenine [19] and/or BH4 pathways [20].

Although epidemiological studies have clearly shown that females are more vulnerable than males to stress-related psychopathologies, preclinical research is still mainly conducted with male animals. Consequently, the lack of preclinical research carried out with females may explain the poorer outcomes of treatments among subjects of this sex [21]. Given that the main source of the stress contributing to the development of mood disorders in humans is social in nature [8], animal models based on social stressors may be the most appropriate, as they represent situations that individuals may face in their daily lives. However, it is necessary to design and conduct experiments that take the ethology (natural behaviors) of the species into consideration, in order to obtain results that can eventually be successfully translated to the clinic [22]. Many experiments have, in the past, been based on the stress generated by agonistic interactions prompted by territorial aggression or dominance, or in other words, the social defeat model [23–25]. However, this model is not suitable for inducing chronic social stress in OF-1 females, as subjects from this population do not display territorial aggression [26]. Consequently, our knowledge of the specific mediators involved in the possible negative effects of social stress in females is very limited. In light of females' social nature, the chronic social instability stress (CSIS) model may be more appropriate and have greater ethological validity for this population. However, the results are not always consistent when applied to female mice [27]. Some authors [26,28,29] have reported an association between the CSIS model and depressive-like behavioral changes, although others have failed to find any such connection [30–32], a discrepancy that may indicate individual differences in intrinsic sociability. In light of this, the aim of the present study was to analyze the role of inherent sociability in behavioral and neurochemical responses to chronic social instability stress among female mice using our previous paradigm [31].

2. Material and methods

2.1. Subjects and husbandry

Eighty-one OF1 outbred female mice (Janvier Labs, France) were purchased at age 8 weeks. They were housed in groups of three in transparent plastic cages (24.5 × 24.5 × 15 cm), with black poplar/aspen shavings as litter bedding, two sheets of tissue as nesting material, and a sheet of cardboard as enrichment. Animals were provided with ad libitum access to water and food. The room was kept at a temperature of between 22°C and 24 °C, with a relative humidity level of 70% and a reversed 12-h light/dark cycle (white lights on from 20:00–08:00 h), including 20 min of progressively increasing light (dawn, 07:40–08:00 h) and 20 min of progressively decreasing light (dusk, 19:40–20:00 h). All procedures involving mice were performed in accordance with that established in the European Directive (2010/63/EU) and were approved by the Animal Welfare Ethics Committee of the University of the Basque Country (CEEA-UPV/EHU; M20/2018/090) and the Gipuzkoa Provincial Council (PRO-AE-SS-062).

2.2. Experimental procedure

After the 10-day acclimation period (day -1), animals were housed in groups of three (non-stressed animals, $n = 33$) or four (stressed animals, $n = 48$). Then, the Social Interaction Test (SIT) was performed to classify subjects into the high-sociability and low-sociability groups. Since the aim was to study the inherent social interaction of the animals, we analyzed this parameter according to the number of animals they would be living with during the experiment; non-stressed 3 and stressed 4. On day 0, the mice were divided into four experimental groups: Non-Stressed/Low-Sociability (NS/LS) ($n = 13$), Non-Stressed/High-Sociability (NS/HS) ($n = 20$), Stressed/Low-Sociability (S/LS) ($n = 20$), and Stressed/High-Sociability (S/HS) ($n = 28$). The S groups were subjected to the Chronic Social Instability Stress (CSIS) model for 28 days, whereas the NS mice remained in the same housing conditions as during the adaptation period (3 animals per cage). Behavioral assessment of all animals ($n = 81$) commenced once the CSIS period had ended, with the Sucrose Preference Test (SPT), the Open Field Test (OFT), and the Novel Object Recognition Test (NORT) being carried out between days 28 and 30. On day 31, blood samples of all animals were collected by a submandibular puncture to determine plasma corticosterone levels. All animals were then sacrificed by cervical dislocation. The brain was removed and the whole hypothalamus, hippocampi, and striata were dissected under sterile conditions and stored at −80 °C for biological determinations (Fig. 1).

2.3. Stress procedure

In order to increase unpredictability, this model was modified from Labaka et al. [26], applying seven variable periods of four days of isolation and overcrowding as previously described [31]. Thus, the S groups (both HS and LS] were exposed to the CSIS model over a 28-day period (Fig. 1). The mice were subjected to a highly unstable and unpredictable social situation, with alternating phases of isolation (1, 2, or 3 days) and crowding (4 per cage, during 1, 2, or 3 days). During each crowding phase, we ensured that four different mice that had no previous contact were placed together in a new clean cage. Meanwhile, control mice (NS) were housed in stable groups of three.

2.4. Behavioral assessment

Each animal's movements and behavior were recorded with an overhead video camera (GZ-MG773; JVC, Yokohama, Japan) for subsequent assessment using the ANY-maze© computerized version 4.96 video-tracking software program (Stoelting Europe, Dublin, Ireland). Behavioral assessment was performed during the dark phase (the

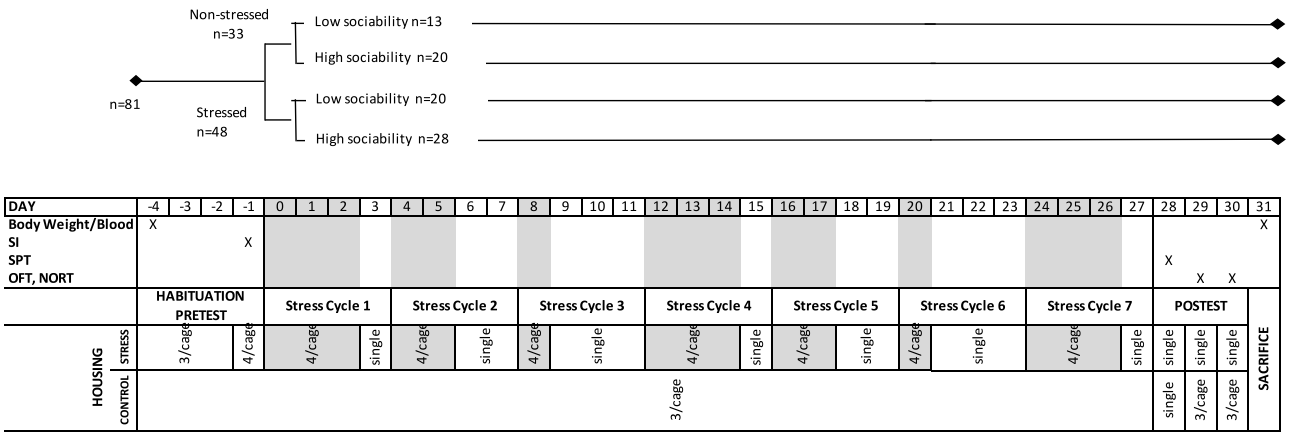

Fig. 1. Experimental procedure.

animals' active period) at 09:00 h and at 12:00 h (darkness under dim red lighting). All behavioral tests were performed in opaque plexiglass open-field box arenas (plexiglass boxes; 40 × 40 × 30 cm), except for the SPT and the SIT, which were conducted in the transparent plastic cages in which the mice were usually housed. Objects were cleaned between trials with a solution of 0.5% acetic acid.

2.4.1. Social interaction test (SIT)

On day -1, the mice were recorded in their home cages for 5 min. The behaviors assessed were: the time each subject spent attempting to maintain social contact with another mouse (interaction emitted), the time other subjects spent attempting to maintain social contact with the subject under study (interaction received), the time spent in non-social exploration and the time spent immobile [31]. Mice that emitted and received more social interaction and spent less time exploring non-social zones were categorized as High Sociability (HS), whereas those that emitted and received fewer social interaction behaviors and spent more time exploring their environment were assigned to the Low Sociability (LS) group. A multivariate discriminant analysis was performed (Wilk's Lambda method with step entry) to confirm the integrity of the groups derived from the cluster analysis and to determine which behavioral variables most efficiently discriminated between the clusters. In the NS group, the multivariate analysis carried out accounted for 100% of the cases obtained by the cluster solution, thereby confirming the statistical validity of the established groups. Social interaction emitted and received and non-social exploration were the variables that best discriminated between clusters ($p < 0.001$), followed by time spent immobile ($p = 0.024$). Regarding the S group, the discriminant model applied accounted for 89.6% of the cases obtained by the cluster solution, thereby confirming the statistical validity of these groups, as well as their behavioral description. Social interaction emitted and non-social exploration were the variables that best discriminated between clusters ($p < 0.001$), followed by social interaction received ($p = 0.002$). However, immobility made no significant contribution to the cluster differentiation ($p = 0.372$).

2.4.2. Sucrose preference test (SPT)

On day 28, all mice were individually housed and water bottles from the cages were removed. In their place, the mice were offered a free choice between two new bottles (both with a volume of 50 ml and identical in appearance to the mice's regular water bottles, to avoid the novelty factor) for a period of 24 h. One of the new bottles contained a 1% sucrose solution, and the other one contained water. The animals were not deprived of food or water before the test and the position of the bottles was counterbalanced to prevent the possible effect of a side preference. The consumption of the sucrose solution and water was measured by weighing the bottles at the beginning and at the end of the test. Each value was then divided by the mouse's body weight to calculate the relative intake. The sucrose preference discrimination index was calculated as follows: (sucrose consumption-water consumption)/total consumption * 100.

2.4.3. Open field test (OFT)

On day 29, the OFT was performed to assess behavioral responses, such as anxiety-like behaviors and mice locomotion. Animals were placed in the arena and were allowed to explore for 5 min [33]. Time spent in the center and time spent in the peripheral zone (area along the walls) of the arena were analyzed, along with locomotor activity (distance traveled).

2.4.4. Novel object recognition test (NORT)

On day 29, 1 hour after the OFT, NORT training was performed. The mice were placed in the open-field arena with 2 identical plastic caps, each measuring 4 cm in diameter. The caps served as familiar objects (F and F). The mice were allowed to explore the arena for 10 min, after which they were returned to their home cage. On day 30, the mice were returned to the same arena, but this time, one of the familiar objects (F) was replaced with a steel triangle that constituted the novel object (N). The total time spent exploring both objects was recorded over a 5 min period. Recognition memory was measured in terms of discrimination index: (time with N - time with F)/(time with N + time with F). Time spent in the F and N areas was also analyzed.

2.5. Physiological determinations

2.5.1. Sample collection

On day 31, blood samples were obtained by facial vein puncture with a lancet. Samples (15 μL) were collected using lithium heparin tubes (BD Microtainer®) between 09:00 h and 10:00 h. Samples were then centrifuged at 1800 g for 15 min at 4°C, and the resulting plasma was collected and stored in cryotubes (ClearLine®) (2 mL) at −80°C. Subsequently, the animals were sacrificed by cervical dislocation. The brain was removed and the hypothalamus, hippocampi, and striata were dissected under sterile conditions and stored at −80 °C.

2.5.2. Determination of plasma corticosterone concentrations

Plasma corticosterone levels were quantified using an enzyme immunoassay (Corticosterone Elisa Kit, ADI-900–097, 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%.

2.5.3. Real-time RT−PCR measurements of mRNA expression

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). The cDNA sequences were obtained from GenBank at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). 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 (Appendix Table A.1). The relative gene expression was determined using the $2^{-D\Delta t}$ method [34]).

2.5.4. Determination of monoamines and their metabolites by high-performance liquid chromatography (HPLC)

Brain structures were weighed and homogenized in a 60 μl solution (1% formic acid in acetonitrile). Zirconia Ceramic Balls (0.5 and 1.0 mm) were inserted into the sample tubes and then placed in the Bullet Blender for 3 min to bust the brain tissue. Immediately afterwards, the tubes were vortexed for 5 min (Vortex Genie-2; Scientific Industries, Bohemia, NY, USA) and, subsequently, the samples were centrifuged for 15 min at 15,000 × g and 4°C. The supernatants were dried for 30 min with compressed air to concentrate the samples and were then reconstituted with 30 μl of 0.05% trifluoroacetic acid. Next, the samples were again centrifuged for 20 min at 15,000 × g and 4°C and placed in the autosampler unit for analysis.

L-Phenylalanine (Phe), L-Tyrosine (Tyr), dopamine (DA), 3,4-Dihydroxyphenylacetic Acid (DOPAC), noradrenaline (NA), 3-methoxy-4-hydroxyphenylglycol (MHPG), Tryptophan (Tryp), kynurenine (Kyn), Kynurenic Acid (Kyna), 3-Hydroxykynurenine (3-HK), serotonin (5-HT), and 5-Hydroxyindoleacetic Acid (5-HIAA) were determined using 20 μL of each sample injected into the HPLC (Hewlett Packard 1100 System). The samples were separated on a Poroshell 120 EC—C18 column (100 × 4.6 mm, 2.7 μm), with an Analytical Guard Column (12.5 × 4.6 mm, 5 μm) being used for protection (Agilent Technologies). The mobile phase for this study comprised 0.05% trifluoroacetic acid (solvent A) and 99.9% acetonitrile (solvent B). The flow was maintained at a constant rate of 0.5 ml/min. The column was maintained at 25°C during the analysis, and the samples were maintained at 4°C in an autosampler unit. The samples were analyzed and monitored either by a fluorescence detector (FLD) at an emission wavelength of 320 nm or by a variable wavelength detector (VWD). The Phe (Excitation wavelength (Ex) 212 nm), NA, Tyr-and 5-HT (Excitation wavelength (Ex) 229 nm), and DA, MHPG and 5-HIAA (Excitation wavelength (Ex) 283 nm) effluents were monitored with the fluorescence detector at an Emission wavelength of 320 nm. The 3-HK, Kyn, DOPAC, Kyna, and Tryp effluent was monitored with a variable wavelength detector set at 230 nm. The total sample analysis time was 27 min. The final data were expressed as ng/ml.

2.6. Statistical analysis

A cluster analysis (Low or High Sociability) was performed using the SPSS 28.0 for Windows software package (SPSS Inc., Chicago, IL, USA). Statistical analyses of the behavioral and physiological variables and graphic visualization and design were performed using GraphPad Prism software (9.0, GraphPad Software, Inc). Study variables were first transformed into Z-scores and outlier values were adjusted to the median in accordance with the boxplot outlier labeling rule. Variables were analyzed using 2-way ANOVAs, the factors were stress and sociability. Specific comparisons between the NS/LS and S/LS groups, and between the NS/HS and S/HS groups 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 of between 0.5 and 0.8 are considered indicative of moderate effects, and values < 0.5 are considered to indicate small effects). Values of $p < 0.05$ were considered statistically significant (95% confidence). Data are expressed as mean ± standard error (SEM). Only significant differences between groups are presented in the results section and small effect sizes are not considered.

3. Results

3.1. Behavioral assessment

No differences were found in either the SPT or the NORT (Fig. 2a, b). In contrast, in the OFT, stressed mice were observed to travel a greater distance ($F_{(3,77)} = 8.741$; $p = 0.004$; $\eta 2 = 0.102$) than their non-stressed counterparts (Fig. 2c). No differences were observed in the time spent in periphery or in the center (Fig. 2d).

3.2. Biological assessment

3.2.1. Neuroendocrine effects

Overall, HS mice had higher corticosterone plasma levels than their LS counterparts ($F_{(3,76)} = 6424$; $p = 0.013$; $\eta 2 = 0.078$) (Fig. 3a). Hypothalamic GR mRNA relative gene expression was lower in stressed than in non-stressed animals ($F_{(3,75)} = 23.49$; $p < 0.001$; $\eta 2 = 0.24$) and the post hoc analysis revealed differences between the NS/HS and the S/HS groups ($p < 0.001$; $d = 1.20$; Fig. 3b). Similarly, MR mRNA

Table A1
PCR Primer specification.

Gene	Function	Primer sequences (5′−3′)	Analyzed structure	Gen Bank accession No.
GR	Glucocorticoid receptor	F: CCCATGGAGGTAGCGATTGT R: TGTAAAGGCTGCCCAATGTGT	Hypothalamus Hippocampus	DQ504162.1
MR	Mineralocorticoid receptor	F: ACCTGCAGAGAGGACCAATGA R: GGAGTAATTCGTGTTTTTCTTTGCT	Hypothalamus Hippocampus	AJ311855.1
IDO	Rate-limiting enzyme of tryptophan catabolism	F: AAAGCAATCCCCACTGTATCCA R: TGCCTTTTCCAATGCTTTCAG	Hippocampus Striatum	BC049931.1
iNOS	Inducible nitric oxide synthase	F: GGATCTTCCCAGGCAACCA R: CAATCCACAACTCGCTCCAA	Hippocampus Striatum	NM_010927.4
GAPDH	Catalyzing enzyme of glycolysis	F: CGGCCGCATCTTCTTGTG R: GTGACCAGGCGCCCAATAC	Hypothalamus Hippocampus Striatum	NM_001289726.1
HPRT	Catalyzing transferase of hypoxanthine	F: TGGGAGGCCATCACATTCT R: TCCAGCAGGTCAGCAAAAGAAC	Hypothalamus Hippocampus Striatum	NM _013556.2

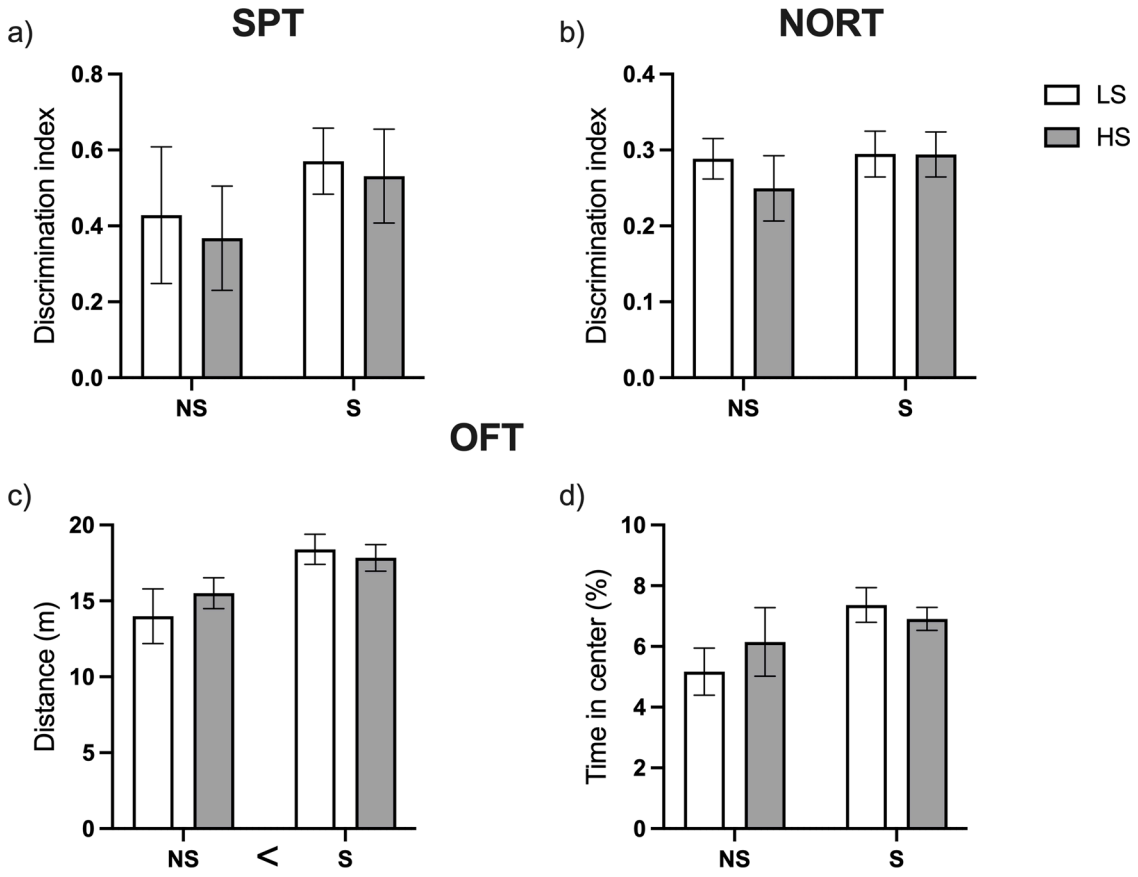

Fig. 2. a) SPT and b) NORT discrimination indexes, c) distance traveled, and d) the percentage of time spent in the center during the OFT. Stress factor significance is expressed as < or > according to directionality. Data are expressed as mean ±*S*.E.M.

expression was lower in stressed than in non-stressed animals ($F_{(3,75)}$ = 5.119; p = 0.027; η2 = 0.064), although in this case, the post hoc analysis did not reveal any differences between groups (Fig. 3c). The MR/GR ratio was higher in stressed than in non-stressed animals ($F_{(3,75)}$ = 7.917; p = 0.006; η2 =0.095), and the post hoc analysis revealed differences between the NS/HS and the S/HS groups ($p < 0.01$; $d = 1.04$; Fig. 3d). As in the hypothalamus, hippocampal GR mRNA relative gene expression was lower in stressed than in non-stressed animals ($F_{(3,75)}$ = 27.85; $p < 0.001$; η2 = 0.27) and the post hoc analysis revealed differences between the NS/LS and S/LS groups ($p < 0.01$; $d = 1.19$) and NS/HS and the S/HS groups ($p < 0.001$; $d = 1.18$; Fig. 3e). MR mRNA expression was higher in HS animals compared to LS ($F_{(3,75)} = 4.359$; p = 0.04; η2 = 0.06), although, the post hoc analysis did not reveal any differences between groups (Fig. 3f). Finally, hippocampal MR/GR ratio was higher in stressed than in non-stressed animals ($F_{(3,75)} = 14.10$; $p <$ 0.001; η2 = 0.16), and the post hoc analysis revealed differences between the NS/LS and the S/LS groups ($p < 0.05$; $d = 1.07$; Fig. 3g).

3.2.2. *Hippocampal and striatal monoamines and their metabolite levels*

3.2.2.1. Catecholamine levels in the hippocampus. Regarding catecholamines, the levels of both precursors, Phe ($F_{(3,72)} = 12.03$; $p < 0.001$;; η2 = 0.143; Fig. 4a) and Tyr ($F_{(3,73)}$ = 5.972; p = 0.017; η2 = 0.076; Fig. 4b), were significantly lower in stressed subjects than in controls. The post hoc analysis revealed that S/LS subjects had lower Phe-levels than their counterparts in the NS/LS group (p = 0.05; d = 1.00). No differences were observed in DA (Fig. 4c) or DOPAC levels (Fig. 4d), although the DOPAC/DA ratio was lower in the stressed group (4667.752 ± 742.232) than in the non-stressed one (16,388.747 ± 4320.515) ($F_{(3,69)} = 9.315$ $p = 0.006$; η2 = 0.119). Furthermore, stressed animals were found to have higher NA levels ($F_{(3,72)} = 8.165$; $p = 0.006$; η2 = 0.102; Fig. 4e) and lower MHPG levels ($F_{(3,72)} = 9.086$; $p = 0.004$; η2 = 0.112; Fig. 4f) than the non-stressed ones. Stressed mice (14.519 ± 1.938) also presented lower MHPG/NA ratio ($F_{(3,72)} = 6.784$; $p = 0.011$; η2 = 0.086) compared with non-stressed subjects (144.562 ± 71.441).

3.2.2.2. Catecholamine levels in the striatum. The statistical analyses revealed no differences in the catecholamine precursors (Fig. 5a,b), although the stressed group (1.072 ± 0.045) had a higher Tyr/Phe-ratio than the non-stressed mice (0.907 ± 0.0546) ($F_{(3,74)} = 4.805$; $p = 0.032$; η2 = 0.061). Stressed mice had higher DA levels than their non-stressed counterparts ($F_{(3,75)}$ = 11.35; p = 0.012; η2 = 0.131), and the S/HS group had significantly higher levels than NS/HS subjects ($p = 0.015$; d = 0.85; Fig. 5c). Moreover, although no differences were observed in DOPAC levels (Fig. 5d), stressed mice (7.104 ± 3.472) had a lower DOPAC/DA ratio than the non-stressed mice (283.491 ± 184.607) ($F_{(3,70)}$ = 4.248; p = 0.043; η2 = 0.057). Regarding NA, although no differences were observed in relation to the stress factor, they were observed in relation to the sociability factor ($F_{(3,74)} = 8.156$; $p = 0.006$; η2 = 0.099), with NS/LS and S/LS animals having higher NA levels than their NS/HS and S/HS counterparts ($t = 2.631$; $p = 0.013$ and $t = 2.040$; $p = 0.047$, respectively; Fig. 5e). No differences were observed in MHPG levels (Fig. 5f). Interestingly, stressed mice (32.295 ± 4.008) were observed to have a lower MHPG/NA ratio than the non-stressed mice (102.596 ± 16.882) ($F_{(3,73)} = 15.873$; $p < 0.001$; η2 = 0.179).

3.2.2.3. Indolamine levels in the hippocampus. Regarding indolamine levels, stressed animals had lower Tryp levels ($F_{(3,73)} = 13.91$; $p < 0.001$; η2 = 0.160), with differences being observed between the NS/LS and S/LS groups ($p = 0.03$; $d = 1.05$; Fig. 6a). No differences were observed in Kyn levels (Fig. 6b), although stressed animals had lower levels of Kyna ($F_{(3,71)} = 5.077$; $p = 0.027$; η2 = 0.67; Fig. 6c) and 3-HK ($F_{(3,73)} = 5.760$;

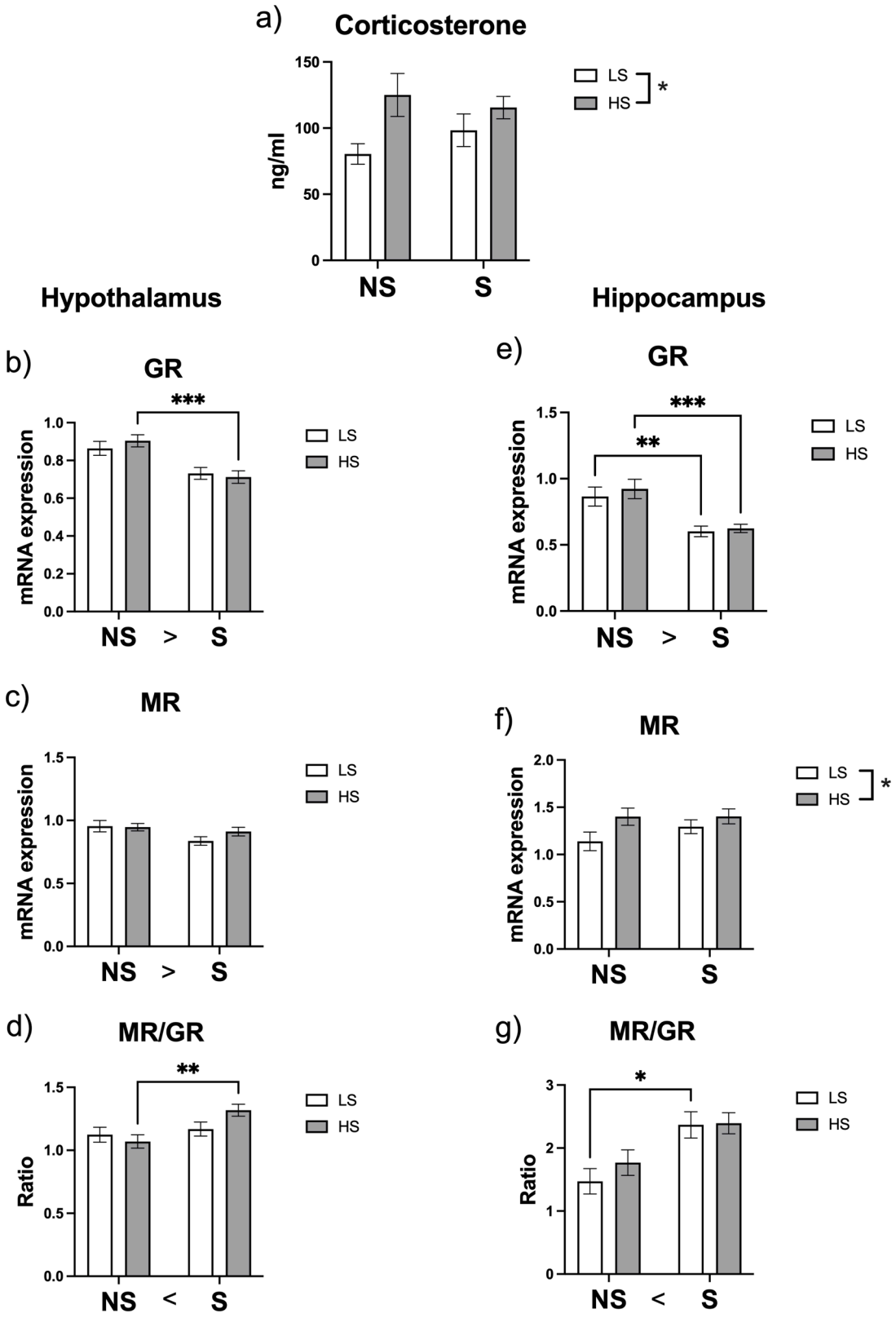

Fig. 3. a) Plasma corticosterone levels (ng/ml) at day 31. Hypothalamic b) GR and c) MR mRNA gene relative expression levels, and d) MR/ GR ratio. Hippocampal e) GR and f) MR mRNA gene relative expression levels, and g) MR/ GR ratio. Stress factor significance is expressed as < or > according to directionality. Data are expressed as mean ±*S*.E.M. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

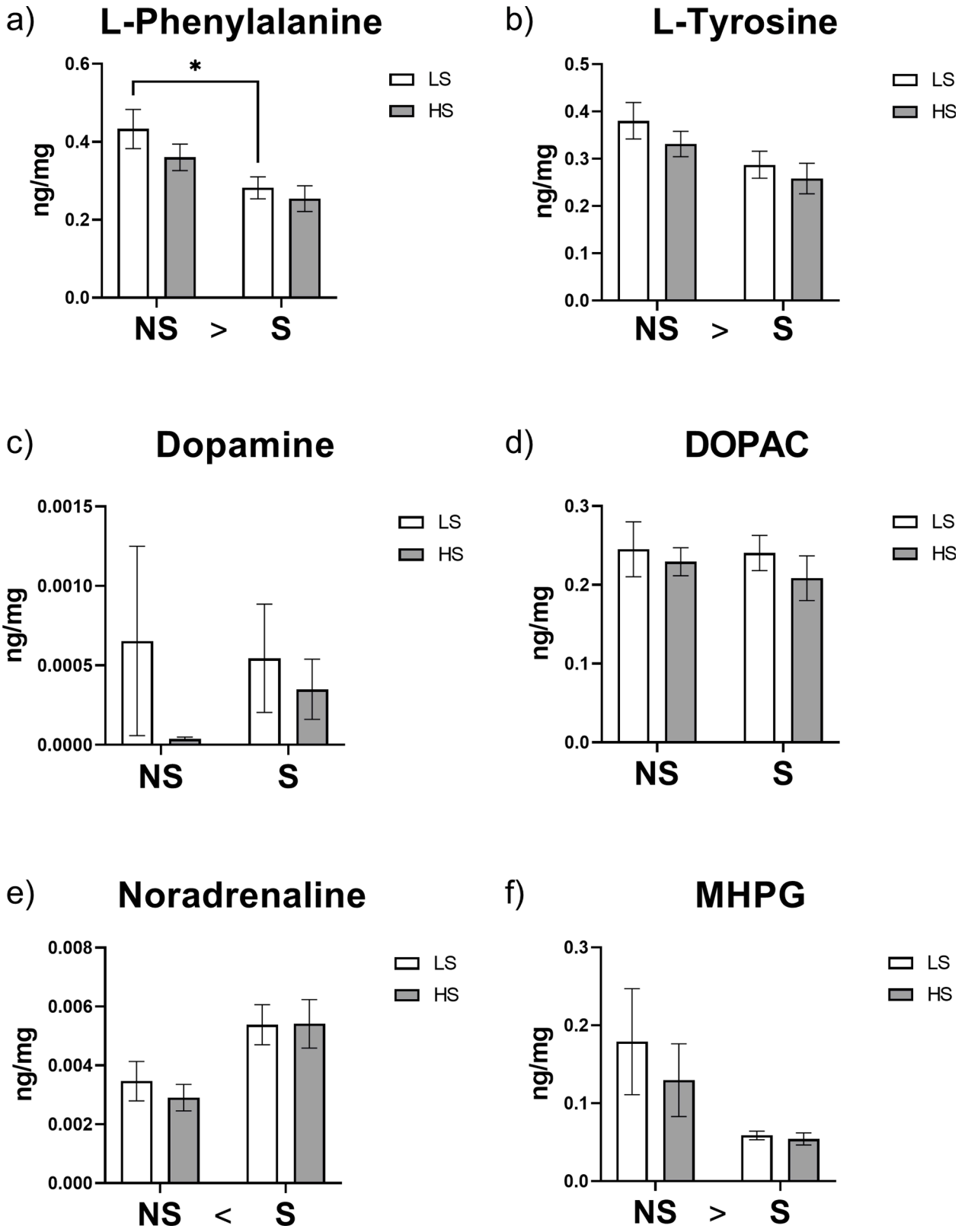

Fig. 4. Hippocampal a) L-Phenylalanine, b) L-Tyrosine, c) Dopamine, c) DOPAC, e) Noradrenaline, and f) MHPG levels expressed in ng/mg. Stress factor significance is expressed as < or > according to directionality. Data are expressed as mean ±*S*.E.M. *$p < 0.05$.

$p = 0.019$; $\eta 2 = 0.073$; Fig. 6d). They (0.010 ± 0.003) also showed a lower Kyna/3-HK ratio in comparison with non-stressed mice (0.017 ± 0.004) ($F_{(3,71)} = 3.998$; $p = 0.049$; $\eta 2 = 0.053$). Interestingly, the sociability factor was also significant for 3-HK ($F_{(3,73)} = 4.044$; $p = 0.048$; $\eta 2 = 0.052$), with LS animals having higher expression levels than their HS counterparts. Stressed mice had higher 5-HT levels ($F_{(3,72)} = 24.602$; $p < 0.001$; $\eta 2 = 0.255$), and differences were observed between the NS/LS and S/LS groups ($p = 0.001$; $d = 1.28$) and between the NS/HS and S/

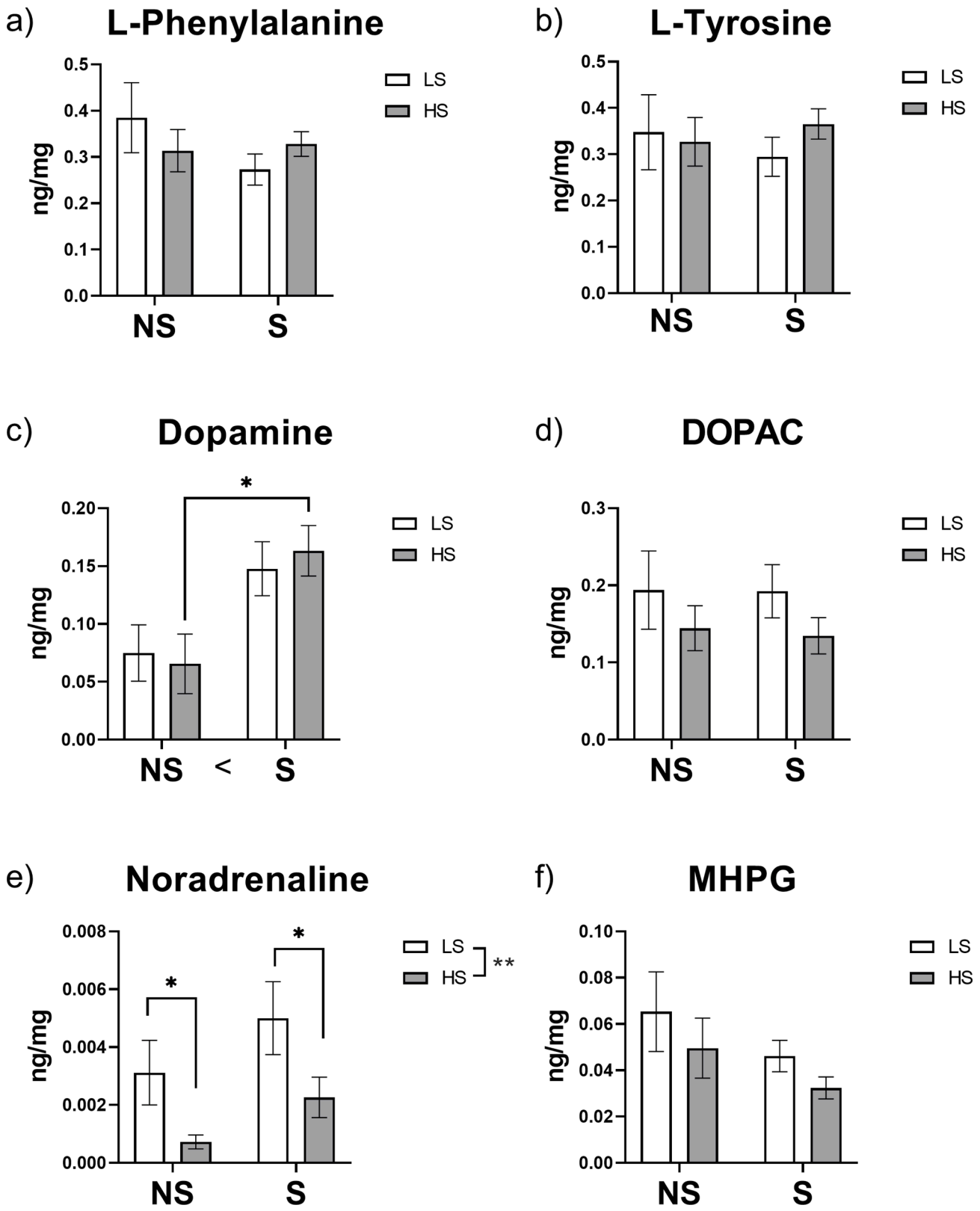

Fig. 5. Striatal a) L-Phenylalanine, b) L-Tyrosine, c) Dopamine, c) DOPAC, e) Noradrenaline, and f) MHPG levels expressed in ng/mg. Stress factor significance is expressed as < or > according to directionality. Data are expressed as mean ±*S*.E.M. *$p < 0.05$.

HS groups ($p = 0.04$; $d = 1.10$) (Fig. 6e). In contrast, stressed mice had lower 5-HIAA levels ($F_{(3,72)} = 5.692$; $p = 0.020$; $\eta 2 = 0.073$; Fig. 6f). Remarkably, stressed mice (0.232 ± 0.0417) were also found to have a higher 5-HT/Tryp ratio than non-stressed mice (0.189 ± 0.083) ($F_{(3,72)} = 6.676$; $p = 0.012$; $\eta 2 = 0.85$). Stressed group (0.228 ± 0.073) also showed a lower 5-HIAA/5H-T ratio compared with non-stressed subjects (1.338 ± 0.445) ($F_{(3,72)} = 10.009$; $p = 0.002$; $\eta 2 = 0.122$).

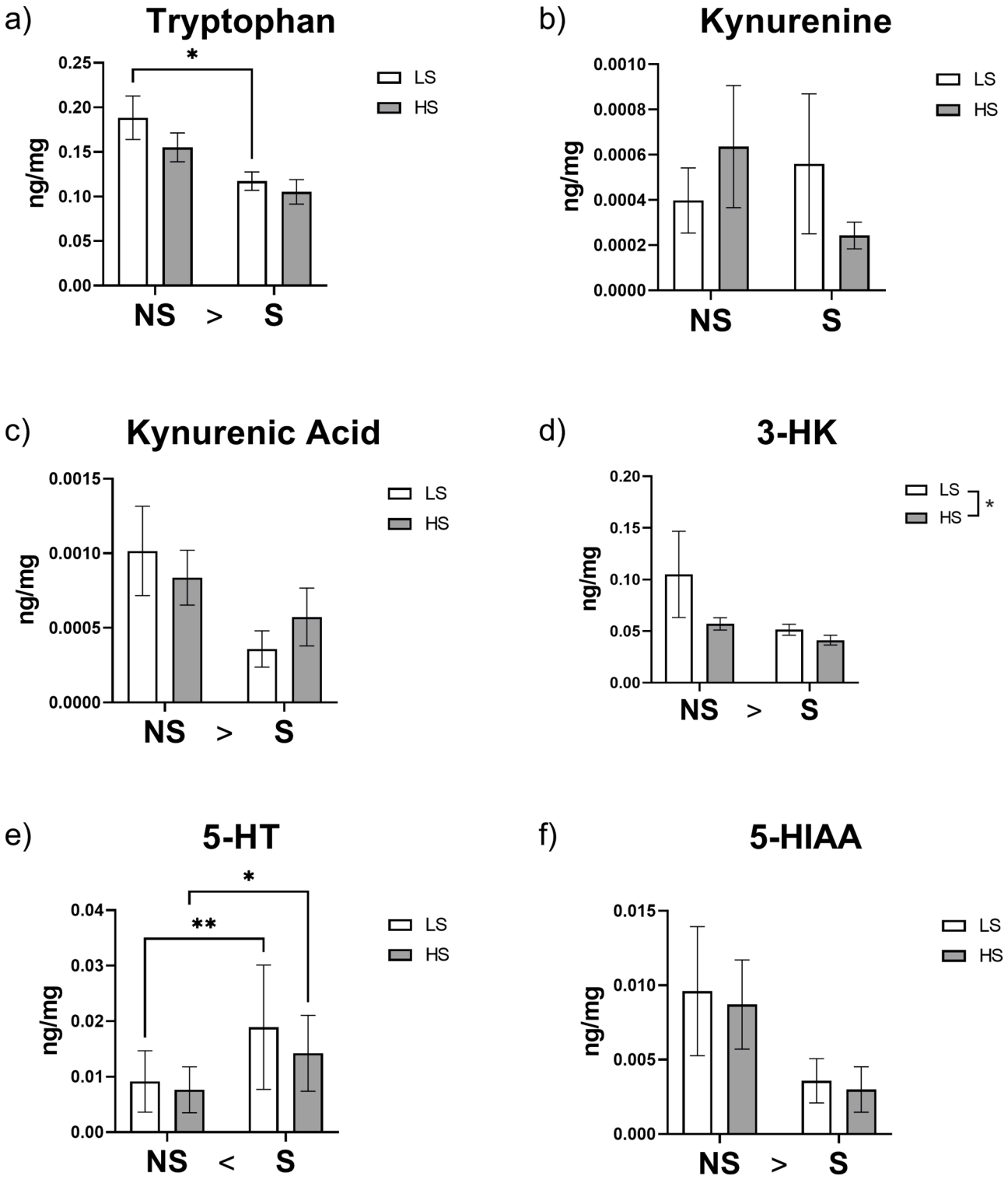

Fig. 6. Hippocampal a) Tryptophan, b), Kynurenine, c) Kynurenic Acid, d) 3-HK, e) 5-HT, and f) 5-HIAA levels expressed in ng/mg. Stress factor significance is expressed as < or > according to directionality. Data are expressed as mean ±*S*.E.M. *$p < 0.05$, and **$p < 0.01$.

3.2.2.4. Indolamine levels in the striatum. Regarding the indolamine pathway, no differences were observed in Tryp (Fig. 7a), Kyn (Fig. 7b) or Kyna levels (Fig. 7c), although stressed mice had higher 3-HK levels ($F_{(3,75)} = 8.483$; $p = 0.005$; $\eta2 = 0.105$) than the non-stressed ones. Moreover, stressed group ($0.003 \pm < 0.001$) showed a lower Kyna/3-HK ratio compared to non-stressed ones (0.007 ± 0.002) ($F_{(3,75)} = 4.139$; $p = 0.049$; $\eta2 = 0.103$; Fig. 7d). Similarly, stressed subjects had higher 5-HT levels than non-stressed mice ($F_{(3,74)} = 13.561$; $p < 0.001$; $\eta2 = 0.155$), and the post hoc analysis revealed differences between the NS/HS and the S/HS groups ($p = 0.01$; $d = 1.07$; Fig. 7e). Stressed mice (0.172 ± 0.013) also had a higher 5-HT/Tryp ratio than the non-stressed subjects (0.092 ± 0.017) ($F_{(3,72)} = 6.676$; $p = 0.012$; $\eta2 = 0.85$). In contrast, stressed mice had lower 5-HIAA levels ($F_{(3,73)} = 8.791$; $p = 0.004$; $\eta2 = 0.107$) and, interestingly, differences were also found for the stress x sociability interaction ($F_{(3,73)} = 4.839$; $p = 0.031$; $\eta2 = 0.062$). Post hoc analyses revealed significant differences between the NS/LS

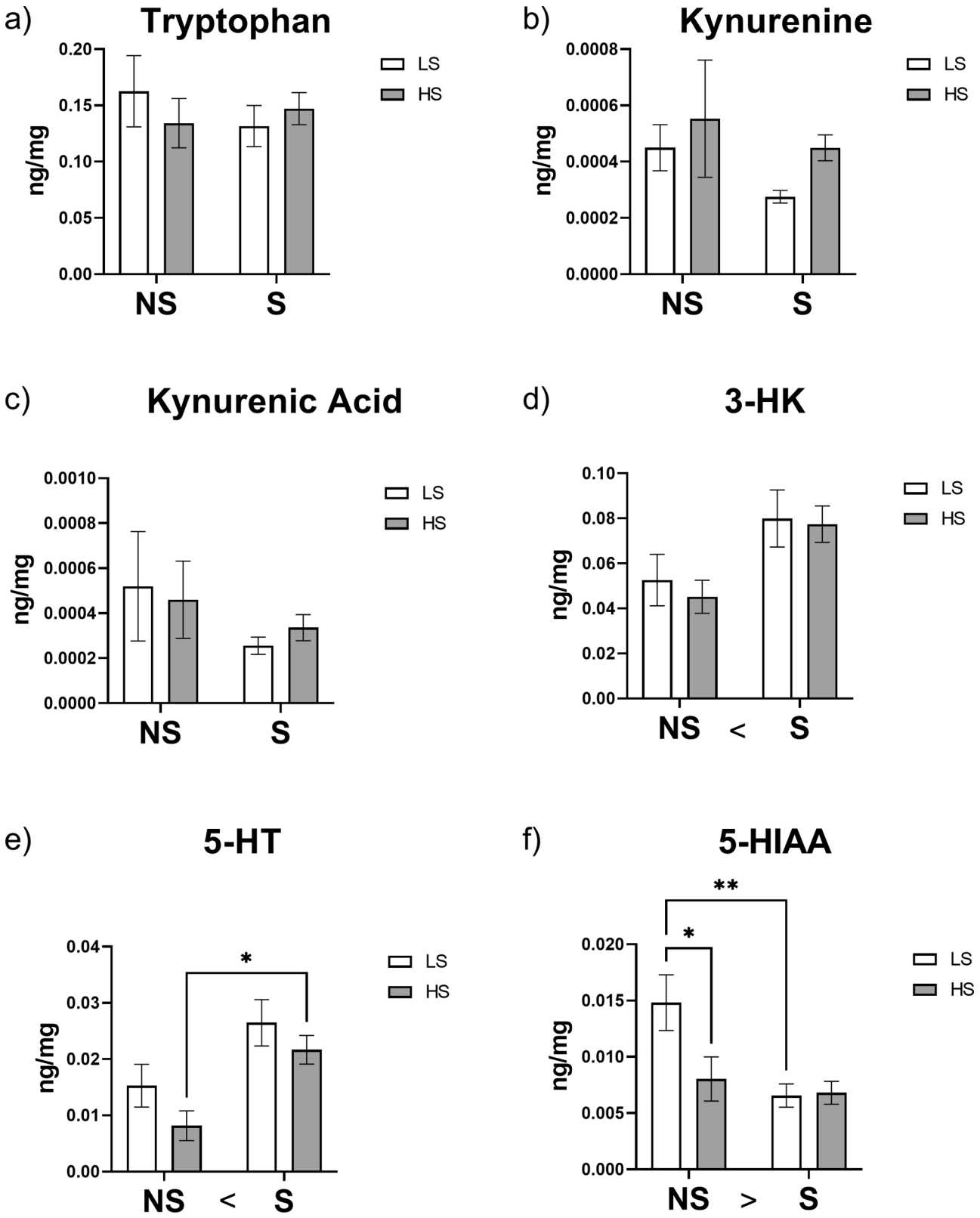

Fig. 7. Striatal a) Tryptophan, b), Kynurenine, c) Kynurenic Acid, d) 3-HK, e) 5-HT, and f) 5-HIAA levels expressed in ng/mg. Stress factor significance is expressed as $<$ or $>$ according to directionality. Data are expressed as mean $\pm$*S*.E.M. *$p < 0.05$, and **$p < 0.01$.

and NS/HS groups ($p = 0.036$, $d = 0.784$), as well as between the NS/LS and S/LS groups ($p = 0.008$; $d = 1.217$; Fig. 7f). In terms of the ratio, stressed mice (0.553 ± 0.141) had lower 5-HIAA/5-HT levels than their non-stressed counterparts (2.895 ± 0.855) ($F_{(3,73)} = 6.638$; $p = 0.012$; $\eta 2 = 0.83$).

3.2.3. Hippocampal and striatal IDO and iNOS mRNA relative gene expression

Regarding the hippocampus, stressed mice had lower IDO levels than non-stressed mice ($F_{(3,76)} = 10.133$; $p = 0.002$; $\eta 2 = 0.118$), and significant differences were observed between the NS/HS and S/HS groups ($p < 0.05$; $d = 1.02$; Fig. 8a). iNOS mRNA expression was also lower in stressed mice ($F_{(3,76)} = 6653$; $p = 0.012$; $\eta 2 = 0.80$; Fig. 8b). In the striatum, no differences were observed between the NS and S groups in terms of either IDO or iNOS mRNA expression, although the sociability factor was significant for IDO mRNA expression levels, with HS mice having higher IDO levels than their LS counterparts ($F_{(3,76)} = 4.109$; $p = 0.046$; $\eta 2 = 0.051$; Fig. 8c, d).

4. Discussion

4.1. CSIS in female mice with high or low sociability: effects on behavior

The social instability stress model applied during 4 weeks did not produce the expected behavioral alterations in female mice, and no behavioral effects were observed as a function of sociability. We observed that females seem to be able to cope without inducing a depressive phenotype. These results underline the need, already pointed out by other authors [27,35], to establish a standardized CSIS protocol in order to achieve a more predictive model.

Individual differences are a fundamental aspect to consider when studying the negative effects of social stress. Although there is evidence to suggest that higher sociability levels may have a protective effect, particularly under conditions of social stress [36,37], we found no effect of either high or low sociability on any of the behavioral tests performed after the application of a chronic social instability stress model in female mice. The absence of statistically significant results may be due to the fact that none of these post-stress behavioral tests assess sociability specifically; also (and perhaps more likely), it may be that the CSIS model applied was not sufficient to elicit an allostatic load reflecting changes in behavior [31]. Although substantial evidence suggests that stressful life events predispose individuals to depression and anxiety-like behaviors [38,39], our CSIS model did not reveal anhedonia, a key index of depression-like behaviors [40]. The application of this stress paradigm has revealed positive [41] and negative [42,43] anhedonic effects, probably due to methodological (stress or anhedonia protocol, light or

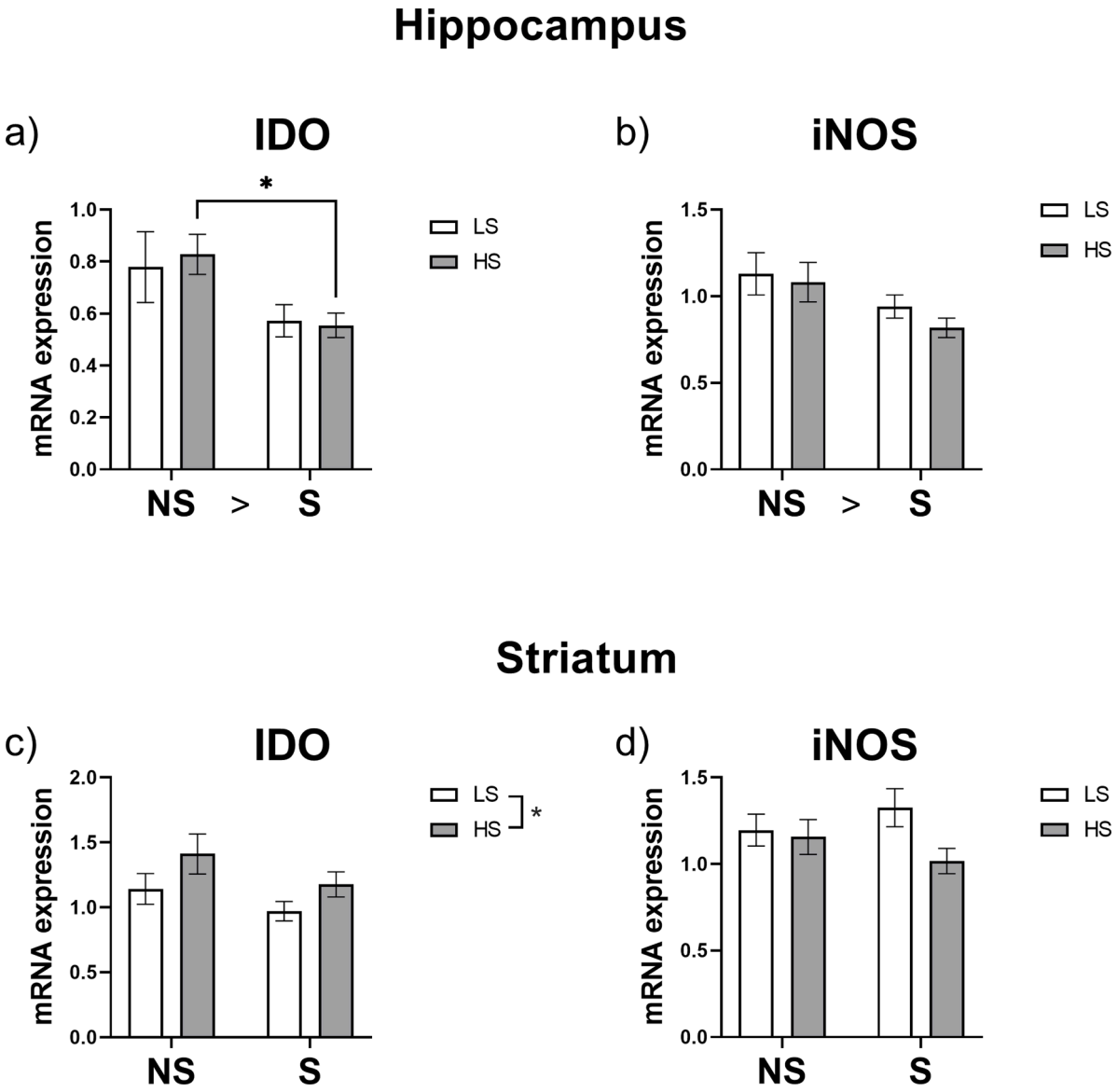

Fig. 8. a) IDO and b) iNOS mRNA expression levels in the hippocampus, and c) IDO and d) iNOS mRNA expression levels in the striatum. Stress factor significance is expressed as < or > according to directionality. Data are expressed as mean ±*S*.E.M. *$p < 0.05$.

dark phase behavioral testing) and individual (species, sex, stress coping strategies, phase of the females' estrous cycle) differences. In this regard, our lack of results in the sucrose preference test should also be interpreted with caution, since i) passive sucrose consumption tests without operant protocols may not be a good indicator of depressive-like behavior in laboratory mice [44], and ii) we did not use additional tests, such as forced swimming, because of their severity and because it is not clear whether immobility reflects a depressive phenotype or is a learned adaptive behavior [45].

Although stressed female mice displayed increased locomotor activity in the OFT, their lower thigmotaxis does not allow us to establish this observation as a symptom of anxiety-like behavior. Therefore, a more thorough assessment would have been necessary to rule out anxious behavior, using different test as elevated plus maze, zero maze or light and dark box [46]. Nevertheless, the active behavioral profile of female mice exposed to CSIS observed previously in our laboratory [26, 31], and by other authors [28], has been interpreted as indicative of higher arousal and precludes us from ruling out the possibility of our findings being indicative of anxiety-like behaviors. 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 [47], or alternatively, as an adaptive coping strategy designed to manage and regulate pressures, demands, and emotions in response to stress [48]. Furthermore, although previous work in our laboratory found no effect of the estrous cycle on behavior following social stress due to instability [26], this variable was not considered in this study, and we cannot, therefore, rule out the possibility of the estrous cycle and estrogen levels having some effect on the active behavioral profile observed in female mice exposed to CSIS [49,50].

4.2. CSIS in female mice with high or low sociability: neuroendocrine effects

Consistently to that reported by other authors, stress did not alter corticosterone levels during exposure to CSIS [30,41,51]. As in previous studies conducted in our laboratory [31], among female mice, exposure to CSIS increased the MR/GR ratio, decreasing GR levels. 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 [52]. Sex differences in the GR function also appear to make females more susceptible to dysregulation after a stressful event [53]. 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 [26,31] may attenuate the negative feedback process in response to a situation of chronic stress. Considering corticosterone plasma levels at the end of the CSIS model, our paradigm may have caused habituation of the HPA axis response that is often observed upon repeated exposure to the same stressor. In this sense, we cannot rule out a down-regulation of GR receptors expression in stressed subjects caused by glucocorticoids. The differential expression of receptors observed between stressed and non-stressed mice could indicate regulation of the HPA axis, with different consequences, since it has been associated with both, deleterious [54] and protective effects [55] in stressful situations. Furthermore, it is important to note that in the only work in which the effects of GR receptor deletion were studied in both sexes, they observed only deleterious effects in males, suggesting alternative mechanisms of GR regulation by females [56].

We did observe an effect of social connectedness, with high-sociability subjects having higher corticosterone levels and MR expression in the hippocampus. This receptor at the hippocampal CA2 region has been associated with social behavior [57]. However, as we have measured the expression at the whole hippocampus, we cannot determine if there are different expression patterns in the different regions of the hippocampus between groups. These results support the hypothesis that sociability may play a modulatory role in HPA activity, especially in situations of social stress [58–60]. If this were the case, the results obtained here would indicate that this change in female mice (allostatic load) depends on sociability. Considering that lower MR receptors expression has been commonly associated with mood disorders [61], and taking into account that we did observe a lower expression of MR receptors in the hypothalamus, the observed increased expression of these receptors in the hippocampus only in subjects with high sociability does not allow us to rule out a protective effect of high sociability in female mice submitted to CSIS model.

4.3. CSIS in female mice with high or low sociability: neurochemical effects

Although the release of catecholamines is a key initial event in response to stressors, it is now clear that different types of stressors and coping strategies elicit specific responses [62]. In our case, social instability resulted in an increase in NA in the hippocampus, as well as in a decrease in the levels of its metabolite MHPG and its precursors Phe-and Tyr, which rules out an over-activation of this pathway induced by social instability. In the striatum, we observed increased dopamine levels in subjects exposed to social instability, which may explain the increased locomotor activity observed in the OFT. However, as in the hippocampus, the reduced DA turnover observed in stressed subjects rules out the social instability-induced hyper-activation of the dopaminergic pathway. Interestingly, this increase in dopamine levels was only observed in subjects with high sociability. In this sense, sociability has been described as a behavioral characteristic that reflects a tendency to affiliate and is associated with positive affect, as well as with differences in the sensitivity of brain DA systems. Furthermore, in the striatum, and independently of stress, NA levels were significantly reduced in those subjects with high sociability, supporting the idea of the protective effect of this factor.

It is also well known that stressful situations can impact the Tryp-Kyn pathway, through glucocorticoids and cytokine-induced activation [63]. The stress model applied in the present study did not produce the expected changes in relation to the Tryp metabolic pathways. Social instability increased 5-HT levels in both the hippocampus and striatum, while reducing the levels of its metabolite 5-HIAA in both structures and its precursor Tryp in the hippocampus. Whereas in the hippocampus this effect was observed in subjects with high and low sociability, in the striatum it was only observed in subjects with high sociability, again highlighting the importance of this factor in the study of the negative effects of social stress. Although social stress has been commonly associated with an imbalance of the Tryp metabolic pathway, favoring Kyn synthesis over 5-HT, and therefore with an increase in its metabolites [64], our results reveal the opposite effect after the application of the social instability stress model. Interestingly, while no effect was observed in the Kyn/Tryp ratio, an activation biomarker of the Kyn pathway [65], increased activation of the 5-HT pathway (5-HT/Tryp ratio) was found in female mice subjected to CSIS. However, this increased serotoninergic production with respect to Kyn did not result in higher serotoninergic activity, since, similarly to that observed in the noradrenergic and dopaminergic pathways, a lower 5-HT turnover (5-HT/5-HIAA ratio) was observed in stressed subjects. Overall, these results do not allow us to rule out the possibility of a lower level of monoaminergic transmission in female mice subjected to social instability.

Interestingly, although social instability was not observed to affect Kyn levels in any of the structures, a reduction in both Kyna and 3-HK levels was found in the hippocampus. Although an increase in 3-HK in the striatum was observed after the application of the social instability model, the opposite effect observed in the hippocampus, together with the behavioral, neuroendocrine, and neurochemical results outlined above, allows us to rule out the possibly that the social instability model applied to female mice for 4 weeks does not have a deleterious effect at

any of the levels analyzed.

Stress-induced inflammatory activation is one of the main hypotheses in the study of the negative effects of social stress on health. Many studies have focused on the impact of pro-inflammatory cytokines on monoaminergic function, specifically through the activation of IDO. Firstly, this enzyme catalyzes the initial and rate-determining step of the Tryp metabolism via the Kyn pathway. And secondly, the nitric oxide (NO) produced by iNOS inhibits IDO activity by directly interacting with it and promoting its degradation. Contrary to the expected results, social instability stress decreased the synthesis of the IDO and iNOS enzymes when applied to female mice for 4 weeks. Consistently with the behavioral and neuroendocrine findings, these results support the idea that the social instability model applied did not elicit allostatic load. Although we did not observe any change in IDO or iNOS expression in the striatum, the lower expression of these enzymes in the hippocampus may reflect a resilient response to a mild stressor, regulating the IDO-mediated Tryp-degrading pathway. If this were indeed the case, it would indicate that the application of our psychosocial stress model did not promote the conversion of Tryp to Kyn and Kyna or 3-HK. The administration of IDO and iNOS inhibitors has been shown to alleviate the neurochemical and behavioral effects associated with chronic stress [66–68], and it has been suggested that social support may influence the expression of IDO [69]. Similarly, our results indicate that this reduction in IDO expression was significant only in those subjects with high sociability.

According to the behavioral and neuroendocrine results presented above, the neurochemical profile observed after the application of the CSIS model is not in line with the expected results and does not rule out that the level of stress was insufficient. Although there is extensive evidence, that stress and corticosterone are able to change the biosynthesis, release, and reuptake of monoamines [70–72], the absence of behavioral changes and effects on corticosterone levels following the application of the social instability stress model makes it difficult to establish a relationship between these variables (behavior, neuroendocrine and neurochemical).

Finally, we would like to point out the need to have predictive and translatable animal models of chronic social stress. To this end, we believe that it is essential to study both sexes but applying the same social stressor. In this sense, it would be interesting to explore the model of social chronic defeat in both males [73] and females [74]. Likewise, it is necessary to do a deeper phenotyping, since in neuroscience we tend to use relatively simple and quick tests [75], but we should focus more on the ethogram of the mice and expand the battery of tests for each behavioral domain to be studied.

In conclusion, although female mice exposed to CSIS showed increased arousal, there was no evidence of depressive-like behavior. Exposure to CSIS also did not affect corticosterone levels, although it increased the MR/GR ratio by decreasing GR expression. As a result of increased arousal, female mice exposed to CSIS had higher levels of NA and DA in the hippocampus and striatum respectively, although lower monoaminergic turnover was also observed. Contrary to the expected results, CSIS increased 5-HT levels in both the hippocampus and striatum, which may explain the lack of results in anhedonia. Similarly, and contrary to the expected results, CSIS was found to reduce kynurenic acid and 3-HK levels in the hippocampus, probably due to the observed decrease in the synthesis of the enzymes IDO and iNOS. Interestingly, the observed decrease in IDO synthesis and the increased 5-HT and DA levels in the striatum were only found in highly sociable subjects. Overall, our model has produced neuroendocrine and neurochemical but not behavioral changes, so it has not allowed us to study sociability in depth. Therefore, a model that induces both molecular and behavioral phenotypes should be applied to determine the role of sociability.

Funding

This study was supported by the Spanish Ministry of Science and Innovation RTI2018-098264-B-I00 (MCIU/AEI/FEDER, UE), the UPV/EHU GIU18/103 and the PIBA 2019-22 Project Grants.

Ethical approval

All procedures involving mice were performed in accordance with that established in the European Directive (2010/63/EU) and were approved by the Animal Welfare Ethics Committee of the University of the Basque Country (CEEA-UPV/EHU; M20/2018/090) and the Gipuzkoa Provincial Council (PRO-AE-SS-062).

Availability of data and materials

The study data will be made available upon reasonable request to the corresponding author.

CRediT authorship contribution statement

Alina Díez-Solinska: Investigation, Formal analysis, Data curation, Visualization, Writing – original draft. **Garikoitz Azkona:** Conceptualization, Writing – original draft, Visualization, Supervision. **Maider Muñoz-Culla:** Investigation, Writing – original draft. **Garikoitz Beitia-Oyarzabal:** Validation, Writing – original draft, Funding acquisition. **Olatz Goñi-Balentziaga:** Methodology, Supervision, Writing – review & editing. **Eneritz Gómez-Lazaro:** Supervision, Writing – review & editing, Funding acquisition. **Oscar Vegas:** Conceptualization, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

The authors thank SGIker (UPV/EHU) for the technical and human support provided.

Appendix A

Table A1

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SECTION III: RESULTS, DISCUSSION AND CONCLUSIONS

Synopsis of findings across studies, General Discussion and Conclusions

7. SYNOPSIS OF FINDINGS ACROSS STUDIES

7.1. CHAPTER 1. Study #1: Individualized Housing Modifies the Immune-Endocrine System in CD1 Adult Males

In this study, isolated mice exhibited higher levels of fecal CORT metabolites at the first week, although these levels normalized by the fourth week. Both the experimental and control groups showed an increase in body weight, with no significant differences between them. In addition, while there were no significant variations in WBC count between the groups at the beginning and end of the study, isolated mice exhibited a decrease in these cells over time. Specifically, both monocytes and granulocytes showed a similar trend and were reduced after the experimental procedure. There were no significant differences in RBC or platelets. These findings suggest that while the experimental procedure did not significantly influence the measured variables, isolation induces a physiological response that could interfere with the responses typically observed following a stress procedure in a laboratory.

7.2. CHAPTER 2. Study #2: Chronic defeat stress induces monoamine level dysregulation in the prefrontal cortex but not in the hippocampus of OF1 male mice

In this study, two distinct coping strategies were identified among male mice facing another male mice during the first encounter of the resident-intruder paradigm of the CSDS. The behavioral characteristics of these male mice were assessed and mice were divided into two different clusters: AA and PR. These clusters were based on the frequency with which different behaviors, such as

threat, non-social exploration, and exploration from a distance, were displayed by mice during physical interactions.

Regarding neuroendocrine variables, stressed mice presented higher CORT plasma levels, particularly at day 9, indicating heightened stress response. Concerning brain neurochemistry, changes were observed in both the HC and PFC, although the alterations in the HC were considerably weaker. Stressed mice had lower levels of Tyr, DA and DOPAC in the PFC, along with higher levels of NA and lower levels of MHPG. Alterations in the indolamine pathway were also observed, including lower Kyn and Kyna levels, higher levels of 5-HT, and lower levels of 5-HIAA. These altered levels resulted in changes in the ratios between monoamines and their metabolites. In the HC, only a reduction of 3-HK was observed. Stressed mice also consumed less sucrose in the SPT, which is indicative of depressive-like symptoms. This group also showed lower weight gain compared to controls, but they recovered by the end of the experiment.

Concerning coping strategies, no significant differences were found when comparing AA with PR mice. However, both groups showed certain alterations in brain neurochemistry and body weight compared to the control group, as well as differences in behavior when compared to the non-stressed group.

7.3. CHAPTER 3. Study #3: Chronic social instability stress down-regulates IL-10 and up-regulates CX3CR1 in tumor-bearing and non-tumor-bearing female mice

This study investigated the effects of stress and tumor presence on various physiological and behavioral parameters in female mice. At the

neuroendocrine level, stressed non tumor-bearing animals showed higher CORT levels on day 31, along with lower GR levels and higher MR/GR ratio in the HC and HT and lower MR in the HT. On the other hand, tumor-bearing mice showed decreased CORT plasma levels, higher MR levels and MR/GR ratio in the HC, and lower GR and MR levels in the HT.

In terms of the immune system, stressed mice displayed decreased expression of pro-and anti-inflammatory cytokines in both the HC and the ST. This group also showed an altered CX3CL1-CX3CR1 pathway (higher CX3CR1 and lower CX3CL1/CX3CR1 ratio) in the mentioned brain structures alongside with the PFC. Tumor-bearing mice exhibited variations in the anti-inflammatory cytokine IL-10, although the variations in the pro-inflammatory cytokines were only significant in the HC with lower IL-1β. Interestingly, the CX3CL1-CX3CR1 pathway was also altered in a similar way to the stressed group, although in a less consistent way. Finally, stressed mice presented an increased arousal, as indicated by greater distances covered in the OFT and higher mobility in the NORT, and showed a reduction in body weight, whereas tumor-bearing mice presented no differences in these variables.

Despite the mentioned alterations in the neuroendocrine and immune functions and in behavior, stressed mice did not exhibited an increased tumor development.

7.4. CHAPTER 4. Study #4: The role of sociability in social instability stress: Behavioral, neuroendocrine and monoaminergic effects

In this study, no differences were observed in CORT levels, although stressed mice exhibited lower GR levels and higher MR/GR ratios in both the HT and the HC, and lower MR levels only in the HT.

Moreover, alterations in brain neurochemistry were observed in stressed mice. Stressed mice exhibited reduced levels of catecholamine precursors, including Phe and Tyr, higher NA and lower MHPG, which lead to changes in the ratios between the monoamines and their metabolites. In the ST, an increase in DA levels was observed, together with differences in the ratios between the monoamines and their metabolites. The indolamine pathway was also altered in both the HC and the ST. In both structures, the kynurenic and the serotoninergic pathways were altered as a result of changes in the precursors and the metabolite levels and the ratios.

The study also explored the influence of sociability on stress responses in female mice. Highly sociable mice exhibited elevated plasma CORT levels and MR levels in the HC. Only minor differences were observed at the neurochemical level, with the most notable being lower levels of 3-HK in the HC and lower levels of NA in the ST among highly sociable mice.

8. GENERAL DISUSSION

8.1. Chronic Social Stress in male and female mice: effects on the neuroendocrine system and brain neurochemistry (Table 1)

8.1.1. The neuroendocrine response

The results of these studies have indicated that chronic stress lasting from 3 to 4 weeks, induced either by social isolation or CSDS in males, or by CSIS in females, can elicit neuroendocrine changes, although in different ways.

Regarding males, study #1 demonstrated that social isolation resulted in an increased fecal CORT metabolites in the first week, although, after four weeks, a normalization of these levels was observed, reaching similar levels to those observed in group-housed males (Díez-Solinska et al., 2023b). This normalization may suggest habituation to isolation, which is consistent with what is often observed after repeated exposure to the same stressor or to a new environment (Swan et al., 2023). However, this habituation could be dependent on the specific stimulus to which an individual is exposed. In fact, in the study #2 in which males were submitted to CSDS, this habituation did not occur. In the study #2, individuals submitted to CSDS showed higher plasma CORT levels than those of non-stressed mice on both the 9th and 21st days of the stress procedure (Díez-Solinska et al., 2024), what indicates a lack of CORT normalization. These findings could be potentially attributed to the difference in severity between social isolation and CSDS. If such were the case, isolated individuals could have experienced acute stress in the first week rather than chronic stress over the four weeks. However, this hypothesis cannot be confirmed since it cannot be determined when the CORT normalizes in study #1, as CORT

plasma levels were not analyzed during the intermediate (2nd and 3rd) weeks, being this one of the limitations of the study #1.

Based on these data obtained in male mice, two main conclusions could be inferred. Firstly, group housing could represent a favorable condition for individuals, as it does not induce alterations in CORT levels. Secondly, CSDS appears to emerge as a more stressful condition for male mice as the HPA axis remains activated over time. However, CORT levels were measured using different samples (fecal CORT versus plasma CORT levels, respectively), and thus strict comparisons of these results must be taken with caution.

With respect to HPA reactivity in female mice, although the results in scientific literature are not consistently homogeneous, the use of the CSIS model has been associated with elevated CORT levels (Baranyi et al., 2005; Herzog et al., 2009b; Jarcho et al., 2016; Labaka et al., 2017; Schmidt et al., 2010). However, in contrast to these findings, studies #3 and #4 (Díez-Solinska et al., 2022; Díez-Solinska et al., 2023a) show that the CSIS model does not trigger the expected response in female mice as no increase in plasma CORT levels were observed. It is important to consider that the time point of blood collection in males and females varies and this may have influenced the differences observed. In males, blood samples were collected 5-10 minutes after the FST, which is a procedure that can induce a certain level of stress in animals, whereas in females, blood was collected the day after the behavioral assessment. Besides these differences in the timing of blood collection, another explanation for the absence of differences among females would be environmental habituation, which would entail a normalization within the HPA axis. However, in addition to CORT plasma

levels, GR and MR expression levels were also assessed and, indeed, differences were observed between stressed and non-stressed females in these receptors. The alteration in both GR and MR receptors in HC and HT, would suggest a dysregulation of the HPA axis as they are crucial in the HPA axis negative feedback. Similar to other studies in which female mice showed lower GR levels after CSIS (Labaka et al., 2017), in the studies #3 and #4 (Díez-Solinska et al., 2022; Díez-Solinska et al., 2023a), stressed females exhibited lower GR levels in both the HC and the HT. Additionally, and contrary to Labaka et al., (2017), stressed females also presented lower levels of MR in the HC (Díez-Solinska et al., 2022) and the HT (Díez-Solinska et al., 2023a), and a higher MR/GR ratio in both structures (Díez-Solinska et al., 2022; Díez-Solinska et al., 2023a). The lower GR levels and the higher MR/GR ratio suggest a relatively higher proportion of MR compared to GR, which could have significant implications for the HPA axis, as MRs have a higher affinity for GCs.

On the basis of the studies conducted and despite the fact that chronic stress has commonly been associated with the dysregulation of the neuroendocrine system, the effects of chronic stress may depend on the nature of the stressor itself.

8.1.2. Brain neurochemistry

The results of studies #2 and #4 have shown that chronic stress, induced either by CSDS in males or CSIS in females, can elicit changes in brain neurochemistry. Nevertheless, differences can be distinguished when comparing results in both sexes.

The brain structures analyzed differed between sexes due to the intrinsic differences in male and female mice. The HC was analyzed in both, males and females, as it is a fundamental structure not only in the processing and multisensory encoding of information, but also in the learning of adaptive behaviors to the environment they encode (Zemla & Basu, 2017), thereby constituting an important structure in processing a stressful condition. However, the PFC was only analyzed in males, as this structure has been extensively related to aggressive behaviors (Nelson & Trainor, 2007), and it has been linked to dominant behaviors, such as aggressiveness, in mice (Wang et al., 2011). Conversely, the more social behaviors displayed by females prompted the study of the ST, as this structure has emerged as a relevant brain structure in social behaviors (Dölen et al., 2013; Henschen et al., 2013; Solié et al., 2022). Overall, psychosocial stress has been shown to alter brain neurochemistry in both the PFC and the ST in human studies (Pruessner et al., 2008), making its analysis relevant when studying CS in mice.

Regarding the HC, both males and females exhibited a distinct physiological response regarding catecholamines and indolamines.

In terms of catecholamines, in the study #2, no significant differences were observed in any of the catecholamines or their metabolites between stressed male mice and controls (Díez-Solinska et al., 2024), which is consistent with findings in previous studies (Favoretto et al., 2020; Venzala et al., 2013). However, in the study #4, differences were observed between stressed and non-stressed female mice (Díez-Solinska et al., 2023a). Regarding the dopaminergic pathway, stressed females exhibited similar results to what was found in males,

as no differences were found in DA and DOPAC levels between stressed and unstressed females, and thereby, a dopaminergic activation could not be determined. However, an alteration in the metabolism towards DA cannot be ruled out as, with lower Phe and Tyr levels, DA levels remain the same in stressed and non-stressed females. Additionally, stressed females also presented a lower DOPAC/DA ratio, suggesting a potential accumulation of DA and an altered metabolisation of DA into DOPAC. Concerning the noradrenergic pathway, and contrary to what was observed in study #2 in males, stressed female mice showed higher levels of NA and lower levels of MHPG than non-stressed mice. These differences in catecholamine levels were in turn observed in the ratios, as stressed female mice exhibited a lower MHPG/NA indicating an altered metabolism from NA to MHPG. The higher levels of NA, lower levels of MHPG, and the lower MHPG/NA ratio in female mice could suggest, on the one hand, a more pronounced NA synthesis in response to stress, which is quite remarkable considering that stressed females also presented lower Phe and Tyr levels (precursors of NA); and, on the other hand, a possible alteration in the metabolic process involved in the conversion of NA to MHPG. Indeed, this alteration in the noradrenergic pathway, coupled with the higher levels of DA in the ST discussed below, could explain the increased arousal observed in stressed females in the OFT.

Concerning indolamines, in the study #2, stressed male mice exhibited a lower Kyn/Tryp ratio and a higher Kyna/3-HK ratio compared to control mice, likely due to lower levels of 3-HK. However, no differences were observed in the serotonergic pathway, neither in 5-HT nor in its metabolite 5-HIAA. In contrast, in the study #4, stressed females showed lower levels of Tryp, Kyna, and 3-HK,

albeit higher levels of 5-HT. These alterations in the metabolite levels, together with the lower 5-HT/Tryp ratio might indicate a deviation of Tryp metabolism towards the 5-HT pathway, resulting in higher levels of 5-HT, and suggesting an increased serotonergic activity. Furthermore, the elevated 5-HT levels persist due to reduced degradation into 5-HIAA, as evidenced by the lower 5-HIAA/5-HT ratio. Thus, unlike the males in study #2, which exhibited anhedonia in the SPT, these results in indolamine levels in the HC in female mice, along with the higher 5-HT levels in the ST, could explain the absence of anhedonia in stressed females, thus suggesting a more adaptive monoaminergic turnover. However, on the other hand, stressed females also exhibited a lower Kyna/3-HK ratio, which could indicate that, although the levels of both metabolites are lower in stressed animals, Tryp metabolism would be diverted towards 3-HK production rather than Kyna. 3-HK demonstrated neurotoxic properties (Eastman & Guilarte, 1989; Guilarte & Wagner, 1987), while Kyna has been related to neuroprotective effects (Hilmas et al., 2001; Parsons et al., 1997), and thereby, stressed females would be diverting Kyn metabolism to the neurotoxic pathway. However, although 3-HK has been shown to have neurotoxic effects, stressed females do not show behavioral nor cognitive alterations. They may be reversing the possible deleterious effects of 3HK by increasing the serotoninergic activity or, alternatively, they could begin to develop behavioral or cognitive alterations with a longer exposure, as the effect on them may take time to occur.

Concerning the analysis of monoamines in the PFC in the study #2, differences were observed between stressed and non-stressed males in both the catecholamines and the indolamines.

Regarding catecholamines in the PFC, while no differences were found in NA levels, stressed mice did show lower MHPG levels, which may indicate a lower NA degradation, and thus an alteration in the NA metabolism. This potential alteration can also be inferred by differences in the levels of Tyr and DA, which are NA precursors. Although both groups, stressed and controls, exhibited similar levels of Phe, stressed animals presented lower levels of Tyr, DA, and DOPAC, suggesting a decreased dopaminergic activity in this area, which could potentially affect the production of NA. This decrease in DA, and therefore, in DOPAC, levels could be attributed to two factors: the reduced levels of its precursor Tyr and the elevated NA/Tyr ratio, indicating an increase in the release of NA relative to Tyr. Considering that DA is essential in regulating different cognitive, emotional and behavioral functions, as well as in reward and hedonic states (Ressler & Mayberg, 2008; Wise, 2008), the altered catecholamine levels in the PFC could affect reward behavior and motivation and therefore be a potential explanation for the anhedonia shown by stressed males in the SPT. This would support the dopaminergic role in mood-related disorders such as depression, consistent with previous research (Gorwood, 2008; Kelley et al., 2005).

In terms of indolamines in the PFC, stressed males also exhibited lower levels of certain metabolites, such as Kyn and Kyna, with no changes in 3-HK. Additionally, although there were no significant differences in 5-HT levels, the diminished levels of 5-HIAA and a reduced 5-HIAA/5-HT ratio might suggest that the released 5-HT was not metabolized. These findings differ from previous studies, which either indicate a decrease (Favoretto et al., 2020) or no alterations (Fuertig et al., 2016; Venzala et al., 2013) of 5-HT in the PFC. However, our results align with the reported 5-HT levels in the HC from these studies.

Regarding the study of monoamines in the ST in the study #4, differences were also found between stressed and non-stressed females in both the catecholamines and the indolamines.

Consistent to what was observed in the HC, stressed female mice also presented alterations in the catecholamine levels in the ST. For instance, they exhibited an altered dopaminergic pathway by exhibiting higher DA levels and a lower DOPAC/DA ratio, suggesting, again, an accumulation of DA in this region and thereby a higher dopaminergic activity. As mesolimbic DA has been related to locomotion (Balleine, 2019; Salamone & Correa, 2012), this increased dopaminergic activity could explain the increased arousal of stressed female mice in the OFT. Furthermore, concerning the noradrenergic pathway, stressed females exhibited a higher Tyr/Phe and a lower MHPG/NA ratios, suggesting a similar alteration to which was found in the HC.

Concerning indolamines in the ST, again similarly to what was observed in the HC, 5-HT levels were higher in stressed mice, while 5-HIAA levels were lower, resulting in a lower 5-HIAA/5-HT ratio in stressed females. Despite the common association of social stress with an alteration in the Tryptophan metabolic pathway, leading to increased Kyn synthesis over 5-HT (Bergamini et al., 2018), the findings in study #4 suggest the opposite effects. These findings are consistent with the higher 5-HT/Tryp ratio in stressed females, indicating a higher proportion of 5-HT when compared to Tryp. However, despite the increased serotoninergic production, it cannot be concluded a higher serotoninergic activity as a lower 5-HT/5-HIAA ratio was also observed. Stressed

females showed higher levels of 3-HK and lower Kyn/3-HK ratio compared to non-stressed ones.

Overall, hippocampal activation in stressed males and females shows significant differences in neurochemical response. In stressed males, no significant differences in catecholamine levels were observed compared to controls, indicating a relatively similar activation of dopaminergic and noradrenergic pathways regardless stress. However, in stressed females, a different response was observed, with an altered noradrenergic pathway, suggesting an increased synthesis of NA and possible alterations in its metabolism. For indolamines, both stressed males and females show changes in Tryp metabolism. However, changes in males were limited to changes in the Kyn pathway, while changes in females were much more pronounced in both the Kyn and 5-HT pathways. A differential hippocampal response was observed; although, the variances might stem not only from the sex factor but also from the nature of the stressor itself. Regarding the PFC of stressed males, it undergoes alterations in dopaminergic, noradrenergic activity and tryptophan metabolism, suggesting a complex and adaptive response to stress. Regarding the ST in females, although the levels of monoamines and their metabolites did not show as significant differences as in the HC, the ratios indicate an alteration of both catecholamine and indolamine pathways, suggesting a higher monoaminergic turnover in the ST. The increased activity in this structure has often been associated with physical stress, while psychosocial stress has been linked to decreased activity (Kogler et al., 2016). This could explain the less pronounced changes in this structure compared to the alterations found in the HC, although activation of this structure cannot be ruled out. Further investigation into these

pathways within this structure is needed, including an analysis of the enzymes and cofactors involved in the metabolism of these monoamines.

It is worth noting, however, that these differences in brain neurochemistry cannot be solely attributed to sex. While both males and females underwent chronic stress, the nature of the stressors differed. Indeed, it has been observed that the nature of the stressor can alter physiological responses in the differently (Li et al., 2019) suggesting that these findings may be influenced by methodological variations rather than sex differences themselves.

Table 1

Effects of CSS on neuroendocrine and neurochemical parameters (Chapters 1, 2, 3 and 4)

EFFECTS OF CHRONIC SOCIAL STRESS ON PHYSIOLOGICAL AND BEHAVIORAL OUTCOMES				
	MALES		FEMALES	
	CHAPTER 1 (Isolation)	CHAPTER 2 (CSDS)	CHAPTER 3 (CSIS)	CHAPTER 4 (CSIS)
Neuroendocrine parameters (i.e. CORT, GR, MR)	↑ Fecal CORT (week 1) = Fecal CORT (week 4)	(S & NS/C) = Plasma CORT (day 1) (S & NS/C) ↑ Plasma CORT (days 9 and 21)	↑ Plasma CORT (day 31) (only in S/NT animals) HC: ↓ GR ↑ MR/GR HT: ↓ GR, ↓ MR ↑ MR/GR	= Plasma CORT HC: ↓ GR ↑ MR/GR HT: ↓ GR, ↓ MR ↑ MR/GR
Neurochemical parameters (i.e. SAM axis, monoamines and their metabolites)	-	HC: Catecholamines: = Indolamines: ↓ 3-HK ↓ Kyn/tryp ratio ↑ Kyna/3-HK ratio PFC: Catecholamines: ↓ Tyr, ↓ DA, ↓ DOPAC, ↑ NA, ↓ MHPG ↑ NA/tyr Indolamines: ↓ Kyn, ↓ Kyna, ↑ 5-HT, ↓ 5-HIAA ↓ Kyn/tryp, ↑ 5-HT/tryp, ↓ 5-HIAA/5-HT ratios	-	HC: Catecholamines: ↓ Phe, ↓ Tyr, ↑ NA, ↓ MHPG ↓ DOPAC/DA, ↓ MHPG/NA ratios Indolamines: ↓ Tryp, ↓ Kyna, ↓ 3-HK, ↑ 5-HT, ↓ 5-HIAA ↓ Kyna/3-HK, ↑ 5-HT/tryp, ↓ 5-HIAA/5-HT ratios ST: Catecholamines: ↑ DA ↑ Tyr/Phe, ↓ DOPAC/DA, ↓ MHPG/NA ratios Indolamines: ↑ 3-HK, ↑ 5-HT, ↓ 5-HIAA ↓ Kyna/3-HK, ↑ 5-HT/tryp, ↓ 5-HIAA/5-HT ratios

(- = Not measured variable; ↑ = higher; ↓ = lower; C = controls; NS = non-stressed; S = stressed; S & NS/C = stressed and non-stressed groups present the same trend compared to controls). The reference group for the expression of the data is the S group. Only significant differences ($p < 0.05$) are presented.

8.2. Chronic Social Stress in male and female mice: effects on the immune system and tumor development (Table 2)

In studies #1, #3 and #4, both, stressed males and females, exhibited a dysregulation in the immune system after CS exposure (Díez-Solinska et al., 2022; Díez-Solinska et al., 2023a; Díez-Solinska et al., 2023b), although in a different way. Furthermore, this did not lead to an increase in tumor development in female mice, as shown in study #3.

Regarding males, a decrease in WBC levels over time, particularly in monocytes and granulocytes, was observed in study #1 (Díez-Solinska, et al., 2023b). This could be interpreted as a response of the immune system to CS exposure. In contrast to acute stress, CS has been related to immunosuppression. When submitted to CS, the immune system can react by inhibiting the release or well-functioning of the immune cells, which has been often referred to as stress-related immunosuppression. Indeed, it has been observed that alongside activation of the HPA axis and subsequent release of CORT, a decreased number of WBC can occur (Dhabhar & McEwen, 1997). However, given the inherent nature of the procedure employed for measuring CORT (week 1 and 4), it cannot be identified when exactly the CORT normalized in study #1, and therefore, while a potential relationship between HPA axis activation and immunosuppression could be suggested, this relationship cannot be definitively affirmed or dismissed.

A dysregulation of the immune system was also observed in stressed females compared to their non-stressed counterparts in studies #3 and #4 (Díez-

Solinska et al., 2022; Díez-Solinska et al., 2023a). While the parameters measured were not the same to those studied in socially isolated males in study #1, females also exhibited differences on the immune system considering the stress factor. This was reflected in the alteration of cytokine, chemokine and enzyme levels. In stressed females, a decrease in pro-inflammatory cytokines and an even more pronounced decrease of the anti-inflammatory cytokine IL-10 was observed in both the HC and the ST, resulting in a pro-inflammatory trend (Díez-Solinska et al., 2022). The alteration of cytokine levels, including the decrease in IL-10, following exposure to CS is something that has been documented in female mice (Labaka et al., 2017; Voorhees et al., 2013). However, the trend to downregulate both pro and anti-inflammatory cytokines and the greater decrease observed in IL-10, leading to a pro-inflammatory trend, is a different physiological response from what is commonly observed in males, as they frequently exhibit an increase in pro-inflammatory cytokines (Zhao et al., 2019). This could indicate that changes in IL-10 levels would affect females more deeply than males. Moreover, these findings in female mice are in line with the ones observed in the study #4, in which stressed female mice showed a decrease of IDO and iNOS enzymes (Díez-Solinska et al., 2023a). It has been shown that pro-inflammatory cytokines, such as IL-1β or TNF-α, can activate the expression of IDO and iNOS enzymes (Suschek et al., 2005), and thus, lead to a lower inflammatory state. A decrease in these cytokines, as it happens in study #3, could be interfering with IDO and iNOS activity. However, in the study #4, cytokine levels were not analyzed and, therefore, it cannot be concluded that the decrease in IDO and iNOS is due to a reduction in pro-inflammatory cytokines.

Regarding chemokine levels, in study #3, an imbalance in the CX3CL1/CX3CR1 pathway was observed in the HC, the ST and the PFC in female mice submitted to CSIS. The decrease in CXRCL1/CX3CR1 ratio in HC and ST may be due to the significant increase in CX3CR1 expression in both structures. Higher levels of CX3CR1 were also found in the PFC, although the increased levels of CX3CL1 in this structrue likely prevented the ratio from showing an imbalance in this structure. The increased CX3CR1 levels suggest a heightened communication between neurons and microglia in stressed females, possibly indicating an increased need for CX3CL1 to regulate microglial activation. These chemokines have been shown to participate in a complex interplay with cytokines, and thus, they have been related to inflammatory response in different brain regions. The reduced IL-10 levels observed in the HC and the ST could be maintaining higher CX3CR1 levels in these structures (Ramos et al., 2010), which in turn could be mitigating inflammation by decreasing pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α, as CX3CR1 is known for inhibiting the pro-inflammatory activity of the microglia. For instance, mice with CX3CR1 deficiency are less susceptible to develop stress-induced depressive-like (Milior et al., 2016) and anxiety-like behaviors (Wohleb et al., 2013), which are mood disorders widely related to neuroinflammation (Dowlati et al., 2010; Hiles et al., 2012).

Therefore, chronic stress, in addition to exhibiting immunosuppressive effects in males (study #1), which is consistent with other studies in the scientific literature (Dhabhar & McEwen, 1997), also impacts inflammation in females (study #3), although in a different way to what is commonly observed (Rohleder, 2019). These immune system dysregulations can create a favourable context for

the development of diseases such as cancer (Dai et al., 2020; Parker et al., 2004). In study #3, CSIS exposure did indeed affect the immune system of female mice, as previously discussed, but this altered immune response did not affect tumor development, which contrasts with previous research in male mice (Vegas et al., 2006). Although a potential reason for the diversity of findings documented across scientific literature could be the differences in the applied methodology or the differences among individuals, a recent study indicate that psychosocial stress, in fact, can inhibit tumor growth in female mice (Dawes et al., 2020). Thereby, this underscores the importance of considering sex differences and the need to further investigate the mechanisms through which stress could impact tumor development in females.

Thus, given all the aforementioned points, CS alters the immune system in both males and females. In males, the expected immunosuppressive effect was observed. Howeever, in females, the inflammatory trend was different to what is usually observed in males (Vegas et al., 2006). Therefore, its impact on the development of diseases, such as cancer, can also vary significantly and the lack of an increased tumor development in stressed females evidences this. However, it cannot be ruled out a possible effect of the different chronic stress model employed in females.

Table 2

Effects of CSS on the immune system and tumor development (Chapters 1, 2, 3 and 4)

EFFECTS OF CHRONIC SOCIAL STRESS ON PHYSIOLOGICAL AND BEHAVIORAL OUTCOMES				
	MALES		FEMALES	
	CHAPTER 1 (Isolation)	CHAPTER 2 (CSDS)	CHAPTER 3 (CSIS)	CHAPTER 4 (CSIS)
Immune system (i.e. blood cells, cytokines, chemokines and enzymes)	↓ WBC, ↓ monocytes, ↓ granulocytes over time	-	HC: ↓ IL-1β,↓ IL-6,↓ TNF-α (↓ pro-inflammatory cytokines) ↓↓ IL-10 (↓ anti-inflammatory cytokine) ↓ IL-1β/IL-10 ratio ↑ CX3CR1 ↓ CX3CL1/CX3CR1 ratio ST: ↓ IL-1β, ↓ TNF-α (↓ pro-inflammatory cytokines) ↓↓ IL-10 (↓ anti-inflammatory cytokine) ↑ IL-6/IL-10 ratio ↑ CX3CR1 ↓ CX3CL1/CX3CR1 ratio PFC: ↑ CX3CR1,↑ CX3CL1	HC: ↓ IDO, ↓ iNOS ST: =
Tumor development	-	-	= Tumor area and tumor foci	

(- = Not measured variable; ↑ = higher; ↓ = lower). The reference group for the expression of the data is the stressed group. Only significant differences ($p < 0.05$) are presented.

8.3. Chronic Social Stress in male and female mice: effects on behavior and body weight (Table 3)

In terms of behavior, differences can be observed in the effects of CS on the behavior of males and females in studies #2, #3 and #4 (Díez-Solinska et al., 2022, 2024; Díez-Solinska et al., 2023a). While in study #2, males submitted to CSDS exhibited anhedonia in the SPT, thus suggesting a depressive-like behavior; in studies #3 and #4, females submitted to CSIS did not present anhedonia, although they showed a higher arousal in the OFT and the NORT. However, in none of the studies stressed animals showed anxiety-like behavior, as no thigmotaxis was observed in the OFT, which is an index of anxiety-like behavior in mice (Simon et al., 1994).

The results regarding anhedonia in male mice are consistent with previous studies examining the effects of chronic stress on male mice (Francis et al., 2015; Friedman et al., 2014; Gómez-Lázaro et al., 2012). The increased susceptibility to developing depressive symptoms could be linked to the physiological response of males to CS. Indeed, depressive symptomatology has been associated with alterations in the PFC and the HC (MacQueen & Frodl, 2011; Price & Drevets, 2010), structures in which, in study #2, stressed males exhibited changes in catecholamine and indolamine levels. Females, on the other hand, did not show signs of anhedonia in studies #3 and #4, which may indicate that females could be more resilient to the effects of CS in terms of depressive-like behaviors. This would contradict the commonly observed trend that women are significantly more prone to developing depression, as evidenced in human studies (Bromet et al.,

2011; Salk et al., 2017). However, when examining male and female rodent models of depression, disparate findings have emerged. Several studies have reported varying findings regarding animal models, some of them indicating that females showed more (Goodwill et al., 2019; Konkle et al., 2003; Page et al., 2016) or less (Bai et al., 2014; Burke et al., 2016; Dalla et al., 2005) depressive-like behaviors in females compared to males; while others revealed no differences between sexes (Eltokhi et al., 2021). However, given that depression is often linked to neuro-inflammation (Miller et al., 2009), the fact that females exhibit lower pro-inflammatory cytokine levels than non-stressed females in study #3, contrasting to the trend usually observed in males, could explain the absence of depressive-like behaviors. Additionally, the increased CX3CR1 levels, through its involvement in neuro-inflammation, may act as a protective factor, making female mice less susceptible to developing symptoms of stress-related mood disorders, as CX3CR1 deficiency has been related to depressive-like symptomatology (Corona et al., 2010). On the other hand, females do show greater mobility and arousal in studies #3 and #4, which in fact could be explained, in study #4, by the increase in DA in the ST, which has been found to be related to arousal and activity (Capuron et al., 2012).

Regarding body weight, differences were observed across studies #1, #2 and #3 (Díez-Solinska et al., 2022, 2024; Díez-Solinska et al., 2023b), although none of them followed the same trend. In the study #1, all the males, regardless if they were stressed or non-stressed, increased their body weight during the experiment, thus showing a similar weight at the end of the stress procedure with no significant differences among them. In the study #2, a similar pattern was observed, with no differences in weight at the end of the experimental procedure.

However, in this study, differences in body weight gain were observed at the midpoint of the experiment (day 9), with stressed males showing lower body weight gain than non-stressed ones. This, along with what was observed in the SPT, which revealed anhedonia in stressed males, could indicate that CS influenced both normal food intake and the intake of hyperpalatable substances. Nevertheless, there was a recovery of weight by the end of the experiment, indicating certain habituation to CSDS. This recovery could be due to a return to normal food intake at the end of the experiment or, alternatively, to increased mobilization of glucose as a result of the observed elevation in plasma CORT levels, as GCs can induce glucose synthesis in order to maintain the metabolism of an organism (Remage-Healey & Romero, 2000; Sapolsky et al., 2000b). A different pattern was observed in females. In the study #3, stressed females showed lower body weight at the end of CSIS, which could be either a direct physiological effect of the stress, or an indirect consequence of the increased mobility of stressed females in the OFT and NORT. These results reveal how stress affects differently to males and females, being females more susceptible to show differences in weight. However, as weight was not assessed consistently throughout the stress procedure in the three mentioned studies, fluctuations in body weight could have occurred and therefore a constant downward trend cannot be determined. This fact also prevents males and females from being reliably compared in terms of weight.

Table 3

Effects of CSS on behavior and body weight (Chapters 1, 2, 3 and 4)

EFFECTS OF CHRONIC SOCIAL STRESS ON BEHAVIOR AND BODY WEIGHT				
	MALES		FEMALES	
	CHAPTER 1 (Isolation)	**CHAPTER 2 (CSDS)**	**CHAPTER 3 (CSIS)**	**CHAPTER 4 (CSIS)**
Behavior (i.e. depressive-like, anxiety-like behaviors and cognitive impairments)	-	↓ Sucrose consumption (SPT) = ↑ Anhedonia	↑ Distance (OFT) and ↑ time mobile (NORT) = ↑ Arousal	↑ Distance (OFT)
Body weight	= body weight (increased in both)	↓ Weight gain (day 9) = weight (day 21)	↓ Body weight (Final-Basal)	-

(- = Not measured variable; ↑ = higher; ↓ = lower). The reference group for the expression of the data is the stressed group. Only significant differences ($p < 0.05$) are presented.

8.4. Tumor in female mice: effects on physiology and behavior (Table 4)

The presence of tumor cells had neuroendocrine and immune effects, but did not affect behavior nor body weight in female mice in study #3 (Díez-Solinska et al., 2022).

Tumor-bearing mice presented lower CORT levels as well as altered MR and GR levels in the HC and the HT, although they did not follow the same trend in both structures. While in the HC, the MR and the MR/GR ratio were higher, in the HT both receptors, the GR and the MR, were lower. These results suggest an effect of tumor on the HPA axis, possibly affecting its negative feedback by altering the expression levels of the receptors. The MR in the HC has been widely related to beneficial effects, including memory formation (Dorey et al., 2012; Groch et al., 2013), which would fit with what we found in the NORT, as stressed tumor-bearing mice showed a greater discrimination index than non-stressed non-tumor-bearing mice. However, as contradictory results have been found regarding MR's effects on cognition and its involvement in different pathologies (Gomez-Sanchez & Gomez-Sanchez, 2014), it cannot be concluded that the tumor is beneficial solely based on its association with the increase of MRs. In fact, the tumor affects the immune response by altering the expression levels of cytokines. In the HC, a reduction in the expression of IL-1β and IL-10 was observed, although no pro-inflammatory tendency was found in this structure probably due to a lower effect of IL-10. Contrary to this, in the ST, a pro-inflammatory imbalance could be observed, probably mediated by the decrease in IL-10. Additionally, tumor development also disrupts the balance of the CX3CL1/CX3CR1 axis differently in these brain regions. While in the HC, the

tumor reduces the ratio CX3CL1/CX3CR1 by increasing CX3CR1 and decreasing CX3CL1 expression levels; in the ST, the increase in both the receptor and the ligand did not alter the ratio. The decrease in CX3CL1 in the HC could indicate a loss of neuronal control of microglia in the HC (Ferretti et al., 2014; Limatola et al., 2005), which may explain the lack of a pro-inflammatory trend in this structure. In contrast, in the PFC, the ratio is increased probably due to the upregulation of CX3CL1 expression. These results show that the tumor has opposite effects on the analyzed structures. It is possible that, once more, the differences that exist on the basis of sexual dimorphism in microglial-induced inflammation (Han et al., 2021) are influencing the variability of the results found, so it would be necessary to study these chemokines in greater depth in females.

Table 4

Effects of tumor presence on physiological outcomes and behavioral traits (Chapter 3)

EFFECTS OF TUMOR ON PHYSIOLOGICAL AND BEHAVIORAL OUTCOMES	
	FEMALES
	CHAPTER 3 (CSIS)
Neuroendocrine parameters (i.e. CORT, GR, MR)	↓ Plasma CORT HC: ↑ MR ↑ MR/GR HT: ↓ GR, ↓MR
Immune system (i.e. cytokines and chemokines)	HC: ↓ IL-1β (↓pro- inflammatory cytokine) ↓ IL-10 (↓anti-inflammatory cytokine) ↑CX3CR1, ↓CX3CL1 ↓CX3CL1/CX3CR1 ratio ST: ↓ IL-10 (↓anti-inflammatory cytokine) ↑ IL-1β/IL-10 ratio ↑CX3CR1, ↑CX3CL1 PFC: ↑CX3CL1 ↑CX3CL1/CX3CR1 ratio
Behavior (i.e. depressive-like, anxiety-like behaviors and cognitive impairments)	= Behavior (OFT, NORT SPT, ST)
Body weight	= Body weight

(- = Not measured variable). The reference group for the expression of the data is the tumor-bearing group.

8.5. Behavioral traits in male and female mice: effects on physiology and behavior (Table 5)

Both male and female mice were divided regarding their differential intrinsic behavioral traits in order to examine if these differences could be interfering in the effects of chronic stress on physiological parameters and behavior. Given the existence of an ethogram established by Brain, McAllister, and Walmsley (1989), and subsequently modified by Vegas et al. (2006), in study #2, males were categorized according to these behaviors and their occurrence frequency (Díez-Solinska et al., 2024). However, due to the lack of a similar ethogram for females, and considering the previously mentioned sociable, non-territorial, and less aggressive nature of females compared to males, in study #4, behavioral differences in females were assessed based on their social interactions with other females (Díez-Solinska et al., 2023). Thus, male mice were divided into AA and PR mice, while females were divided into High Sociable (HS) and Low Sociable (LS).

Regarding the neuroendocrine aspects, in the study #2, the difference in CORT levels between stressed and non-stressed groups was not dependent on the coping strategy employed, meaning that both, AA and PR mice showed no differences in CORT levels in none of the measured CORT points (basal, day 9 and day 21) (Díez-Solinska et al., 2024). According to this, PR mice do not appear to have a less adaptive or efficient response with a more pronounced physiological alteration when compared to AA mice, as it has been suggested in other studies (Ballestín et al., 2021; Hawley et al., 2010; Russo et al., 2012; Wood & Bhatnagar, 2015). Indeed, it could be expected that learned

helplessness may occur in situations of stress that cannot be modified by the individual. In such cases, the passive coping strategy would turn even more adaptive and of greater benefit than an active strategy, which could be more energy consuming with no reward in return. This would be consistent with the statement that there is no absolute rule about the appropriateness of one strategy or the other, but rather that it is the specific circumstance that determines its appropriateness (Dingemanse & Wolf, 2010). In study #2, although exposure to chronic stress had effects on the HPA axis in male mice, differences regarding coping remain unclear. Frequently, an active coping strategy has been linked to a lower HPA axis response (Koolhaas, 2008; Koolhaas et al., 2007), while a passive coping has been related to higher CORT levels (Gómez-Lázaro et al., 2012; Pérez-Tejada et al., 2013). However, not all studies support this finding (Hodes et al., 2014; Jochems et al., 2015; Murra et al., 2022), and thus, study #2 follows the same trend of the later studies. Contrary to findings in males, where differences in stress coping behavior were not associated with CORT levels, in study #4, the degree of sociability in females had an effect on both plasma CORT levels and hippocampal MR levels, increasing both in highly sociable females. These higher levels of CORT and increased MRs suggest that sociability upregulates the HPA axis. These findings are contrary to what is generally found in the literature, where attachment and social interaction can modulate stress response (Carter, 1998), both in humans, where social support reduces cortisol when facing a psychosocial stressor (Kirschbaum et al., 1995; Thorsteinsson & James, 1999), and in animals (Sachser et al., 1998). However, the benefits of sociability may be limited to species where animals live in social groups. Therefore, given the nature of the

stress to which females were subjected, i.e. CSIS, it could be hypothesized that sociability did not serve as a protective factor for them, leading to higher reactivity of the HPA axis and higher levels of CORT. However, it is worth mentioning that, basal submandibular blood was not collected from females in study #4 in order to avoid a potential interference of animal handling with subsequent SIT administration, which determined females' high or low sociability. Consequently basal CORT levels were not assessed. This limitation prevents us from knowing whether the CORT levels of HS females were already higher at baseline or if the increase in these levels was the result of chronic stress exposure.

In terms of brain neurochemistry, although different studies have found that dopaminergic, serotonergic and noradrenergic activity are related to behavior, such as levels of aggression (Nelson & Trainor, 2007), no significant differences were found in either monoamines or their metabolites as regards of coping in male mice in study #2. Both, AA and PR mice exhibited differences in specific monoamines in the PFC and in some of the ratios in both the HC and the PFC when compared to the control group. Contrary to other studies in which mice with a passive coping strategy presented lower NA (Pérez-Tejada et al., 2013), in study #2, only the NA/Tyr ratio was significantly different between AA and PR animals. Passive-reactive mice showed a higher NA/Tyr ratio, which could suggest an increased noradrenergic activity of this group in the PFC when compared to AA mice. Indeed, PR mice also showed higher NA levels compared to the control group in this brain structure, which would support the idea of an increased noradrenergic activity, that could be interpreted as a physiological response to a chronic state of stress. The increase of NA is usually related to neuroinflammation (Feinstein et al., 2016) and, although in study #2

inflammatory factors were not measured, other studies suggest that CSDS in male mice can increase pro-inflammatory cytokine levels in passive male mice (Gómez-Lázaro et al., 2012; Joana et al., 2016). These findings would align with the increased NA levels in the PFC in PR mice. In study #4, similar findings were observed indicating that sociability in females failed to exert a significant impact on brain neurochemistry. High sociable mice exhibited lower 3-HK and NA levels in the HC and the ST, respectively. This could indicate that LS female mice exhibit a similar trend to PR male mice by showing higher NA levels and thus, an increased noradrenergic activity. On the other hand, the lower levels of 3-HK in the HS females could suggest that, despite an increase in IDO levels, Kynurenine is not being metabolized into 3-HK, which is more toxic than the Kyna pathway. Thereby, a trend towards a less neurotoxic profile could be deduced HS females compared to LS females, which may become more evident over time.

Regarding behavior, and similarly to what reported by other studies (Goñi-balentziaga et al., 2020), in study #2, PR male mice exhibited higher immobility and lower distance travelled and swimming in the OFT and the FST respectively. Therefore, PR mice presented a similar behavior to the behavior they displayed during the behavioral assessment in the first social interaction, in which AA mice showed higher exploration and mobility compared to PR mice. These results suggest that male mice behavior remains stable over time regardless the CSDS exposure. However, despite these differences in terms of mobility, it cannot be concluded that stressed mice exhibited depressive or anxiety-like behaviors according to the coping strategy used. This absence of depressive- and anxiety-like behaviors is evidenced by the absence of anhedonia in the SPT and in the

absence of thigmotaxis in the OFT respectively. In females, by contrast, no differences were observed regarding sociability. These findings in study #4 are of particular interest, as it might be hypothesized that females exhibiting low sociability, which could be interpreted as social withdrawal, would display depressive-like behaviors, as this is a main characteristic of depression (American Psychiatric Association, 2013). Indeed, previous studies have associated reduced interaction time with a stranger mouse with depressive symptoms (Kaidanovich-Beilin et al., 2011). However, results in study #4 suggest that lower sociability does not inherently predispose female mice to heightened susceptibility to display depressive-like behaviors. Similarly, no advantage in anxiety-like behaviors nor in cognitive impairments was observed among either more or less sociable females. Regarding HS females, and based on a recent research that found that oral CORT administration to females may result in reduced sociability (Berger et al., 2019), it could be suggested that elevated CORT levels in HS females may have diminished their sociability levels post-experimental procedure, which is related to depressive symptoms. Nonetheless, given the lack of sociability assessment after CSIS, alongside the absence of behavioral and cognitive alterations in the OFT and the NORT, it could be ruled out that HS females are more susceptible to developing depression following CSIS, despite their higher elevated CORT levels, which have been related to depressive symptoms in male mice (Zhao et al., 2008).

Therefore, although some studies have clearly linked active coping with resilience (Murra et al., 2022) and passive coping as being a more dysfunctional coping style (Cabib et al., 2021; Wood & Bhatnagar, 2015), according to the results obtained in study #2, it is not possible to reach to that conclusion. None

of the coping strategies proposed make an individual more resilient or more susceptible to developing stress-related mood disorders. Neither high nor low sociability assessed in study #4 could be associated with greater resilience or vulnerability in females. Therefore, for both male and female mice, it would be necessary to analyze behaviors in a more specific and precise way in order to build a more accurate behavioral profile. This could allow us to explore whether there are specific behavioral traits that may make individuals more or less vulnerable to physical or mental illness.

Table 5

Effects of behavior (coping in males and sociability in females) on physiology and behavior after CSS exposure (Chapters 2 and 4)

EFFECTS OF INTRINSIC BEHAVIOR ON PHYSIOLOGICAL AND BEHAVIORAL OUTCOMES		
	MALES	**FEMALES**
	CHAPTER 2 (CSDS)	**CHAPTER 4 (CSIS)**
Behavioral traits (i.e. coping strategies in males and sociability in females)	Behavioral assessment during first social interaction (most displayed behaviors): AA: During DPI: + exploration from a distance, non-social exploration and threat. During NPI: non-social exploration PR: During DPI: + Immobility. During NPI: + Immobility	Behavioral assessment before CSIS through SIT (day -1) (displayed social behaviors): HS: + social interaction received and emitted LS: + non-social exploration
Neuroendocrine parameters (i.e. CORT, GR, MR)	= Plasma CORT regarding coping	(HS/LS) ↑ Plasma CORT (HS/LS) ↑ MR in HC
Neurochemical parameters (monoamines and their metabolites)	HC: Catecholamines: = regarding coping Indolamines: (AA/C) ↓ Kyn/tryp ratio PFC: Catecholamines: (PR/C) ↑ NA (PR/AA & C) ↑ NA/tyr Indolamines: (AA/C) ↓ Kyn, ↓ Kyn/tryp ratio	HC: Catecholamines: = regarding coping Indolamines: (HS/LS) ↓ 3-HK ST: Catecholamines: (HS/LS) ↓ NA Indolamines: = regarding coping
Immune system (i.e. enzymes)	-	HC: (HS/LS) ↑ IDO
Behavior (i.e. depressive-like, anxiety-like behaviors and cognitive impairments)	(PR/AA & NS) ↓ distance, ↑ time immobile (OFT) (PR/AA & NS) ↓ swimming, ↑ time immobile (FST)	= Behavior (OFT, NORT and SPT)
Body weight	(AA/NS) ↓ weight gain (day 9) = Weight (day 21) regarding coping	-

(- = Not measured variable; = higher; = lower DPI = Direct physical interaction. NPI = Non-physical interaction). The reference group for the expression of the data is the group before the forward slash (/) compared to the group or groups after it. Only significant differences ($p < 0.05$) are presented.

9. CONCLUSIONS

The following conclusions address the specific objectives and hypotheses of each of the studies presented.

Study #1

1. Chronic social isolation in male mice resulted in a physiological response commonly associated with stress, thereby confirming itself as a source of chronic stress.
2. The physiological activation presented by stressed mice normalized by the end of the stress procedure, which suggests a habituation to isolation.
3. Stressed mice exhibited an immunosuppressed profile after chronic isolation.

Housing condition is important when considering stress outcomes in male mice. Isolation has shown deleterious effects of on mice's physiology, which suggests that this condition may interfere with the physiological measurements of a study.

Study #2

1. Male mice submitted to CSDS exhibited a heightened physiological reactivity during and after stress procedure, as well as a distinct dysregulation on catecholamine and indolamine levels in different brain structures.

2. In addition to the physiological response, stressed mice displayed depressive-like behavior.
3. When exposed to a social challenge, male mice exhibit distinct behavioral profiles. Individual differences emerge in the way individuals cope with the situation. The behavioral profiles can be classified in two different coping strategies: active-aggressive (characterized by heightened exploration and threatening behaviors) and passive-reactive (characterized by increased immobility).
4. Coping strategies remain stable over chronic stress exposure. However, mice did not exhibit a differential physiological and behavioral response to stress based on the coping strategy adopted. Only minor differences were observed in catecholamine and indolamine levels.

Chronic Social Defeat Stress was found to induce neuroendocrine, neurochemical and behavioral effects on male mice. However, contrary to what was expected, coping strategies did not exert a significant influence on the physiological effects or behavior.

Study #3

1. Chronic Social Instability Stress leads to a heightened stress response in female mice.
2. Female mice submitted to chronic stress exhibit an inflammatory trend. However, given the downregulation in both pro- and anti-inflammatory cytokines, the presence of an inflammatory profile cannot be conclusively affirmed.

3. Stressed female mice show an altered neuron-microglia interaction consistent across the analyzed brain structures.
4. Chronic Social Instability Stress did not result in a greater tumor development in female mice.
5. Female mice submitted to stress showed higher arousal, although no depressive-like nor anxiety-like behaviors were observed. Cognitive impairments were also not found.
6. Tumor development results in neuroendocrine and immune alterations, although it does not induce the expected sickness behavior in females.

Chronic Social Instability Stress leads to a differential physiological response when comparing stressed and non-stressed females. However, these alterations did not cause stressed mice to have a more pronounced tumor progression, nor did it induce depressive-like or anxiety-like behaviors. Thus, while CSIS model induces physiological changes, these are not strong enough to foster effects on physical or behavioral disorders development.

Study #4

1. Female mice submitted to CSIS exhibit a physiological stress reactivity as well as a distinct dysregulation in brain neurochemistry in the analyzed brain structures.
2. Chronic Social Instability Stress did not cause females to display depressive-like nor anxiety-like behaviors. Additionally, they did not present cognitive alterations.
3. Female mice show intrinsic behavioral differences prior to the exposure to a stressor. Considering the social interactions with their conspecifics,

females can be categorized into highly sociable (with more social interactions received and emitted) and low sociability (display more non-social exploration).

4. High or low levels of sociability do not determine how female mice cope with chronic social instability stress. Only minor physiological differences were found, with no behavioral differences.

Again, CSIS lead to a differential physiological response when comparing stressed and non-stressed females. Differences in the neuroendocrine system, neurochemical response and immune system were encountered. However, these alterations did not result in behavioral nor cognitive impairments. Additionally, sociability did not determine more resilience or vulnerability to CSIS exposure.

General Conclusion

Based on these findings, CSS can alter physiological and behavioral responses in males and females, and both, coping strategies and sociability, are promising factors when studying these responses. Both stressed males and females have developed distinct physiological responses and behavioral phenotypes compared to their non-stressed counterparts. Additionally, when comparing males and females, differences were also observed. However, given the methodological differences between the followed procedures and given the lack of consistency in the parameters evaluated across studies, stable conclusions cannot be drawn regarding the comparison between studies. Moreover, coping strategies and sociability, appear to modulate some of the measured physiological parameters in response to stress, although they do not have a notable influence on depressive-like nor anxiety-like behaviors. This could

suggest that either the nature or the duration of the stress models employed, or the behavioral traits analyzed, have not been sufficient to identify robust differences in stress response regarding behavior. Thereby, a more comprehensive exploration of behavior should be considered in order to reliably study the effects of behavior in the individual response to stress.

Therefore, not only studying females, but also individual differences is crucial, and efforts should be made to comprehend female-specific physiological and behavioral responses, as well as individual variability.

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ANNEX

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Mediante este escrito los Doctores Oscar Vegas Moreno (NIF 14608879S) y Garikoitz Azkona Mendoza (NIF 44171143B), como directores de la tesis doctoral de la doctoranda Alina Isabel Díez Solinska (NIF 71363611Q), certifican que la tesis doctoral titulada "Effects of Chronic Social Stress on neuroendocrine and neurochemical function, immune response and behavior: insights from experimental models in male and female mice" cumple con las condiciones para ser realizada a través de la modalidad de compendio de publicaciones.

La tesis incluye cuatro artículos publicados, todos en revistas científicas que aparecen en las bases de datos de Journal Citation Reports (JCR). Los cuatro artículos publicados están en el primer o segundo cuartil de sus respectivas categorías. En todos los casos, la doctoranda es la autora principal y, además, su trabajo está en línea con su Plan de Investigación. A continuación, las referencias de los artículos que componen la tesis, con sus respectivos DOI:

Díez-Solinska, A., Lebeña, A., Garmendia, L., Labaka, A., Azkona, G., Perez-Tejada, J., & Vegas, O. (2022). Chronic social instability stress down-regulates IL-10 and up-regulates CX3CR1 in tumor-bearing and non-tumor-bearing female mice. *Behavioural Brain Research*, *435*, 1–12. https://doi.org/10.1016/j.bbr.2022.114063

- <u>Indicadores de Calidad (2022):</u>

  Revista: Behavioural Brain Research

  Factor de Impacto de la Revista: 2.7

Categoría: Behavioral Sciences

Cuartil: Q2

Posición: 20/52

Díez-Solinska, A., Azkona, G., Muñoz-Culla, M., Beitia-Oyarzabal, G., Goñi-Balentziaga, O., Gómez-Lazaro, E., & Vegas, O. (2023). The role of sociability in social instability stress: behavioral, neuroendocrine and monoaminergic effects. *Physiology and Behavior*, *270*, 1–15. https://doi.org/10.1016/j.physbeh.2023.114306

- Indicadores de Calidad (2022):

  Revista: Physiology and Behavior

  Factor de Impacto de la Revista: 2.9

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  Cuartil: Q2

  Posición: 17/52

Díez-Solinska, A., Ortega-Saez, I., Grífols, R., Martí, C., Zamora, C., Muñoz-Culla, M., Vegas, O., & Azkona, G. (2023). Individualized housing modifies the immune – endocrine system in CD1 adult male mice. *Animals*, *13*(6), 1–12. https://doi.org/10.3390/ani13061026

- Indicadores de Calidad (2022):

  Revista: Animals

  Factor de Impacto de la Revista: 3.0

  Catategoría: Veterinary Sciences

  Cuartil: Q1

  Posición: 16/144

Díez-Solinska, A., Goñi-Balentziaga, O., Beitia-Oyarzabal, G., Muñoz-Culla, M., Vegas, O., & Azkona, G. (2024). Chronic defeat stress induces monoamine level dysregulation in the prefrontal cortex but not in the hippocampus of OF1 male mice. *Behavioural Brain*

Research, 1–28. https://doi.org/10.1016/j.bbr.2024.115023

- Indicadores de Calidad (2022):

  Revista: Behavioural Brain Research

  Factor de Impacto de la Revista: 2.7

  Catategoría: Behavioral Sciences

  Cuartil: Q2

  Posición: 20/52

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