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Quality and chronicity of stressors differentially affect psychoneuroendocrine and immune system cross-talk in mouse models

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"*Everybody knows what stress is and nobody knows what it is. The word stress, like success, failure, or happiness, means different things to different people and, except for a few specialized scientists, no one has really tried to define it although it has become part of our daily vocabulary. It is effort, fatigue, pain, fear, the need for concentration, the humiliation of censure, loss of blood, or even an unexpected success that requires complete reformulation of one's life? The answer is yes and no. That is what makes the definition of stress so difficult. Every one of these conditions can produce stress, and yet none of them can be sigled out as being "it" since the word applies equally to all others as well.*"

Hans Selye, 1973, American Scientist, p.692

CONTENTS

ABSTRACT

Stress physiology overlaps to a great extent in humans and mice, thus preclinical models can guide and refine hypothesis-driven clinical research questions. The brain is a primary mediator and target of stress resiliency and vulnerability because it determines what is threatening and regulates behavioural and physiological responses to a given stressor. A growing body of evidence suggests that the sustained activation of the HPA axis, and the consequent suppressed activity of the immune system mediated by glucocorticoid hormones, may increase the susceptibility to disease. The connection between central stress response pathways and peripheral targets involves a number of neurochemical and/or inflammatory factors that ultimately affect neuronal functioning and/or survival as well as adaptive physiological responses that allow fighting against external challenges. In this regard Brain-Derived Neurotrophic Factor (BDNF) represents a crucial factor for the integration of neural, immune, endocrine and metabolic responses to stressful challenges. However, the quality and quantity of stress, and the mechanisms leading to increased susceptibility to disease, are still a matter of intense investigation. The **general objective** of the work presented in this thesis was to characterise how quality and timing of stressors differentially impact on behavioural, neuroendocrine and immune profile in animal models. **To this aim** both central (BDNF levels) and peripheral (CORT, cytokines, splenic apoptosis, leptin and adiponectin levels) responses, as well as endophenotype of depression, were analyzed in mouse models. In particular, in **Chapter 2**, we report about the impact of social deprivation on anxiety and depressive-like behaviours, promoting a disrupted emotional state, while in **Chapter 3** we identify a specific neuroendocrine-immune profile associated to specific changes in central mediators, describing the relevance of the nature and length of stress exposure. Both these studies relied on the assessment of BDNF as the principal mediator in the orchestration of brain and peripheral responses. **Chapter 4** describes research in which both psychophysical and social stressors promote disease in a transgenic mouse model of breast cancer, through a specific neuroendocrine-immune-leptin axis. Analyses of the effects of stressful events in wild type and transgenic mice – described in **Chapter 2**, **3** and **4** - highlight the importance of individual responses to environmental stimuli in defining the outcome of disease. Results are discussed considering that stressful life events are related to a range of physical and emotional health problems and reduced quality of life, social support being a crucial protective factor in disease susceptibility.

CHAPTER 1

1. GENERAL INTRODUCTION

1.1. STRESS IS A RISK FACTOR FOR PSYCHONEUROENDOCRINE REGULATIONS

1.1.1. Stress definition - What is stress?

Stress can be described as any change in the external or internal environment perturbing the homeostasis of an organism. Selye in 1936 described the "general adaptation syndrome" as a complex mechanism of activation of the neuroendocrine system to prepare the body to respond to external challenges. Stress begins in the brain with the perception and elaboration of an external event as stressful and affects the brain itself, as well as the rest of the body, through plastic changes, leading to adaptation (McEwen and Seeman, 1999).

Coping strategies, reflected in adaptive physiological changes, are therefore important components of the stress response. Thus, during stress, attention is enhanced and the brain focuses on the perceived threat, cardiac output and respiration are accelerated, catabolism is increased and blood flow is redirected to provide the highest perfusion and fuel to the aroused brain, heart and muscles to allow an animal to “fright, fight or flight” (Chrousos and Gold, 1992). The connection between central stress response pathways and peripheral targets involves the activation of a number of neurochemical and/or inflammatory mediators that ultimately can affect cell function and/or survival including cytokines and growth factors (Hayley et al., 2005; Cirulli and Alleva, 2009; Moreno-Smith et al., 2011; Capoccia et al., 2013).

The perception of stress is influenced by experiences, genetic background and behaviour. It must be taken into account that there are significant individual differences in stress perception, processing, appraisal and coping, based upon the experience of the individual (Gunnar and Quevedo, 2007; Dhabhar, 2008). Positive or negative experiences at school, work or in interpersonal relationships can prejudice an individual towards either a positive or negative response in a new situation. How the individual reacts may carry over into habits such as smoking, drinking excessively, eating too much, poor sleep, lack of exercise and interaction with friends and family, all of which contribute to allostatic overload (McEwen and Seeman, 1999).

The consequences of stress responses are generally adaptive insofar as they extend for a short time, but can become very harmful when stress is chronic and prolonged (Munck et al., 1984; Dhabhar and McEwen, 1997; McEwen and Seeman, 1999). In this context, it is important to distinguish the duration and intensity of the stress. Acute stress lasts for a period ranging from minutes to hours, while chronic stress persists for days to months. Stress can be quantified by measuring the levels of stress hormones and neurotransmitters, as well as evaluating the extent of concurrent physiological changes, such as increased heart rate and blood pressure. In this regard, the central nervous system (CNS), the hypothalamic-pituitary-adrenal (HPA) axis and also the

immune system (IS) act leading to protection and adaptation of the organism to environmental challenges (allostasis) (Kort, 1994; Kiecolt-Glaser et al., 1996; Avitsur et al., 2009). However, chronic dysregulation (i.e., over-activity or inactivity) of these physiological systems promotes the persistent elevation of stress mediators and the consequent allostatic load (McEwen and Gianaros, 2010).

1.1.2. Stress effects on neuroendocrine function

The neuroendocrine system is pivotal to the allostatic/adaptive responses to stress, employing neuropeptides and hormones as mediators. The HPA axis (which releases the glucocorticoids - GC - hormones) and sympathetic adrenomedullary systems (which releases catecholamines) coordinate the stress response in brain and periphery. They act as integrating units controlling the physiological and behavioural adaptive responses necessary for an organism to cope with stress (in a time-limited fashion) and their functioning is, in turn, affected by stress.

An important signal for the deleterious effects of chronic stress is the disruption of the circadian rhythm of corticosterone in rodents (Dhabhar and McEwen, 1997) and the rhythm of cortisol in humans (Sephton et al., 2000).

Under basal conditions the HPA axis coordinates daily and sleep related events through the secretion of corticotrophin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and GC (Windle et al., 1998; Young et al., 2004). The amplitude of this ultradian adrenal GC rhythm is enhanced during the activity period (the dark phase in rodents). Amplitude and frequency can change during disease and the aging process (Lightman et al., 2008). Activation of the HPA axis, by either psychological or physiological threats, can occur at any time and results in the release from the hypothalamus of the neuropepetides CRH and arginine-vasopressin (AVP) into the hypophyseal portal blood system. The combined action of CRH and AVP stimulates the anterior pituitary gland to secrete peptides derived from the proopiomelanocortin transcript, which includes ACTH; this hormone, acting on the cortex of the adrenal glands, eventually promotes the synthesis and secretion of the GC, the main hormones of stress (Engler et al., 1989; Rivier and Rivest, 1991). Glucocorticoids regulate the secretion of CRH, AVP and ACTH through negative feedback actions on the brain and anterior pituitary gland (Canny et al., 1989). In humans and many mammalian species, the predominant GC is cortisol, whereas in rodents and birds the key GC is corticosterone.

GC are steroid hormones which have both protective and damaging effects on the organism. In the short time they are essential for adaptation, maintenance of homeostasis and survival. Yet, over longer time intervals, they impose a cost (allostatic load) that can accelerate disease processes or participate to pathological changes ranging from immunosuppression, obesity and atherosclerosis (McEwen, 2000b).

Glucocorticoids exert action in the brain via high affinity Type I mineralocorticoid receptors (MR) and low affinity Type II glucocorticoid receptors (GR) (De Kloet et al., 1998). In the brain MR are located in the hippocampus, a region involved in memory formation and retention, and other regions of the limbic system such as lateral septum, amygdala and hypothalamus, while GR have a more extensive distribution within the brain, being also present in the hippocampus, and most abundant in the hypothalamus and in pituitary corticotrophs. At basal levels, GC predominantly bind to MR and only slightly occupy GR. In addition, MR are responsible for much of the effects of basal and low-stress levels of GC at the onset of the stressors, whereas GR largely mediate the effects of high-stress levels of GC, facilitating the re-establishment of homeostasis when stress levels of GC prevail (De Kloet et al., 1998; De Kloet, 2004). Alterations in the effectiveness of negative feedback by GC have profound effects on the activity of the HPA axis and regulation of stress responses, such that aberrant action can increase the vulnerability of the individual to stress-induced disorders or disease (De Kloet et al., 1998). There is also evidence that changes in the effectiveness of negative feedback by GC contribute to attenuate HPA axis responses to stress in some physiological states, thereby resulting in stress hyporesponsiveness.

1.1.3. Stress effects on immune system response

The communication between the central nervous system and the immune system occurs via a complex network of signals linking the nervous, endocrine and immune systems (Glaser and Kiecolt-Glaser, 2005b). The field of psychoneuroimmunology (PNI) has recently produced new insights that help understanding the pathophysiological processes resulting from disrupted immune system function. Works in this field have established that psychological stress disturbs the functional interaction between the nervous and immune systems (Glaser and Kiecolt-Glaser, 2005a; Webster Marketon and Glaser, 2008; Kiecolt-Glaser et al., 2010; Yang et al., 2010), altering some signals, such as cytokines, chemokines and modifying macrophages physiological functions (Yang and Glaser, 2002; Webster Marketon and Glaser, 2008; Yang et al., 2010). In this context, there are two main sources of variation that can influence immune system activity and lead to susceptibility to disease. These are mainly linked to the characteristics of the individual subject (gender, age and species) and to the nature of the stressor itself (intensity, duration and perception).

In order to understand the relationship of psychosocial stressors to the immune system, it is useful to distinguish between innate and acquired immunity (Segerstrom and Miller, 2004). Innate immunity is an immune response characteristic of vertebrates and invertebrates, as well as plants, and the basic mechanisms that regulate its functioning are highly conserved. Cells involved in the innate immunity represent the first line of host defense during infection and, therefore, play a crucial role in the early recognition to destroy

the invading pathogens (Medzhitov and Janeway, 2000; Mogensen, 2009). The cells involved in natural immunity include: mast cells, eosinophils, basophils and the phagocytic cells including macrophages, neutrophils, and dendritic cells, and function within the immune system by identifying and eliminating pathogens that might cause infection. More in detail, these cells assemble at the site of injury or infection, release toxic substances, such as oxygen radicals, that damage invaders, and phagocytose both invaders and damaged tissue. Another cell involved in natural immunity is the natural killer cell. Natural killer cells recognize the lack of a self-tissue molecule on the surface of target cells (characteristic of many kinds of virally infected and some cancerous cells) and induce their direct cytotoxicity (Chan et al., 2014). These responses result important in limiting the early phases of viral infections, before specific immunity becomes effective (Andrews et al., 2003; Andoniou et al., 2006), and in attacking self-cells that have become malignant (immune surveillance of tumors) (Smyth et al., 2000; Smyth et al., 2001).

The acquired immune system, composed of T and B lymphocytes, is characterised by memory or secondary antigen-specific immune responses (Taniguchi et al., 2003). These cells are produced in hematopoietic organs, such as the bone marrow, spleen and thymus (Abbas et al., 2014). T lymphocytes play an important role in cell-mediated immunity. They can be distinguished from NK cells and B lymphocytes by the presence of the T cell receptor on the cell surface (Webster Marketon and Glaser, 2008). T lymphocytes can be divided into subgroups: T-helper cells, T-cytotoxic cells, and T-regulatory cells. The main function of T-helper cells is to produce cytokines that direct and amplify the rest of the immune response. T-cytotoxic cells recognize antigen expressed by cells that are infected with viruses or otherwise compromised (e.g., cancer cells) and lyse those cells. T regulatory cells produce anti-inflammatory cytokines that suppress or downregulate induction and proliferation of effector T cells. B cells are involved in humoral immunity and produce soluble proteins called antibodies that can perform a number of functions, including neutralizing bacterial toxins, binding to free virus to prevent its entry into cells, and opsonization, in which a coating of antibody increases the effectiveness of natural immunity (Segerstrom and Miller, 2004).

The transition from innate to adaptive immunity is mediated by cytokines, inflammatory molecules, that are released by macrophages, and lymphocytes, although they can also be produced by polymorphonuclear leukocytes (PMN), endothelial and epithelial cells, adipocytes, and connective tissue (Butterfield et al., 2006; Arango Duque and Descoteaux, 2014). Cytokines can act in an autocrine, paracrine or endocrine fashion and can be subdivided in to proinflammatory (Th1) and antiinflammatory (Th2) cytokines, which are produced by type 1 and type 2 helper T cells respectively (Webster Marketon and Glaser, 2008). The proinflammatory cytokines include IL-1, IL-2, IL-6, IFNγ and tumor necrosis factor α (TNFα) whereas antiinflammatory cytokines include IL-4, IL-5, IL-10 and IL-13.

Th1 responses result critical for viral clearance, whereas Th2 cells are important for immune responses to parasites in mice and in humans (Romagnani, 1997; Maldonado-Lopez and Moser, 2001). Th1 and Th2 cells can be cross-inhibitory and the balance between Th1 and Th2 cell function or cytokine production may be altered by stressor administration (Moynihan, 2003). Cytokines are the main messengers used by the immune system to communicate directly with the neuroendocrine system. In particular, they stimulate the secretion of hypothalamic CRH and activate the HPA axis, but this effect is blocked by glucocorticoids (Rivest, 2001; Webster et al., 2002; Rhen and Cidlowski, 2005). In this context, chronic exposure to GC inhibits both innate and adaptive immunity reducing circulating leukocyte counts and decreases the production of a large variety of proinflammatory cytokines including IL-1β and TNF-α, forming a negative feedback loop. Dysregulation of this neuroendocrine loop by hyperactivity or hypoactivity of the HPA axis causes systemic changes in inflammation and immunity, increasing susceptibility to infections and cancer (Saul et al., 2005; Dhabhar et al., 2012; Dhabhar, 2014).

1.1.4. Stress modulates brain plasticity through neurotrophins: focus on BDNF

Brain plasticity refers to the brain's ability to respond and adapt to environmental challenges and encompasses a series of functional and structural mechanisms that may lead to neuronal remodelling, formation of novel synapses and birth of new neurons. However, in a broader sense, neuronal plasticity is intimately linked to cellular responsiveness and may therefore be considered as an index of the neuronal capability to adapt its function to a different demand. Failure of such mechanisms might enhance the susceptibility to environmental challenges, such as stress, and ultimately lead to psychopathology. That is to say that the brain, or more specifically, some brain structures or circuits, as a consequence of stress, may become more vulnerable by loosing progressively (or suddenly) the ability to adapt to challenges and maintain their homeostasis.

According to the concept of allostasis and allostatic load, the duration of a stressful experience represents a critical factor in the modulation of brain plasticity (McEwen, 2000a). Indeed the same mediators or system involved in the adaptation to acute challenges, can also participate in pathological effects determined by prolonged repetitive exposure to stressful conditions. In this context, many studies have been focused on two limbic brain regions involved in the neuroendocrine response: the hippocampus, which has high levels of GR and has connections with the amygdala and prefrontal cortex, regions that are more directly involved in emotion and cognition, and the hypothalamus, which represents the final common pathway integrating different stress inputs. For example, a mild stress can enhance learning and memory within hippocampus

(Luine et al., 1996) and can activate hypothalamic neurons (Herman et al., 2008), whereas chronic or severe stressors have detrimental effects leading to neuronal atrophy (Sapolsky, 2003; Herman et al., 2008).

Within this context, neurotrophic factors (NTFs) may play a central role, since they are necessary for the normal development, survival, and plasticity of neurons. More in detail, besides their classical role in supporting neuronal survival, NTFs finely modulate all the crucial steps of network construction, from neuronal migration to experience-dependent refinement of local connections (Poo, 2001). These functions were first reported based on the observation that during the development of the nervous system neuron survival depends on the limited amount of specific NTFs secreted by target cells (Huang and Reichardt, 2001). However, it is now well established that NTFs are important mediators of neuronal plasticity also in adulthood where they modulate axonal and dendritic growth and remodelling, membrane receptor trafficking, neurotransmitter release, synapse formation and function (Lu et al., 2005).

The neurotrophin Brain-derived Neurotrophic Factor (BDNF) is a member of the neurotrophin family that has an important role in development as well as in adult neuronal plasticity and is abundant in lymbic regions that are particularly relevant for mood modulation (Castren et al., 2007).

Modulation of BDNF by stress was originally shown several years ago (Smith et al., 1995). Since then, evidence has been produced demonstrating the complex outcome of stress on the BDNF system. Acute stressors can produce a significant and transient increase of BDNF mRNA levels in the rat (Marmigere et al., 2003) as well as in the prefrontal and cingulate cortex of rats (Molteni et al., 2001) and in the frontal lobe of mice (Molteni et al., 2009) exposed to an acute restraint stress. If the rapid increase of BDNF expression following a single stress may represent a short-term protective mechanism (Rage et al., 2002; Bramham and Messaoudi, 2005; Chao et al., 2006), a prolonged stressful experience may have a detrimental effect on neuroplasticity, because it reduces BDNF expression in limbic regions (Smith et al., 1995; Vollmayr et al., 2001; Roceri et al., 2002; Nair et al., 2007).

Interestingly, BDNF may represent an important element in order to understand the relationship between stress and behavioural changes associated with depression (Castren et al., 2007; Schmidt and Duman, 2007; Cirulli and Alleva, 2009). Specifically, BDNF exerts a main role in the etiopathogenesis of mood disorders since its down-regulation precipitates behaviours associated with anxiety and depression like-behaviours, such as increased behavioural despair in the forced swim test and decreased sucrose preference, a measure of anhedonia, following stress exposure (Snyder et al., 2011).

1.1.5. Animal models of stress and behavioural coping strategies

The development of animal models of stress has led to greater understanding of the role of different stressors and their timing on physiological and behavioural regulations.

Restraint stress results one of the most commonly employed procedures to induce stress-related behavioural, biochemical and physiological changes in laboratory animals, since immobilization models produce an inescapable physical and psychological stress with a low rate of adaptation (Kasuga et al., 1999). The restraint procedure consists in removing subjects from their home cage and putting each of them in a conical 50mL falcon tube, provided with holes for breathing, on a laboratory bench under dim light. After immobilization stress, animals exhibit higher levels of anxiety in the elevated plus maze and other tests of anxiety (Padovan and Guimaraes, 2000; Campos et al., 2010).

As for social stressors, they are effective in triggering powerful physiological and, more importantly, emotional responses in social animals, such as rats and mice (Kudryavtseva et al., 1991; Sheridan et al., 2000; Bartolomucci et al., 2005; Fuchs, 2005; Miczek and de Wit, 2008; Vialou et al., 2010). All mammals interact with other individuals. In the wild, rodents may encounter competition for resources such as territory, food, and access to mates, and even solitary species interact with conspecifics and their chemical cues, if only to avoid them in the future. Both aversive and positive interactions are relevant features of the social environment. Models of social stress used in rodents include social subordination, isolation, and disruption of social hierarchy (Blanchard et al., 2001; Chida et al., 2005; Avitsur et al., 2009). Indeed, in the highly social, group-living rodent species, social competition takes place and specific hierarchies are present that allow discriminating dominant from subordinate subjects (Bartolomucci et al., 2001; Blanchard et al., 2001). In this context, social stress can be obtained disrupting the social structure, for example, by replacing one mouse with a novel unfamiliar subject (Avitsur et al., 2003; Avitsur et al., 2009).

At the opposite extreme, solitary housing can be a potent stressor for highly social species such as rodents (Heinrichs and Koob, 2006). In both rats and mice, extended (weeks-months) solitary housing produces an "isolation syndrome", consisting of hyperadrenocorticism, reduced body weight, altered blood composition, and enhanced pain responsiveness, among other outcomes (Hatch et al., 1965; Valzelli, 1973). These changes coincide with alterations in behaviour including increased aggression, reduced mating behaviour, impaired learning and increased pain sensitivity (Valzelli, 1973).

Within a species, the capacity to cope with environmental challenges largely determines individual survival in the natural habitat. The success of the coping behaviour can be measured by its effectiveness in reducing physiological measures of stress or by its effectiveness in removing an adverse situation. Active coping strategies are used when escape from threat is possible, and the autonomic changes associated with these active strategies are mediated

predominantly by sympathetic activation (hypertension, tachycardia). This is the fight-or-flight response originally described by Cannon (Chrousos and Gold, 1992). Passive coping strategies, such as immobilization or freezing, are usually elicited when threat is inescapable, and are usually characterised by autonomic inhibition (hypotension, bradychardia), and a more pronounced increase in the neuroendocrine response (activation of the HPA axis and increased GC secretion). Under natural conditions, immobility is a highly selected response because movement makes the rodents more detectable to predators and because predators are much more likely to attack moving than still prey (Bolles and Fanselow, 1980). This type of passive response was originally described by Engel and Schmale as a conservation-withdrawal strategy (Engel and Schmale, 1972). Coping strategies concern also displacement/de-arousal behaviours, which may allow an individual temporarily to "cut-off" attention from a threatening stimulus, and this short-term diversion of attention could reduce the negative arousal associated with the stimulus. In this context, rodents perform self-grooming (rubbing the body with paws or mouth and rubbing the head with paws) and digging (animal digs the sawdust with the forelimbs, often kicking it away with the hind limbs) behaviours.

It must be taken into account that acute or chronic stressors affect differentially the capacity of an individual to cope. If the stress response is inadequate or excessive and prolonged, the cost of reinstating homeostasis might become too high, leading to allostatic load. In this context, chronic exposure to inescapable physical and psychological stress, such as restraint and social isolation, results associated to deregulation of HPA axis activity and limbic function, finally promoting endophenotype of depression (McEwen, 2000a; Herman, 2013). Mice exposed to a series of chronic stressors exhibit increased signs of anxiety and depression- like behaviours, including increased passivity in a forced swim test, reduced aggression in a social interaction test, delayed approach to food in a novel environment (Tannenbaum et al., 2002).

1.2. PSYCHONEUROENDOCRINE REGULATIONS AND CANCER

1.2.1. Stress affects cancer progression

Cancer is a multifactorial disorder caused by both internal (inherited mutations, hormones and immune conditions) and environmental factors (smoke, diet, radiation, stress). In this context, although most cancer research is mainly focused on molecular and cellular interactions, there is now clear evidence that psychological factors can influence disease progression. Stressful events, in particular, can unleash a signalling cascade accelerating tumor onset and progression.

Lack of social support, a well known psychosocial stressor, has been associated with pain, fatigue, lethargy and higher inflammation among cancer patients (Gagliardi et al., 2009; Hughes et al., 2014), while in mouse models of

cancer, exposure to social isolation alters tumor and spleen macrophage populations, potentiating tumor growth and metastasis (Hermes et al., 2009; Williams et al., 2009; Madden et al., 2013; Volden et al., 2013). It must be taken into account that 15-50% of breast cancer patients meet diagnostic criteria for anxiety and depression (Burgess et al., 2005) and that the tumor itself can produce depressive symptoms through the expression of cytokines and growth factors, generating a positive feedback loop between coping style and cancer progression (Sephton et al., 2009). Depressed metastatic breast cancer patients are often characterised by blunted cortisol awakening responses and by dysregulation of the HPA axis, reflecting a physiologic profile associated with chronic stress (Sephton et al., 2009). These endocrine changes have the potential to override the immune defense or act directly on the tumor microenvironment, affecting cancer progression.

Many studies suggest that prolonged exposure to stress - with a persistent activation of the HPA axis and the consequent suppressed activity of the immune system (both in humans and in animal models), mediated by glucocorticoid (GC) hormones - can render subjects more susceptible to disease, including neoplastic processes (Cavigelli et al., 2008).

In addition to classic neuroendocrine factors, BDNF is produced by different cell types including immune cells, adipocytes, endocrine and endothelial cells and has a key position in integrating neural, immune and endocrine responses to stress (Cirulli and Alleva, 2009; Capoccia et al., 2013). This neurotrophin is implicated in the pathophysiology of the nervous system, including the etiopathogenesis of depression, while in the periphery it regulates metabolic responses to stress and has been recently involved in tumor development and progression (Cirulli and Alleva, 2009; Yang et al., 2012; Liu et al., 2014).

In addition to the neuroendocrine axis, stress activates the sympathetic nervous system (SNS), which acts through release of the catecholamines norepinephrine (NE) and epinephrine (EPI) from sympathetic noradrenergic nerves and from the adrenal medulla. Beta-adrenergic signalling regulates the biological activity of several cancer-relevant cell types including epithelial cells, adipocytes and most lymphoid and myeloid immune cells. Hypothalamic BDNF can lead to activation of sympathetic innervation of white adipose tissue, which can, in turn, affect tumor growth through the production of multiple mediators such as cytokines, chemokines, adipokines, estrogen and growth factors.

1.2.2. Transgenic mouse models of breast cancer

The pathogenesis of breast cancer involves multiple genetic events, including gain of function mutations in proto-oncogenes, which are involved in supporting cell growth, division and survival, and loss of function mutations in so called 'tumor suppressor' genes, which are involved in preventing unrestrained cellular growth (Hutchinson and Muller, 2000). The majority of gain of function mutations in human primary breast cancers involves

amplifications in one of three chromosomal regions, the c-myc and erbB-2 proto-oncogenes or the chromosomal band 11q13 (Lidereau et al., 1988). Loss of function mutations in primary human breast cancers includes changes in the known tumor suppressor p53 as well as in the familial cancer markers of the BRCA gene family (Hutchinson and Muller, 2000). Many mouse systems exist to address the significance of these mutations in the pathogenesis of breast cancer and a number of transgenic promoters have been employed to target transgene expression to the mammary gland (Hutchinson and Muller, 2000; Fantozzi and Christofori, 2006; Caligiuri et al., 2012).

A consistent number of the transgenics generated have employed the mouse mammary tumor virus long terminal repeat (MMTV) which is active during mammary development (Hutchinson and Muller, 2000; Sakamoto et al., 2012; Peng et al., 2013). One of the known oncogenes, which is expressed under control of the MMTV promoter to initiate or modulate breast carcinogenesis in mice, is ErbB2/Neu (Fantozzi and Christofori, 2006; Ursini-Siegel et al., 2007). ErbB-2 is a member of the epidermal growth factor (EGFR) family of receptor tyrosine kinases (RTKs) (Roskoski, 2014). This family consists of four members (HER1-4) that belong to the ErbB lineage of proteins (ErbB1-4) and which is ubiquitously expressed in epithelial, mesenchymal, and neuronal cells and their cellular progenitors (Olayioye et al., 2000; Roskoski, 2014). The ErbB-2 occurs in 20-30% of human breast cancers and its amplification and subsequent overexpression strongly correlates with a negative clinical prognosis in both lymph node positive (Hynes and Stern, 1994; Mansour et al., 1994; Ravdin and Chamness, 1995; Shah and Chen, 2011) and node-negative (Andrulis et al., 1998; Ozcelik et al., 2007) breast cancer patients. Further evidence that overexpression of ErbB-2 results in an aggressive tumor type stems from studies showing that elevated ErbB-2 expression is observed in many *in situ* and invasive human ductal carcinomas but is rarely observed in benign breast disorders, such as hyperplasias and dysplasias (Allred et al., 1992; Mansour et al., 1994).

Overall thus, multiple transgenic mouse models have confirmed a direct role for ErbB-2 in mammary tumorigenesis, each with their own level of relevance to the human disease. MMTV-driven overexpression of the oncogene neu or an analogous ERbB-2 transgene engineered to possess a similar activating mutation within the transmembrane domain results in the formation of mammary adenocarcinomas that histologically resemble human disease (Bouchard et al., 1989; Guy et al., 1996; Ursini-Siegel et al., 2007). In particular, the FVB-Tg(MMTV-Erbb2) transgenic mice express the activated rat c-neu oncogene (Erbb2) under the control of the MMTV long terminal repeat (LTR) promoter. The activated, or transforming, version of the rat c-neu oncogene has a valine to glutamic acid substitution at acid 664 (Val664 to Glu664) and this transgene is microinjected into fertilized FVB/N eggs (http://jaxmice.jax.org/strain/005038.html). The FVB-Tg(MMTV-Erbb2) transgenic mice develop hyperplasia at 5 months and focal adenocarcinoma and

lung metastases at 7 months, helping to resemble the postmenopausal condition and to allow chronic stress treatment.

1.3. RATIONALE AND OBJECTIVES

1.3.1. Current state of the art and statement of the problem

The literature reviewed so far indicates chronic stress as a vulnerability factor for physical as well as psychological health, although the underlying mechanisms are still unclear. The brain is a primary mediator and target of stress resiliency and vulnerability, because it determines what is threatening and because it regulates the behavioural and physiological responses to a given stressor through its role in HPA axis functioning and feedback. The connection between central stress response pathways and peripheral targets involves a number of neurochemical and/or inflammatory factors that ultimately affect neuronal functioning and/or survival as well as adaptive physiological responses that allow fighting against external challenges. One of the most representative players implicated in these events is BDNF, which is involved in synaptic and morphological plasticity of the brain, both during development as well as at adulthood. This neurotrophin is implicated in the pathophysiology of the nervous system, including the etiopathogenesis of depression, while in the periphery it regulates metabolic responses to stress. While there is now a clear evidence for a potential physiological connection among stressful events, neuroendocrine and immune response and behavioural phenotype, the multiple mediators, the qualitative and temporal features of stress must be studied more extensively in order to clarify the mechanisms through which stress acts on health outcomes.

Stress physiology overlaps to a great extent in humans and mice, thus preclinical models can guide and refine hypothesis- driven clinical research questions. The animal models here used have been selected to identify the key players in a network made up of specific aspects of the social context, copying styles, neuroendocrine mechanisms and immune responses. It is important to consider that chronic stress experience could impinge upon an individual for weeks, months or even years. Thus we used stressors of different lengths and strengths known to affect neuro-immune functioning to model human experiences.

The general aim of the work presented in this thesis was to characterise how quality and timing of stressors differentially impact on behavioural, neuroendocrine and immune profile in animal models (mice).

1.3.2. Rationale of the studies

I. Study the relationship between neuroendocrine activation (circulating corticosterone and central BDNF levels) and a wide array of depression- and anxiety-like behaviours (anhedonia, behavioural despair, generalised and social anxiety) resulting from exposure to chronic stress. To this end, adult C57BL/6J male mice were exposed to either chronic disruption of the social structure (SS), to a stable social structure (SG) or to social deprivation (SD), a condition lacking social stimuli.

II. Characterise central and peripheral effects of different stressors, applied for different time lengths, on neuroendocrine and immune responses in adult male C57BL/6J mice. Specifically, we compared the effects of repeated (7 versus 21 days) restraint stress and social stress on neuroendocrine (circulating corticosterone) and immune (circulating cytokines and splenic apoptosis) function and on a marker of brain plasticity (hippocampal BDNF) in order to identify a specific neuroendocrine profile in response to a selective type of stress.

III. Investigate the effects of social isolation and restraint stress on a transgenic mouse model of breast cancer. Our aim was to characterise the mechanism through which stressful events affect tumor growth, hypothesising that they would activate neuroendocrine pathways, thwarting immune function and modifying the gene expression of central BDNF and of adipose tissue-derived adipokines, finally promoting tumor progression. A further aim of this study was to assess whether changes in tumor biology, resulting from stress exposure, would also be accompanied by an increase in endophenotypes of depression, previously validated in animal models, such as anhedonia.

1.3.3. Experimental approach and outline of the thesis

In **Chapter 2** and **3** we report two studies aimed at characterising a behavioural-neuroendocrine-immune profile in a mouse model of stress. Since natural fluctuations in sex hormones have enormous influences on neuroendocrine function, both these studies were performed on C57BL/6J adult male mice. This strain has been one of the most commonly used in translational research because its genome shares 99% homology with the human genome (Waterston et al., 2002).

Chapter 2 describes a study aimed at assessing the effects of stress on behavioural, neuroendocrine and neurobiological responses. In particular, mice underwent either to a Chronic Social Stress (SS) consisting of a disruption of the social structure, or to Social Deprivation (SD) obtained by prolonged individual housing (Valzelli, 1973). As for social stress, it has been widely reported that the presence of social odour cues emanating from the mouse body, originating largely from their urine, are involved in the advertisement of dominance over a defended territory (Gosling, 1990; Hurst, 1990). Therefore by

disrupting the social group (SS) we attempted to mimic the presence of an intruder in the territory, which might lead to a change in the social structure. In particular, we compared the selected stressful procedures in their ability to induce a depressive-like state by measuring behaviours such as levels of anhedonia (preference for sucrose solution) and behavioural despair (Forced Swim Test). In addition generalised anxiety (Elevated Plus Maze), social anxiety (Social Interaction Test), and the emotional response to a novel environment (Open Field test) were also observed. Levels of BDNF protein as a marker of vulnerability to stress-induced depression were measured in the limbic system.

In **Chapter 3** we focused our attention on the comparison between the response to social stress and the response to a different, psychophysical stress. The main aim of this study was, in fact, to characterise qualitative and temporal features of this two different stressors and to extend the range of mediators analysed and the peripheral targets, to evaluate more extensively the role of acute versus chronic stressors on neuroendocrine and immune function. Specifically, experimental subjects were divided into three groups: 7 days stress (restraint stress or social stress) 21 days stress (restraint stress or social stress), and unhandled controls (CTRL, subjects left undisturbed in their home cage). Corticosterone levels, circulating cytokines, splenic apoptosis and hippocampal BDNF levels were assessed to define a "complete picture" of individual response. The central nervous system and the immune system are known to be engaged in an intense bidirectional crosstalk which can be affected by stress and which involves multiple mediators, including cytokines and growth factors (Hayley et al., 2005). As an example, the immune signalling cytokines, particularly the proinflammatory ones, such as IL-6 or TNF-α, are elevated following stress exposure and can thwart brain plasticity eliciting depressive symptoms, which are amenable to antidepressant treatment (Hayley et al., 2005). However, the directionality of the effects of stress is still a matter of intense investigation: for instance, GC released in response to stress can act both enhancing and inhibiting immune responses and by decreasing or increasing levels of neurotrophins (Smith et al., 1995; Marmigere et al., 2003; Dhabhar, 2008). Factors such as the duration (acute versus chronic) of stress as well as the time of exposure to GC, relative to the activation and time course of the immune response, might differently impact health outcome (Dhabhar, 2008).

In **Chapter 4** we tested the effects of social isolation and restraint stress on tumor progression in a transgenic mouse model of breast cancer. For this study we choose mice of to FVB/NJ (FVB) strain which is more susceptible to mammary tumors than the C57BL/6 strain (Davie et al., 2007; Taneja et al., 2009). More in detail, experimental subjects were ErbB-2(Neu)TgMMTV-ErbB-2 (FVB background) since human breast cancer models using MMTV-LTR mice have often been created in the FVB strain, due to its high productiveness of offspring (Davie et al., 2007; Taneja et al., 2009). In this case

mice were all females because breast cancer incidence is about 100 times more common among women than men. Half of subjects were socially isolated at weaning to model long-term stress that could accompany an individual chronically during life. In addition, to assess whether a further stressful challenge experienced (such as the diagnosis of cancer in humans) would worsen the effects upon a long-term vulnerability, experimental subjects underwent restraint stress by the time they started developing tumors (5 months). At the end of the stress procedures peripheral (HPA axis activity, adipose tissue leptin and adiponectin gene expression, splenocytes apoptosis and cytokines levels) and central (hypothalamic BDNF gene expression) responses were assessed and related to tumor onset and development in order to characterise and clarify the mechanisms through which the environment could act on disease outcome. In addition, it must be taken into account that depressive symptoms often accompany breast cancer patients as a result from situational fear related to diagnosis and prognosis, or may be directly related to the effects of the tumor. In this regard, Lamkin and co-workers have shown an increase in endophenotypes of depression following tumor implantation (Lamkin et al., 2011). Thus a further aim of this study was to assess whether changes in tumor biology, resulting from stress exposure, would also be accompanied by an increase in endophenotypes of depression, previously validated in animal models, such as anhedonia. All data are discussed in **Chapter 5**.

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CHAPTER 2

2. SOCIAL DEPRIVATION STRESS IS A TRIGGERING FACTOR FOR THE EMERGENCE OF ANXIETY- AND DEPRESSION-LIKE BEHAVIOURS AND LEADS TO REDUCED BRAIN BDNF LEVELS IN C57BL/6J MICE

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ABSTRACT
Stress is a main risk factor that can trigger psychiatric disorders, including anxiety and major depression. Neurotrophins, such as Brain-Derived Neurotrophic Factor (BDNF), have been identified as neuroendocrine effectors involved in the response to stress and in the neurobehavioural changes associated with depression. Aim of this paper was to study the relationship between neuroendocrine activation (circulating corticosterone and brain BDNF levels) and a wide array of depression- and anxiety-like behaviours (anhedonia, behavioural despair, generalised and social anxiety) resulting from exposure to chronic stress. To this end, 3-month-old C57BL/6J male mice were exposed to either chronic disruption of the social structure (SS), to a stable social structure (SG) or to social deprivation (SD), a condition lacking social stimuli. Results show that, despite not developing anhedonia (decreased preference for a sucrose solution), SD mice were characterised by increased emotionality and hypothalamic-pituitary-adrenal axis reactivity in addition to reduced BDNF levels. By contrast, SG and SS mice showed increased anhedonia accompanied by no alterations in the behavioural and neuroendocrine profile. The results here reported indicate that mice exposed to different social housing conditions use different behavioural strategies to cope with external challenges. In addition they suggest that social deprivation might represent a stressful condition triggering the emergence of both anxiety- and depression-like behaviours and clearly indicate BDNF as a main neurobiological variable mediating these responses.

Keywords: Stress; Anxiety; Depression; Social deprivation; Anhedonia; BDNF

2.1. INTRODUCTION

Stress is a main risk factor for psychopathology as chronic exposure to severe physical or emotional stimuli can precipitate major depression in vulnerable individuals (Kendler et al., 1999; Cirulli et al., 2009a). Clinical evidence indicates that in depressed patients, emotional arousal, cognitive abnormalities and vulnerability to psychotic episodes are associated to a hyperactive hypothalamic—pituitary— adrenal (HPA) axis (de Kloet et al., 2007). In addition to glucocorticoids (GCs), neurotrophins, such as Brain-Derived Neurotrophic Factor (BDNF), have been identified as neuroendocrine effectors involved in the response to stress and to neurobiological and behavioural changes associated with depression (Pezawas et al., 2004; Duman and Monteggia, 2006; Castren et al., 2007; Schmidt and Duman, 2007; Brunoni et al., 2008; Cirulli and Alleva, 2009; Cirulli et al., 2009b, 2010b). Indeed, decreased blood BDNF levels characterise subjects diagnosed as major depressives, antidepressants reverting this neurobiological change (Shimizu et al., 2003; Karege et al., 2005; Duman and Monteggia, 2006; Sen et al., 2008). In rodents, chronic stress decreases the expression of this neurotrophin, which can lead to neuronal atrophy in the hippocampus and other brain structures (Schmidt and Duman, 2007), while direct hippocampal infusion of BDNF produces anxiolytic and antidepressant effects (Siuciak et al., 1997; Shimizu et al., 2003; Cirulli et al., 2004). In addition, a very recent study reports that knocking-down BDNF in specific rat's brain sites precipitates behaviours associated with depression such as preference for a sucrose solution (Taliaz et al., 2010).

Progress in understanding the pathophysiology of major depression and treatment development would greatly benefit from appropriate animal models (Nestler and Hyman, 2010). The chronic mild stress (CMS) paradigm has been developed in rodents to simulate unpredictable stress that may occur in everyday life (Willner et al., 1987). This procedure is effective in inducing neurobehavioural disturbances, such as a reduction in responsiveness to a reward, which has been compared to anhedonia, one of the core symptoms of human depression (DSM-IV, American-Psychiatric- Association, 1994). Moreover, all the behavioural alterations induced by CMS can be reversed by chronic antidepressant administration (Willner et al., 1987). Although the CMS paradigm appears to be able to reproduce a fair number of depression-like symptoms, data relating specific physiological changes with the behavioural phenotype resulting from exposure to this procedure are scattered and often conflicting. As an example, Larsen and colleagues, despite a behavioural depressive-like phenotype, found an increase, rather than the expected decrease, in BDNF mRNA expression in the hippocampus of rats exposed to chronic unpredictable stress (Larsen et al., 2010). In addition, results from studies assessing anxiety-related behaviours in CMS animal models of depression are rather ambiguous, since some authors report decreased (Kopp et al., 1999) while

others find increased anxiety after this procedure (Kompagne et al., 2008). These discrepancies might depend upon the interplay among different neurobiological variables known to affect anxiety- and depression-like behaviours including HPA axis activation and levels of serotonin (5-HT) and BDNF (Nutt and Stein, 2006) as well as upon the ultimate length of the stress period. Indeed, although there are sufficient and significant differences between anxiety and depression, which support the view that they are independent clinical entities, there is also evidence that these psychopathologies co-exist and that anxiety typically precedes depressive disorders (Ballenger, 1999; Ninan, 1999; Nutt and Stein, 2006). Such co-morbidity is due to the fact that these two pathologies share common neurobiological mechanisms, as increased 5-HT levels, associated with administration of selective 5-HT reuptake inhibitors (SSRIs), are not only able to reduce depressive symptoms but also to reduce anxiety levels (Cryan and Holmes, 2005).

Social stress in humans represents a major etiological factor in the development of emotional disorders, including depression (Leskela et al., 2006; McEwen and Gianaros, 2010). Based on the notion that changing the social structure in a group of mice is a stressful event, especially if this is repeated over time (Avitsur et al., 2002; Schmidt et al., 2010), we chose such paradigm to increase the chance of stimulating behavioural and neuroendocrine responses that have been shaped by evolutionary processes. With this study we aimed at developing a comprehensive and ethologically relevant experimental method to induce chronic stress in mice and to investigate its effects on the interplay among behavioural, neuroendocrine and neurobiological aspects. More in detail, adult male mice (age 3 months), of the C57BL/6J strain, underwent either a Chronic Social Stress (SS), consisting of an experimentally induced disruption of the social structure, or group-housing in a stable social arrangement (SG). It is widely reported that the presence of social odour cues emanating from the mouse body, originating largely from their urine, are involved in the advertisement of dominance over a defend territory (Gosling and McKay, 1990; Hurst, 1990). Therefore by changing the composition of the social group (SS condition) we mimicked the presence of an intruder in the territory, which might lead to a change in the social structure. However, also the maintenance of a stable social structure may represent a source of stress (Avitsur et al., 2003). In fact, it has been shown that group-housed mice show altered behavioural, endocrine and immune indices of stress (Sgoifo et al., 2001). In particular, Bartolomucci et al. (2002) have provided evidence that lack of familiarity/relatedness among cage-mates might be regarded as a main source of stress. Thus, we compared SS and SG to a further condition, i.e. social deprivation (SD). This condition, consisting of prolonged individual housing, is characterised by the lack of social stimuli and, if prolonged, might result in the so-called ‘‘isolation syndrome’’ (Valzelli, 1973).

The effectiveness of such procedures to activate the neuroendocrine system was assessed by measuring the activation of the HPA axis through

plasma levels of corticosterone (CORT). We hypothesised that, following stress, a combination of anxiety and depressive-like behaviours might become manifest in the experimental subjects. Thus we selected those tests which might be more appropriate (according to previous data) to discriminate between these two. To this aim we compared the selected stressful procedures in their ability to induce a depressive-like state was assessed by measuring behaviours such as levels of anhedonia (preference for sucrose solution) and behavioural despair (Forced Swim Test). In addition generalised (Elevated Plus Maze), social anxiety (Social Interaction Test) and the emotional response to a novel environment (Open Field test) were also observed. Levels of BDNF protein were measured in the limbic system as a possible marker of vulnerability to stress-induced depression.

2.2. MATERIALS AND METHODS

2.2.1. Animals

Experimental subjects were 44 adult male mice of the C57BL/6J strain that were purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival all animals were singly housed in the same room provided by air conditioning (temperature 21 ± 1 8C, relative humidity 60 ± 10%), in transparent Plexiglas cages (29 cm x 12 cm x14 cm), under a reversed 12/12 h light/dark cycle with lights off from 0800 to 2000 h. Pellet food (standard diet Altromin-R, purchased from Rieper, Italy) and tapwater were continuously available. Animals were left undisturbed for 1 week before the beginning of the experimental procedure. At the end of this period animals were allowed to habituate to a sucrose solution in order to establish an individual baseline. When the baseline was established mice were assigned to 3 conditions: two group-housing (social stress - SS, n = 16; social group - SG, n = 16) and one individual-housing condition (social deprivation - SD, n = 12); home cages for grouped mice had the following dimensions 37 cm x 21 cm x 19 cm. Sucrose preference was assessed once a week, during 3 weeks (day 0 - baseline, day 7, 14 and 21). At the end of the third week of stress, 8 mice were randomly selected from each social condition to undergo a battery of behavioural tests aimed at assessing the emotional response to a novel environment (Open Field - OF), depressive-like behaviours (Forced Swim Test - FST), generalised (Elevated Plus Maze - EPM) and social anxiety (Social Interaction Test - SIT). During the behavioural assessment the groups/housing conditions (SD,
SG and SS) were maintained until the brief (24 h) social isolation preceding the SIT. In particular, on the first day of behavioural testing mice underwent Open Field (0930 - 1130 h) and four hours later experimental subjects were assessed in the Elevated Plus Maze (1530 - 1730 h). On the next day a first Forced Swim Test session was performed, followed by a second session 24 h later (0930 -

1030 h). On the following day mice underwent an acute social challenge (Social Interaction Test) between 1700 and 1800 h. Before the beginning of behavioural tests, all subjects were moved to the testing room (by the experimenter) and left there to habituate for 1 h. Behavioural performances were video recorded and the behavioural analysis was carried out from the videotape by an observer blind to the social condition, using commercial software (''The Observer 3.0''). At the end of each behavioural session apparatuses were cleaned by a cotton pad wetted with a 50% solution of ethanol and water. All behavioural tests were conducted under dim red light (1 lx) between 0930 and 1730 h (i.e. during mice's active-period) except for the Social Interaction Test which was used as a social challenge to assess CORT levels during the circadian trough (i.e. between 1700 and 1800 h). In addition, CORT levels were assessed in response to the Social Interaction Test. Ten minutes following the end of this test (i.e. 30 min from the beginning) subjects were sacrificed (between 1730 and 1830 h), brains were dissected out and BDNF levels measured in the hypothalamus, hippocampus, frontal cortex, striatum and midbrain. Moreover, adrenal glands were also dissected out, and the ratio of adrenal weight to body weight was calculated in order to provide indirect evidence of hypercorticism-related hypertrophy. All behavioural tests, CORTand BDNF measurements were conducted on 8 subjects per group randomly chosen. The number of subjects was defined on the basis of the distribution of the dependent variables to be assessed and the experimental design, with a level of significance Alpha = 0.05 in a two-tailed test and a power of 1- Beta = 0.80.

Animal handling and experimental procedures were performed in accordance with the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (Decreto L.vo 116/92).

2.2.2. Experimental procedures

2.2.2.1. Sucrose Preference Test - SPT. One week following arrival, experimental subjects underwent 7 days of familiarisation with a novel sucrose solution in order to establish the individual preference. During this phase (familiarisation) mice were housed individually and each cage was provided by two bottles, one containing fresh tap-water and one containing a 4% sucrose solution (Pothion et al., 2004; Lewis et al., 2005). Bottles were daily weighed in order to monitor liquid consumption and switched to balance the effect of side preference in drinking behaviour, which was reported to be of importance for the correct evaluation of sucrose preference (Kant and Bauman, 1993; Strekalova et al., 2004).

The sucrose preference was calculated only on the last day (day 7) of the sucrose exposure period following this procedure: mice were deprived of food and water for 5 h (from 0900 to 0200 h), soon after they were provided again with two bottles either with tap-water or with a fresh sucrose solution for 1 h (in the absence of food). Sucrose preference was then calculated as follows: %

sucrose preference = (sucrose solution intake x 100)/(water intake + sucrose solution intake). Sucrose consumption was derived from bottles' weight. Following the establishment of the sucrose consumption baseline (day 0), on the days anhedonia was assessed (day 7, 14 and 21), SG and SS mice were singly housed for 5 h in order to score individual consumption, while SD mice did not change their social condition.

2.2.2.2. Chronic Social Stress - CSS. Mice were assigned to 3 different conditions: SS, SG or SD. More in detail, SS mice (n = 16) were ear-marked and housed into 4 cages (4 mice/cage) and social structure was disrupted twice a week for three weeks by replacing one mouse with a novel unfamiliar one selected randomly from another cage. Sawdust was replaced at the same time in each cage. Mice in the SG condition (n = 16) were also housed in groups of 4 mice/cage however members of each group remained always the same, possibly leading to a stable social structure. Cages were cleaned and sawdust replaced twice a week mimicking the handling procedure which the SS group was subjected to. SD mice (n = 12) were individually housed and cages also cleaned and sawdust replaced once a week.

2.2.2.3. Open Field - OF. Mice were individually placed in the centre of a cubic arena (open field box 44 cm x 44 cm x 44 cm) made of grey Plexiglas and allowed to freely explore for 20 min; during the last 5 min of the test a novel object (a 50 ml glass beaker) was introduced in the centre of the arena. The open field box was ideally divided into 25 squares and ideally partitioned into a central portion (26.4 cm x 26.4 cm) and a peripheral one, identified as the remaining part of the arena. When data were analysed, each session was subdivided in 4 time blocks (tb) and the time spent in each portion of the arena as well as latency, frequency and duration of locomotion (*crossings* of squares), exploratory activity (*sniffing* and *rearing*), risk assessment (*stretch-attend posture - SAP*), self-directed behaviours (*self-grooming*) and object exploration (*sniffing* and *touching*) were scored. Behaviours were defined as follows:
- *crossing*, crossing the square limits with all paws;
- *sniffing*, self explanatory;
- *rearing*, standing on the hind paws;
- *stretch-attend posture*, exploratory posture in which the body is stretched forward and then retracted to the original position without any forward locomotion;
- *self-grooming*, rubbing the body with paws or mouth and rubbing the head with paws.

When the stimulus object was present, latency to the first contact and sniffing and frequency of contacts and sniffing of the object were also scored.

2.2.2.4. Elevated Plus Maze - EPM. The Elevated Plus Maze was made of two open arms (30 cm x 5 cm x 0 cm) and two closed arms (30 cm x 5 cm x 15 cm)

that extended from a common central platform (5 cm x 5 cm). Each arm was ideally divided into 4 portions in order to facilitate scoring of locomotor activity (*crossings*, see below). The apparatus, made of Plexiglas (grey floor, clear walls), was elevated to a height of 60 cm above the floor. Mice were individually placed on the central platform facing an open arm and allowed to freely explore the maze for 5 min. Behavioural parameters observed were: frequencies of total, open and closed entries (arm entry = all four paws into an arm), % open entries [(open/total) x 100], and time spent in open and closed parts of the maze (File, 2001). Furthermore, we also scored latency, frequency and duration of *crossing*, *sniffing*, *rearing*, *self-grooming* (for a detailed description see Section 2.2.2.3.), *head dipping* (*HED*, exploratory movement of head and shoulders over the edge of the maze) and *SAP*.

2.2.2.5. Forced Swim Test - FST. Mice were tested according to the Porsolt's procedure (Porsolt et al., 1977). Each experimental subject was gently placed into a cylindrical glass (20 cm Ø, 40 cm height), filled with 25 cm of water at a temperature of 26 ± 1 °C for 6 min on 2 consecutive days with a dim light illumination (1 lx). When removed from the water, mice were allowed to dry for 5 min under red light. Twenty-four hours later, a second session took place and latency, frequency and duration of the following behavioural responses were scored: *struggling* (vigorous attempts at climbing the walls of the cylinder); *swimming* (active swimming around) and *floating* (total absence of movement).

2.2.2.6. Social Interaction Test - SIT. A Social Interaction Test was used as a social stressful challenge in order to test the activity of the HPA axis and to assess social anxiety. The night before the test all subjects were placed in a holding cage to stimulate social interactions (Terranova et al., 1993; Cirulli et al., 1996; Panksepp et al., 2007). Experimental subjects were placed in a novel cage, identical to the holding cage, ideally subdivided in three equal parts, with an unfamiliar conspecific of the same strain, weight and sex that had been previously isolated (standard opponent). Standard opponents were marked by a yellow, scentless and nontoxic paint 30 min before testing, in order to discriminate the experimental subject from the unfamiliar conspecific during data collection. The maximum length of social encounters was 20 min; by the time of behavioural observation, each session was subdivided in 4 tb and frequency and duration of *environmental exploration* (sum of *crossing*, *sniffing* and *wall rearing*), *social investigation* (sum of body, nose and ano-genital *sniffing*), *affiliative behaviours* (*allogrooming* and *following*) and *displacement/ de-arousal behaviours* (sum of *digging* and *self-grooming*) were scored. Behaviours were defined as follows:
- *wall rearing*, animal stands on its hind limbs and touches the walls of the cage with the forelimbs;
- *body sniffing*, sniffing any other area of the body of the opponent;
- *nose sniffing*, sniffing the head and the snout region of the opponent;

- *ano-genital sniffing*, sniffing the ano-genital area of the opponent;
- *allogrooming*, grooming the opponent;
- *following*, experimental subject follow the standard opponent;
- *digging*, animal digs the sawdust with the forelimbs, often kicking it away with the hind limbs.

For *crossing*, *sniffing* and *self-grooming* see Section 2.2.2.3.

Animals were blood sampled by tail nick, for basal CORT levels, the night (1730 - 1830 h) before the last Sucrose Preference Test. At the end of the social challenge (1730 - 1830 h), all subjects were sacrificed, trunk blood was collected for CORT assessment and tissues dissected out (brain regions and adrenals) and immediately frozen until quantification of BDNF.

2.2.2.7. Radioimmunoassay for corticosterone determination - RIA. Blood samples (100 ml, approximate volume) were collected individually in potassium EDTA coated tubes (1.6 mg EDTA/ml blood, Sarstedt, Germany). All samples were kept on ice and later centrifuged at 3000 rpm for 15 min at +4 °C. Blood plasma was transferred to Eppendorf tubes for CORT determination and stored at -20 °C until further analysis. CORT was measured using a commercially available radioimmunoassay (RIA) kit containing 125iodine labelled CORT; 5 µl of plasma were sufficient to carry out CORT measurement. Sensitivity of the assay was 0.125 µg/dl, inter- and intraassay variation was less than 10 and 5%, respectively (MP Biomedicals Inc., CA, USA). Vials were counted for 2 min in a gamma-scintillation counter (Packard Minaxi Gamma counter, Series 5000).

2.2.2.8. BDNF measurement. BDNF evaluation was carried out with an enzyme-linked immunosorbent assay kits (Emaxtm ImmunoAssay System number G6891, by Promega, Madison, Wisconsin, USA) following the instructions provided by the manufacturer. Following sacrifice brains were quickly removed and the frontal cortex, hippocampus, hypothalamus, striatum and midbrain were dissected out and immediately stored at -80 °C until used. Brain tissues were homogenised in a lysis buffer prepared according to the kit instructions and centrifuged at 8500 rpm, and the supernatant was used for BDNF analyses. Briefly, BDNF standard and brain samples were distributed in 96-well immunoplates precoated with monoclonal antimouse BDNF antibody (100 µl/well) and incubated for 2 h at room temperature. After washing plates were incubated with an anti-human BDNF antibody for 2 h at room temperature. The plates were washed again and then incubated with an anti-IgY horseradish peroxidase (HRP) for 1 h at room temperature. Tetramethylbenzidine (TMB)/peroxidase substrate solution was added to the wells to produce colorimetric reaction measured at 450 nm with a microplate reader (Dynatech MR 5000, Dynatech Laboratories, Chantilly, Virginia, USA). BDNF concentrations were determined from the regression line for the BDNF standard incubated under similar conditions in each assay. The sensitivity of the assay was about 15 pg/mg of BDNF, and the cross-reactivity with other related

neurotrophic factors (NGF, NT-3, and NT-4) is considered nil (Aloe et al., 1999).

2.2.2.9. Statistical analysis. Data were analysed using parametric analysis of variance (ANOVA) with ‘‘social condition’’ as between-subjects factor and ‘‘time blocks’’ and ‘‘zone’’ as within-subject repeated measures, when appropriate (Sucrose Preference Test, Open Field, Elevated Plus Maze, Forced Swim Test, Social Interaction Test, CORT assessment and BDNF). Post hoc comparisons were performed using the Tukey's test. In analysing data on social investigation (Social Interaction Test), this test was used in the absence of significant ANOVA effects according to the indications given by Wilcox (Wilcox, 1987). Statistical analysis was performed using Statview II (Abacus Concepts, CA, USA). Data are expressed as mean + SEM. A significance level of 0.05 was chosen.

2.3. RESULTS

2.3.1. Sucrose Preference Test - SPT

Social condition affected the preference for a sucrose solution both in the SG and SS groups but not in SD mice (main effect of the social condition: $F(2,41) = 253.791$; $p < 0.0001$, post hoc SD vs. SG and SS $p < 0.01$; SG vs. SS $p < 0.05$). The three groups showed the same basal preference for the sucrose solution which did not decrease in the SD group. After the first week the SG group developed a strong anhedonic profile which did not change from day 7 to 21. By contrast, the SS group developed anhedonia only during the last week of the stress procedure (day 21) (interaction between social condition and days: $F(6,123) = 10.561$; $p < 0.0001$, post hoc comparisons: SD vs. SG and SS $p < 0.01$ on days 7, 14 and 21; SG vs. SS $p < 0.01$ on days 7 and 14; see Fig. 1).

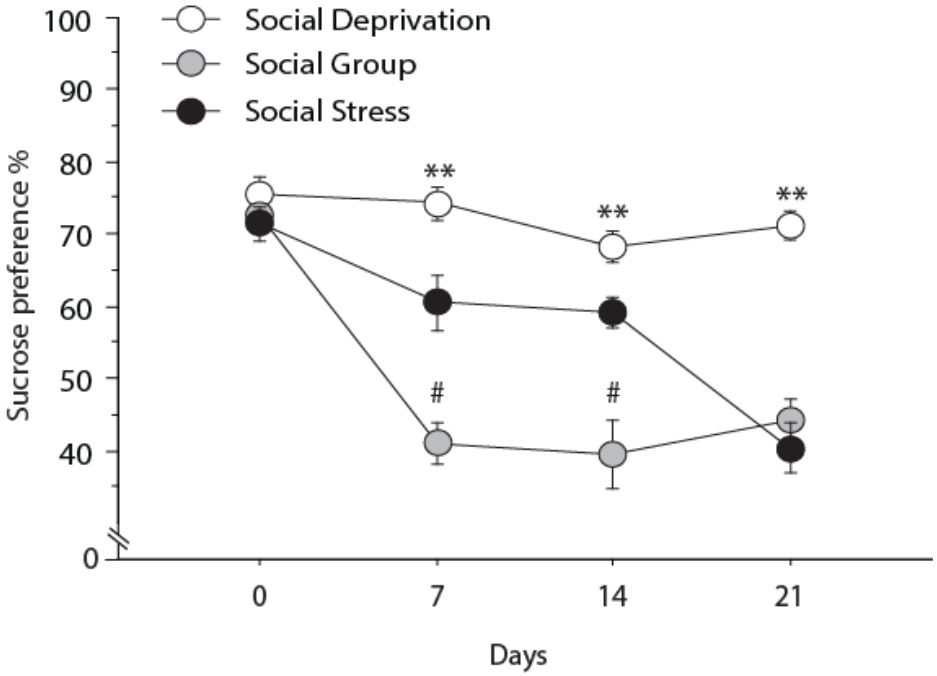

Figure 1. **Assessment of anhedonia in Sucrose Preference Test**. Anhedonia increased over time in the social group (SG) and social stress (SS) conditions only. Results are presented as mean ± S.E.M. (social deprivation - SD, n = 12; SG and SS, n = 16), **p < 0.01 SD vs. SG and SS (days 7, 14 and 21), #p < 0.01 SG vs. SS (days 7 and 14).

2.3.2. Open Field - OF

All subjects spent significantly more time in the peripheral part of the arena (main effect of the zone: $F(1,21) = 202.673$; $p < 0.0001$), this was particularly evident for the SD group (interaction between zone and social condition: $F(2,21) = 9.093$; $p = 0.0006$, post hoc SD vs. SG and SS $p < 0.05$ (Fig. 2A). Locomotor activity overall did not differ among groups (main effect of the social condition: $F(2,21) = 0.042$; $p = 0.9585$) and decreased for all subjects from tb 1 to 3 and increased again during tb 4, i.e. when a novel object was introduced in the arena (effect of time: $F(3,63) = 10.416$; $p < 0.0001$, post hoc tb 1 vs. tb 2 and 3 $p < 0.05$; mean ± standard error: 40.2 ± 2.5; 36.1 ± 2.5; 34.7 ± 2.7; 37.5 ± 2.6, respectively for tb 1, 2, 3 and 4). The SD group performed more rearing than the other groups (effect of the social condition: $F(2,21) = 12.112$; 9.204; $p < 0.0001$; $p = 0.0006$, respectively for frequency and duration, post hoc SD vs. SG and SS $p < 0.01$) and this behaviour persisted over time during the test (interaction between tb and social condition: $F(6,63) = 3.750$; 2.274; $p = 0.0020$; 0.0416, respectively for frequency and duration, post hoc SD vs. SG and SS tb 2, 3 and 4 $p < 0.05$; mean ± standard error: 51.2 ± 5.2; 65.4 ± 5.3;

60.7 ± 5.2; 36.8 ± 4.2, for the SD group for each tb respectively; 38.5 ± 3.4; 42.3 ± 5.8: 34.5 ± 5.7; 14.1 ± 2.9, for the SG group for each tb respectively; 34.9 ± 4.9; 31.4 ± 5.6; 32.4 ± 7.2; 20.3 ± 4.5, for SS group for each tb respectively). Overall, SD mice also showed a higher self-grooming frequency (effect of social condition: ($F(2,21) = 4.857$; $p = 0.0134$, post hoc SD vs. SG and SS $p < 0.05$), while the duration of this behaviour was not significant ($F(2,21) = 2.340$; $p = 0.1105$).

2.3.3. Elevated Plus Maze - EPM

All subjects spent more time in the closed arms of the maze (effect of the zone: $F(1,21) = 36.952$; $p < 0.0001$, post hoc closed arms vs. open arms $p < 0.01$), however this behaviour was particularly evident for the SD group (interaction between zone and social condition: $F(2,21) = 4.463$; $p = 0.0185$, post hoc SD vs. SG and SS $p < 0.05$, see Fig. 2B) that was characterised by a decreased locomotion (effect of social condition: $F(2,21) = 6.125$; $p = 0.0051$, post hoc SD vs. SG and SS $p < 0.05$), a higher frequency and duration of rearing ($F(2,21) = 3.873$; 3.436; $p = 0.0300$; 0.0431, post hoc SD vs. SS $p < 0.05$, respectively for frequency and duration) and by a shorter latency and higher frequency of SAP (effect of social condition: $F(2,21) = 3.355$; 4.337; $p = 0.0461$; 0.0205, respectively for latency and frequency, post hoc SD vs. SG $p < 0.05$, for latency; SD vs. SG and SS $p < 0.05$, for frequency). In addition, SD together with the SS group, also showed a higher frequency and duration of self-grooming (effect of social condition: $F(2,21) = 4.694$; 3.134; $p = 0.0154$; 0.0556, post hoc SD and SS vs. SG $p < 0.05$, respectively for frequency and duration).

2.3.4. Forced Swim Test - FST

SD mice were overall characterised by a higher floating duration (effect of social condition: $F(2,21) = 4.014$;$p = 0.0334$, post hoc SD vs. SS $p < 0.01$, see Fig. 2C) associated to a lower struggling frequency (effect of social condition: ($F(2,21) = 4.461$; $p = 0.0243$, post hoc SD vs. SG $p < 0.05$). No difference was found among the three groups as for floating frequency ($F(2,21) = 2.659$; $p = 0.935$), struggling duration ($F(2,21) = 0.717$; $p = 0.4996$) and frequency and duration of swimming (effect of social condition: $F(2,21) = 2.106$; 1.665; $p = 0.1468$; 0.2131, respectively for frequency and duration).

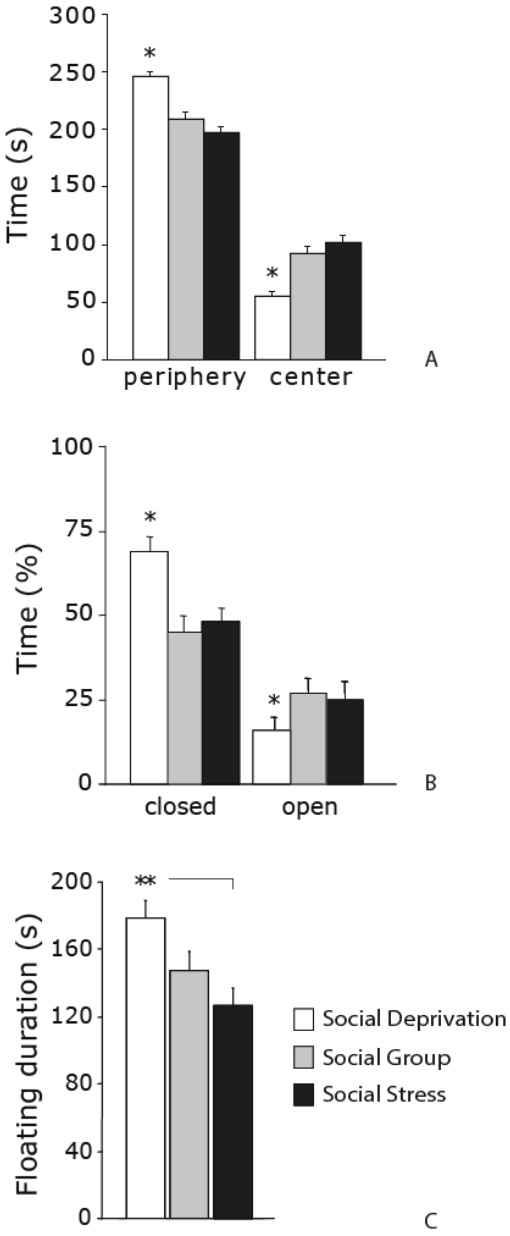

Figure 2. **Emotional and depression-like behaviours**. Socially deprived (SD) mice were characterised by higher emotional responsiveness towards a novel environment as well as by increased anxiety since they spent more time respectively in the periphery of the Open Field arena (A) and in the closed arms of the Elevated Plus Maze (B). In addition, socially deprived mice showed the longest floating duration in the Forced Swim Test indicating a depression-like behavioural profile (C). Data shown are mean + S.E.M. (n = 8 for each experimental group). *$p < 0.05$, **$p < 0.01$.

2.3.5. Social Interaction Test - SIT

Despite the prolonged social deprivation (3 weeks) SD mice did not show any signs of aggressiveness while were characterised by a higher frequency and duration of social investigation (main effect of social condition: $F(2,21) = 3.755$; 3.206; $p = 0.0403$; 0.0510, respectively for frequency and duration, post hoc SD vs. SG $p < 0.05$, both for frequency and duration, Fig. 3A) and spent a lower amount of time exploring the environment when compared to both SG and SS (effect of social condition on duration: $F(2,21) = 6.467$; $p = 0.0065$, post hoc $p < 0.05$, see Fig. 3B). Frequency of environmental exploration did not differ among the social conditions ($F(2,21) = 1.075$; $p = 0.3594$). SS mice were characterised by a lower frequency and duration of displacement behaviours (effect of social condition: $F(2,21) = 8.592$; 4.067; $p = 0.0019$; 0.0321, respectively for frequency and duration, post hoc SS vs. SD and SG $p < 0.05$, see Fig. 3C).

No difference was found as for frequency and duration of affiliative behaviours ($F(2,21) = 2.462$; 1.890; $p = 0.1095$; 0.1759, respectively for frequency and duration).

2.3.6. Corticosterone

Basal CORT levels did not differ as a result of social condition ($F(2,20) = 0.442$; $p = 0.6487$). The Social Interaction Test was effective in inducing the activation of the HPA axis in all the social conditions (effect of repeated measures: $F(1,20) = 295.963$; $p < 0.0001$, post hoc basal vs. stress $p < 0.01$), however CORT levels showed a higher increase in the SD group as a result of the social challenge (interaction between social condition and repeated measures: $F(2,20) = 5.569$; $p = 0.0120$, post hoc SD vs. SG $p < 0.05$ and SD vs. SS $p < 0.01$, Fig. 4A).

2.3.7. Adrenals/body weight ratio

Despite no difference in body weight among groups ($F(2,21) = 0.385$; $p = 0.6849$), SD mice showed the highest values for adrenals/body weight ratio ($F(2,21) = 7.103$; $p = 0.0044$, post hoc SD vs. SG and SS $p < 0.05$, Fig. 4B).

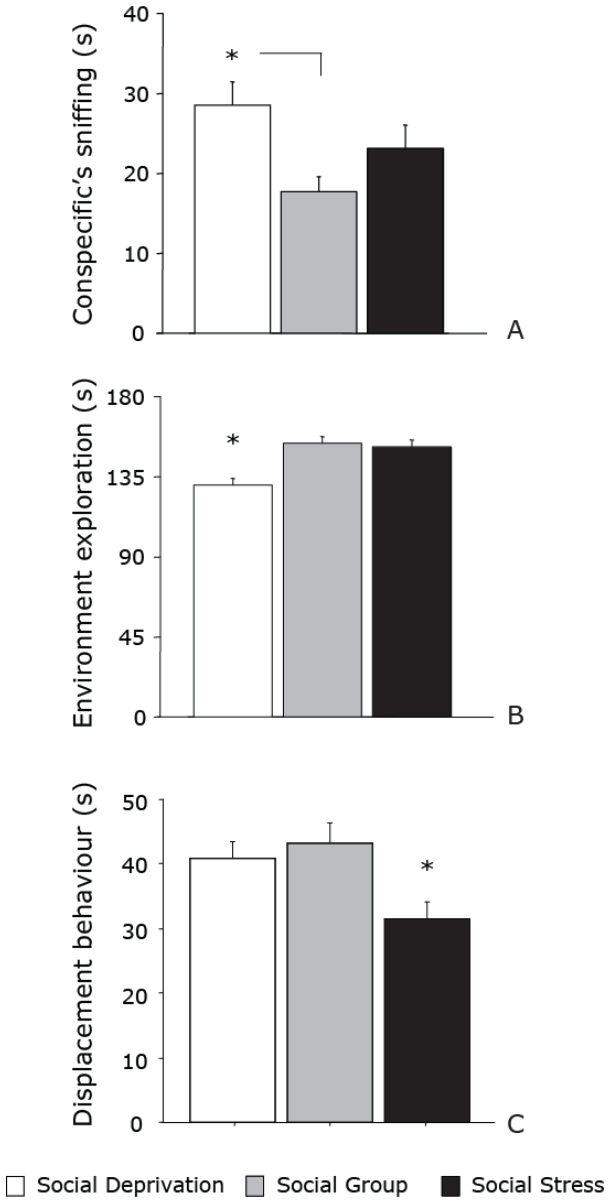

Figure 3. Social anxiety. Results from the Social Interaction Test allowed a fine discrimination of the effects produced by chronic exposure to the different social contexts on social behaviour. Socially deprived (SD) mice spent more time investigating an unknown conspecific partner (A) than the new environment (duration of environmental exploration) (B). The social stress (SS) group spent less time in displacement behaviours than socially deprived and social group (SG) mice (C). Data show mean + S.E.M. (n = 8 for each experimental group). *$p < 0.05$.

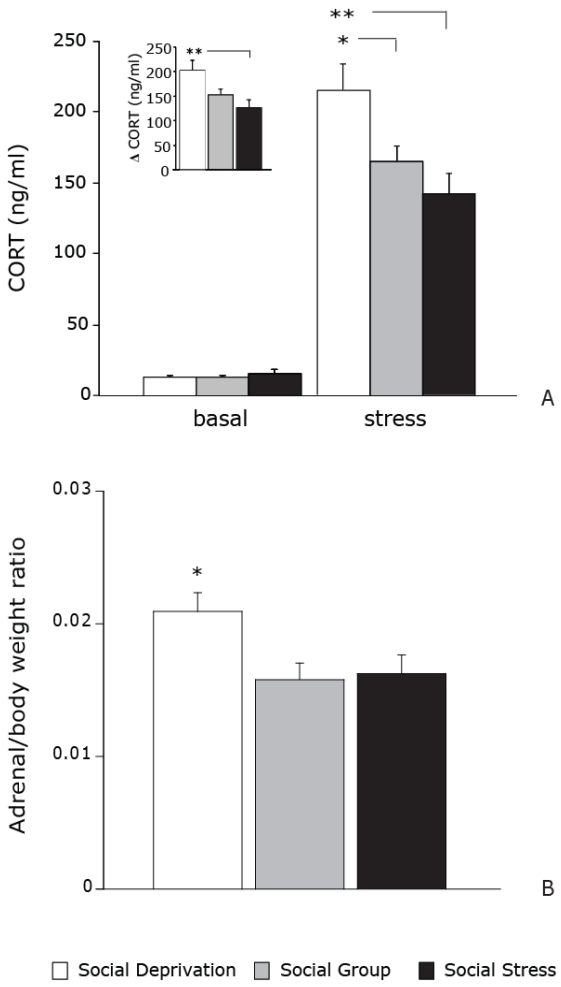

Figure 4. Neuroendocrine activation following a social challenge. Although all subjects showed an activation of the HPA axis following the social challenge, socially deprived (SD) mice were characterised by a greater increase in corticosterone levels. In the inset the difference between basal and stress corticosterone levels (delta) (A). (SD and social group - SG, n = 8; SS, n = 7). In addition, the social deprivation group showed the highest ratio adrenal/body weight, suggesting a chronic stress-induced hypertrophy of the glands (B). Data show mean + S.E.M. (n = 8 for each experimental group). *$p < 0.05$, **$p < 0.01$.

2.3.8. BDNF

Social condition was able to affect BDNF levels as measured in the frontal cortex, hippocampus, hypothalamus and midbrain ($F(2,21) = 16.091$; 12.667; 12.764; 4.937; $p = 0.0001$; 0.0002; 0.0002; 0.0175, respectively for each area, see Fig. 5A-D). In particular the SD group was characterised always by lower

levels of this neurotrophin (post hoc SD vs. SG $p < 0.05$, for frontal cortex and hippocampus; SD vs. SG and SS $p < 0.05$, for hypothalamus and midbrain). SD mice were also characterised by a tendency to reduced BDNF levels in the striatum when compared to SG subjects (effect of social condition: $F(2,21) = 2.822$; $p = 0.0821$). No difference was found among social conditions when adrenals levels of BDNF were analysed ($F(2,21) = 0.942$; $p = 0.4056$).

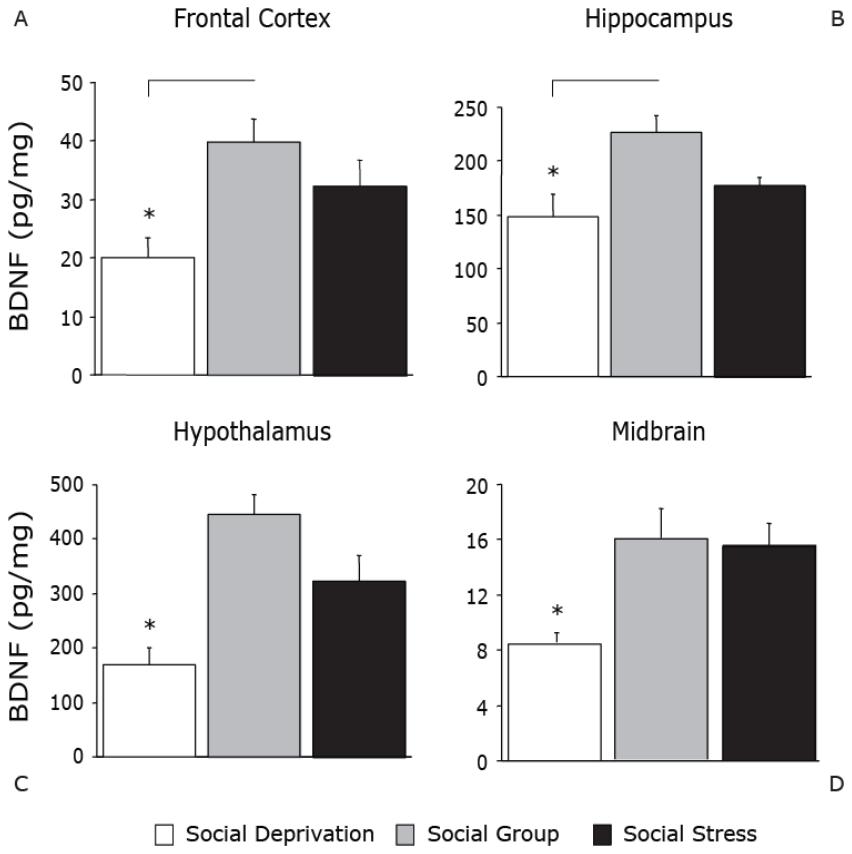

Figure 5. Brain levels of BDNF. The social deprivation (SD) group was characterised by lower BDNF levels when compared only to the social group (SG) in the frontal cortex (A) and hippocampus (B); by contrast, post hoc comparisons showed decreased BDNF levels in the hypothalamus (C) and midbrain (D) of SD mice when compared to both the group-housing conditions (SS and SG). Data show mean + S.E.M. (n = 8 for each experimental group). *$p < 0.05$.

2.4 DISCUSSION

Data from this study show that social deprivation, rather than social stress, leads to increased anxiety and depressive-like behaviours, accompanied by higher levels of corticosterone and reduced brain BDNF levels, all traits indicative of a disrupted emotional state. The results here reported also indicate that mice exposed to different social housing conditions use different behavioural strategies to cope with external challenges.

When emotional behaviours were assessed, SD mice were indeed characterised by the most anxious phenotype, compared to SG and SS mice, as they spent more time in the periphery and showed a higher self-grooming frequency in the Open Field. As for the Elevated Plus Maze test, SD mice spent more time in the closed arms of the maze and were characterised by reduced locomotor activity and by a lower latency and higher frequency of stretched-attend posture, a behaviour indicative of risk assessment. In addition, in the Forced Swim Test, SD subjects showed the highest floating duration and the lowest struggling frequency, commonly interpreted as measures of depressive-like behaviour (Porsolt et al., 1977).

In agreement with these behavioural data, when we investigated the effects of a social challenge on HPA axis activity, we found SD mice to be characterised by the highest CORT levels confirming an increased emotional profile in response to social stimuli (Gavrilovic and Dronjak, 2005). This result finds further support in data showing a higher adrenal/body weight ratio in SD subjects, a measure which is often associated with a hyperactive HPA axis (Ulrich-Lai et al., 2006). Worth noticing, adrenal enlargement, in association with increased glucocorticoids levels, have been found in many patients suffering from major depression (Rubin et al., 1987; Nemeroff et al., 1992).

Data from the Social Interaction Test confirm and enlarge the profile previously described. In addition, observations from this test allow a fine discrimination of the effects produced by chronic exposure to different social contexts on the social behaviour of the two group-housed conditions (SG and SS), so far characterised by similar behavioural (Open Field and Elevated Plus Maze) and neuroendocrine (CORT levels) profiles. In fact, although SG and SS groups reacted similarly to the environmental novelty (clean cage), SS mice were characterised by a lower arousal (displacement behaviours) than the SG and by an overall social profile intermediate between SG and SD subjects. Socially deprived mice, in fact, showed a greater social alertness, possibly due to the prolonged deprivation, as suggested by the association between increased exploration of the unknown conspecific and the high amount of displacement behaviours (digging and self-grooming). By contrast, it is possible to hypothesise that SS mice, which were exposed to a highly variable and unpredictable social context, were better prepared than SG and SD to cope with a social challenge (Pardon et al., 2004). These data also seem to suggest that for laboratory rodents, changes in an established social structure, at least as

assessed in the C57BL/6J strain, which is characterised by relatively low aggressiveness, might represent a form of social enrichment acting as boredom breaking, rather than a source of stress (Siegfried et al., 1981; Jones and Brain, 1987; Parmigiani et al., 1999).

A growing body of evidence shows that stress decreases the expression of BDNF, a neurotrophin involved in the neuronal plasticity of brain structures underlying mood circuitry, contributing to the atrophy of these areas, and that antidepressant treatment reverses or blocks these effects (Duman and Monteggia, 2006; Castren et al., 2007).

Our data show that social deprivation lowers BDNF in a number of limbic regions, including the hippocampus and frontal cortex in line with previous data indicating lower BDNF levels as a result of chronic stress (Tsankova et al., 2006). The hypothalamus, in particular, was characterised by the largest difference in BDNF levels between socially deprived and grouphoused mice. This piece of data is in agreement with previous reports suggesting a specific role of hypothalamic BDNF in regulating neuroendocrine responses to stress (Tapia-Arancibia et al., 2004). As for the midbrain, this area is central to antidepressant action as it is characterised by high levels of 5-HT, a neurotransmitter involved in mood disorders, which is directly related to BDNF function (Thoenen et al., 1991; Lindholm et al., 1994). In our study, disruption of the social structure was used as a chronic stressor capable to induce anxiety- and/or depressive-like symptoms as is often reported in stressprecipitated major depression (Cryan and Holmes, 2005; Nutt and Stein, 2006). At the end of the stressful procedure, SS mice developed a condition of anhedonia while SD mice did not change their preference for the 4% sucrose solution. Interestingly, the SG group showed a decrease in sucrose preference similar, and even stronger, to that observed in the SS group. On the days anhedonia was assessed (day 0 - following 1 week of social deprivation, 7, 14 and 21), SG and SS mice underwent an acute social deprivation, being exposed to single-housing condition for 5 h, in order to score individual sucrose consumption. By contrast, SD mice did not change their social condition. Several studies have suggested that social experiences are intrinsically valuable, even when there is no clear opportunity for the approaching individual to benefit (Panksepp and Lahvis, 2007; Trezza et al., 2010). For instance, subordinate mice express a strong preference for contact with a familiar dominant as long as a barrier prevents fighting behaviour (Van Loo et al., 2001). By contrast, many studies report a higher response to stimuli associated with reward in isolated rodents (Jones et al., 1990; Consorti et al., 1992; Coudereau et al., 1999). In line with these data, our results show that only subjects which underwent the Sucrose Preference Test, following separation from the social group, developed anhedonia suggesting that an acute social deprivation (5 h) is able to affect this behaviour in mice (D'Andrea et al., 2010). These same considerations apply to the fact that, despite most of the studies assess baseline preference for sucrose consumption in individually housed mice, this could represent a bias and should

be compared with baseline preference in group-housed mice (see for example Haenisch et al., 2009).

It is important to emphasise that anxiety and depression co-exist and that anxiety symptoms typically precedes depressive disorders (Ballenger, 1999; Ninan, 1999; Nutt and Stein, 2006). Thus, we cannot exclude that, with a longer social deprivation period, changes in anhedonia might ensue also in SD mice, while anxiety might become less evident.

Taken together, our findings indicate that mice exposed to different social housing conditions use different behavioural strategies to cope with external challenges. In addition they suggest that chronic social deprivation — rather than social instability — might trigger the emergence of anxiety, depression-like behaviours and neuroendocrine activation in association to reduced central levels of BDNF, at least as assessed in C57BL/6J adult male mice. Social isolation and lack of social support have deleterious effects on health, being

regarded as one of the most relevant causes of diseases in human and other mammalian species. Overall, our data are in line with previous findings suggesting social deprivation as valid model for isolation-induced psychopathologies in human beings (Bartolomucci et al., 2003). By contrast, mice living in social groups (SG and SS) displayed higher levels of neural plasticity markers, such as BDNF, underlying the pivotal role of a stimulating social context for the prevention of psychopathology and giving further support to the neurotrophi theory of depression (Wood and Rebec, 2009; Cirulli et al., 2010a).

Finally, results from this study indicate that anhedonia is a complex behavioural trait, which can be influenced by a number of variables, including the social context. Indeed, since social experiences are able to shape the sensitivity to rewards, a note of caution has to be raised when using the sucrose preference protocols used in many animal models of depression.

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CHAPTER 3

3. QUALITY AND TIMING OF STRESSORS DIFFERENTIALLY IMPACT ON BRAIN PLASTICITY AND NEUROENDOCRINE-IMMUNE FUNCTION IN MICE

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ABSTRACT
A growing body of evidence suggests that psychological stress is a major risk factor for psychiatric disorders. The basic mechanisms are still under investigation but involve changes in neuroendocrine-immune interactions, ultimately affecting brain plasticity. In this study we characterised central and peripheral effects of different stressors, applied for different time lengths in adult male C57BL/6J mice. We compared the effects of repeated (7 vs. 21 days) restraint stress (RS) and chronic disruption of social hierarchy (SS) on neuroendocrine (corticosterone) and immune function (cytokines and splenic apoptosis) and on a marker of brain plasticity (Brain-derived Neurotrophic Factor - BDNF -). Neuroendocrine activation did not differ between SS and control subjects, by contrast, the RS group showed a strong neuroendocrine response characterised by a specific time-dependent profile. Immune function and hippocampal BDNF levels were inversely related to hypothalamic-pituitary-adrenal axis activation. These data show a fine modulation of the crosstalk between central and peripheral pathways of adaptation and plasticity and suggest that the length of stress exposure is crucial to determine its final outcome on health or disease.

Keywords: Stress; HPA axis; Cytokines; Apoptosis; BDNF; Brain plasticity; Animal model; Mice.

3.1. INTRODUCTION

Stressful events are well known risk factors that can promote neurochemical changes ultimately involved in the pathophysiology of psychiatric disorders such as major depression (Kendler et al., 1999; de Kloet et al., 2007; Cirulli et al., 2009). Any change of the internal or external milieu may represent a source of stress triggering a complex and coordinated set of physiological responses involving (among others) the activation of the hypothalamus-pituitary-adrenal (HPA) axis (McEwen, 2000; Eskandari and Sternberg, 2002; McEwen, 2004). Although adaptive on the short run, prolonged exposure to glucocorticoids hormones (GC) secreted following stress, may exhaust the capacity of an organism to cope with further stressors and, given the catabolic nature of these adrenal glucocorticoids, lead to an impairment in brain plasticity (McEwen, 2004, 2008; Cirulli and Alleva, 2009).

Stress begins in the brain with the perception and interpretation of the stressful event and affects the brain itself as well as the rest of the body through plastic changes, leading to adaptation. The connection between central stress response pathways and peripheral targets involves the alteration of a number of neurochemical and/or inflammatory factors that ultimately affect neuronal functioning and/or survival (Tapia-Arancibia et al., 2004; Cirulli and Alleva, 2009). One of the most representative players implicated in these events is the neurotrophin Brain Derived Neurotrophic factor (BDNF), which is involved in synaptic and morphological plasticity of the brain both during development (with maximal levels during times of neuronal growth, differentiation and synaptogenesis) as well as at adulthood (Thoenen, 1995; Cirulli et al., 2010; Naert et al., 2011; Griesbach et al., 2012). High levels of this neurotrophin are found in the hippocampus, a brain region expressing also high levels of receptors for GC (GR) and playing a main role in the negative feedback regulation of the HPA axis, a pathway often disinhibited in depressed subjects (Sapolsky, 2001). A growing body of evidence shows that chronic stress decreases the expression of BDNF contributing to neuronal atrophy in the hippocampus and that antidepressant treatment reverses or blocks these effects, restoring brain plasticity (Tapia-Arancibia et al., 2004; Duman and Monteggia, 2006; Castren et al., 2007; Cirulli and Alleva, 2009).

By being able to directly affect HPA axis activity (Tapia-Arancibia et al., 2004; Jeanneteau et al., 2012) and being produced by cells outside the nervous system (including immune cells, adipocytes, endocrine and endothelial cells) BDNF has a key position in integrating neural, immune and endocrine responses to stress (Aloe et al., 1986; Nockher and Renz, 2005; Cirulli and Alleva, 2009). Indeed, the central nervous system and the immune system are known to be engaged in an intense bidirectional crosstalk which can be affected by stress and which involves multiple mediators, including cytokines and growth factors (Hayley et al., 2005). As an example, the immune signalling cytokines, particularly the pro-inflammatory ones such as interleukin-6 (IL-6) or

tumor necrosis factor-alpha (TNF-α) are elevated following stress exposure and can thwart brain plasticity eliciting depressive symptoms, which are amenable to antidepressant treatment (Hayley et al., 2005). However, the directionality of the effects of stress is still a matter of intense investigation: for instance, GC released in response to stress can act both enhancing and inhibiting immune responses and by decreasing or increasing levels of neurotrophins (Barbany and Persson, 1992; Smith et al., 1995; Marmigere et al., 2003; Dhabhar, 2009). Such opposite effects might coexist in the light of the fact that, during stress, multiple interacting mediators are activated in a nonlinear network influencing different systems and functions (McEwen and Gianaros, 2010). Factors such as the duration (acute vs. chronic) of stress as well as the time of exposure to GC, relative to the activation and time course of the immune response might differently impact on health outcome (Dhabhar, 2009). Progress in understanding the pathophysiology of stress would greatly benefit from further preclinical studies incorporating both the permissive as well as the inhibitory role of GC in immune-endocrine interactions and mimic conditions experienced in everyday life (McEwen et al., 1997)

Restraint stress (RS) and the chronic disruption of the social hierarchy (SS) are two of the most widely used experimental paradigms that can induce stress in mice. The first relies on a combination of psychological and physical stimuli and is considered a reliable model of severe stress in humans (Singh et al., 1999; Chiba et al., 2012); the latter represents a comprehensive and ethologically relevant paradigm inducing chronic stress and leading to anxiety and/or depressive-like symptoms as is often reported in stress-precipitated major depression (Cryan and Holmes, 2005; Nutt and Stein, 2006; Berry et al., 2012).Thus, the main aim of the present study was to characterise central and peripheral effects of different stressors, applied for different time lengths on neuroendocrine and immune responses in adult male C57BL/6J mice. Specifically, we compared the effects of repeated (7 vs. 21 days) RS and SS on neuroendocrine (circulating corticosterone) and immune (circulating cytokines and splenic apoptosis) function and on a marker of brain plasticity (hippocampal BDNF) in order to identify a specific neuroendocrine profile in response to a selective type of stress.

3.2. MATERIALS AND METHODS

3.2.1. Animals

Experimental subjects were adult male C57BL/6J mice purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival all animals were group-housed in the same room provided by air conditioning (temperature 21 ± 1°C, relative humidity 60 ± 10%), in transparent Plexiglas cages (29 cm x 12 cm x 14 cm), under a reversed 12/12 h light/dark cycle with lights off from

0800 to 2000 h. Pellet food (standard diet Altromin-R, Rieper, Italy) and tap water were continuously available. All stressors were administered randomly throughout the active phase of the day. A Social Interaction Test was used as a challenge to assess HPA axis response following the social stress procedure and took place between 1700-2000 h, i.e. during the corticosterone (CORT) circadian trough. All subjects were sacrificed at the end of the stress procedure. Animal handling and experimental procedures were performed in accordance with the EC guidelines (EC Council Directive 86/609 1987) and with the Italian legislation on animal experimentation (Decreto L.vo 116/92).

3.2.2. Experimental Procedures

3.2.2.1. Experiment I: effects of restraint stress on neuroendocrine and immune responses. Experimental subjects were 15 mice divided into three groups: 7 days restraint stress (RS7, n=5); 21 days restraint stress (RS21, n=5) and unhandled controls (CTRL, 5 subjects left undisturbed in their home cage). All subjects were group-housed with subjects undergoing the same treatment condition. The restraint procedure consisted in removing subjects from their home cage and putting each of them in a conical 50 ml falcon tube, provided with holes for breathing, on a laboratory bench under dim light for 3 consecutive hrs/day. The stress was administered each day at random times in order to prevent habituation to the procedure. Animals from the RS21 were used to assess stress-related changes in CORT levels so to have repeated measures for each subject during days 1, 7 and 21. On these days the procedure was administered at a fixed times in order to take into account circadian rhythm, i.e. from 1700-2000. Blood samples were collected by tail nick at 0 (basal) and 180 min from the onset of stress (i.e. at 2000). At the end of stress all mice (CTRL, RS7 and RS21) were sacrificed, trunk blood was collected to assess levels of the pro-inflammatory cytokines Interleukin 6 (IL-6) and Tumor Necrosis Factor-alpha (TNF-α), and of the anti-inflammatory cytokine Interleukin 10 (IL-10), (Frick et al., 2008; Frick et al., 2009). Brains and spleen were dissected out in order to assess respectively hippocampal BDNF levels and lymphocyte apoptosis.

3.2.2.2. Experiment II: effects of social stress on neuroendocrine and immune responses. Experimental subjects were 48 adult male mice divided into three groups: 7 days social stress (SS7, n=16), 21 days social stress (SS21, n=16) and controls (CTRL, 16 subjects group-housed). All mice undergoing the SS procedure were ear-marked and housed into 4 cages (4 mice/cage) and social structure was disrupted twice a week for one or three weeks by replacing one mouse with a novel unfamiliar selected randomly from another cage (Avitsur et al., 2003; Avitsur et al., 2006). Sawdust was replaced at the same time in all cages. Control mice were also ear-marked and housed in stable groups of 4

mice/cage. Cages were cleaned and sawdust replaced twice a week mimicking the handling procedure of the SS groups (Berry et al., 2012).
The activity of the HPA axis was assessed in response to a 20 minutes acute stress (Social Interaction Test) and blood samples for CORT evaluation were collected from 8 subjects per group (CTRL, SS7, SS21) right before (basal) and 30 min following the end of stress. Briefly, the night before the Social Interaction Test all subjects were individually housed to stimulate social interactions (Terranova et al., 1993; Cirulli et al., 1996; Panksepp et al., 2007). On the day of test, mice were placed in a novel cage, identical to the holding cage, ideally subdivided in three equal parts, with an unfamiliar conspecific of the same strain, weight and sex that had been previously isolated (standard opponent). Standard opponents were marked with a yellow, scentless and nontoxic paint (Berry et al., 2012).
At the end of the test mice that did not undergo the Social Interaction test (5 mice for each group, randomly chosen) were sacrificed, trunk blood was collected to assess also levels of IL-6, TNF-a and IL-10 (Frick et al., 2008; Frick et al., 2009). Brains and spleen were dissected out, in order to assess respectively hippocampal BDNF levels and lymphocyte apoptosis.

3.2.2.3. Radioimmunoassay for corticosterone determination – RIA. Blood samples (100 µl, approximate volume) were collected individually in potassium EDTA coated tubes (1.6 mg EDTA/ml blood, Sarstedt, Germany). All samples were kept on ice and later centrifuged at 3000 rpm for 15 min at +4°C. Blood plasma was transferred to Eppendorf tubes for CORT determination and stored at -20°C until further analysis. CORT was measured using a commercially available radioimmunoassay (RIA) kit containing 125Iodine-labeled CORT; 5 µl of plasma were sufficient to carry out CORT measurement. Sensitivity of the assay was 0.125 mg/dl, inter- and intra-assay variation was less than 10 and 5%, respectively (MP Biomedicals Inc., CA, USA). Vials were counted for 2 min in a gamma-scintillation counter (Packard Minaxi Gamma counter, Series 5000).

3.2.2.4. BDNF measurement. BDNF evaluation was carried out with an enzyme-linked immunosorbent assay kit (BDNF Emax® ImmunoAssay System number G7610, Promega, Madison, Wisconsin, USA) following the instructions provided by the manufacturer. Following sacrifice brains were quickly removed and the hippocampus was dissected out and immediately stored at -80 °C until used. Brain tissues were homogenized in a lysis buffer and centrifuged at 14000 rpm, and the supernatant was used for BDNF analyses. Briefly, BDNF standard and brain samples were distributed in 96-well immunoplates pre-coated with monoclonal anti-mouse BDNF antibody (100 mµ/well) and incubated for 2 h at room temperature. After washing plates were incubated with an anti-human BDNF antibody for 2 h at room temperature. The plates were washed again and then incubated with an anti-IgY horseradish peroxidase (HRP) for 1 h at room temperature. Tetramethylbenzidine (TMB)/peroxidase substrate solution was

added to the wells to produce colorimetric reaction measured at 450 nm with a microplate reader (Dynatech MR 5000, Dynatech Laboratories, Chantilly, Virginia, USA). BDNF concentrations were determined from the regression line for the BDNF standard incubated under similar conditions in each assay. The sensitivity of the assay was about 15 pg/mg of BDNF, and the cross-reactivity with other related neurotrophic factors (NGF, NT-3, and NT-4) is considered nil (Aloe et al., 1999).

3.2.2.5. Cytokines determination. Quantitative evaluation of TNF-α, IL-6 and IL-10 in sera from trunk blood of stressed and control mice was determined by ELISA kits (R&D Systems, Inc. Minneapolis, USA) according to the manufacturer's instructions. Briefly, standards, controls and sera were placed into the wells and incubated 2 h at room temperature. After washing 5 times, the enzyme-linked polyclonal antibody specific for mouse cytokines was added to the wells and then, after washing, the substrate solution was added. The enzyme reaction was read at 450nm (correction wavelength set at 570nm). The samples values were read off the standard value.

3.2.2.6. Splenocytes apoptosis. Spleens were gently removed and suspended in ice–cold culture RPMI-1640 medium (GIBCO BRL, Grand Island, NY). Splenocytes were isolated from mice spleen by flushing 5 ml of RPMI-1640 medium into spleen by needle and syringe. Cells were then centrifugated at 1200 rpm in order to remove cellular debris. Cells were resuspended in supplemented RPMI 1640 and counted on a hemocytometer in trypan blue to ensure viability. Average viability was >90%. Splenocytes were then cultured in RPMI-1640 medium with 10% FBS (Euroclone, Pero, Italy), 2mM glutamine (Sigma, St Louis, MO) and 50μg/ml gentamycin (Sigma). Apoptosis was measured after 1h of culture. Apoptosis was quantified using FITC-conjugated annexin V (AV) and propidium iodide (PI) apoptosis detection kit (Marine Biological Laboratory, Woods Hole, MA) according to the manufacturer's protocol. Reported data are referred to AV-positive apoptotic cells. AV binds to phosphatidylserine which is exposed at the outer surface of the cell membrane already at early stages of apoptosis and remains so during the subsequent process of apoptosis. By defining apoptotic cells as those cells staining with AV, irrespective of PI staining, we were able to detect early (AV+/PI- cells) as well as late (AV+/PI+ cells) apoptotic cells. In this study we analyzed specifically "early apoptosis" in which the nuclear changes are observed first, in contrast to the changes seen in the later stages of apoptosis and then in the necrosis, which usually begin with cell membrane damage (Wyllie et al., 1980; Perandones et al., 1993). Acquisition was performed on a FACSCalibur cytometer (BD Immunocytometry Systems) and 50.000 events per sample were run. Data were analyzed using the Cell Quest Pro (BD Immunocytometry Systems) software.

3.2.2.7. Statistical analysis. Data were analyzed using parametric analysis of variance (ANOVA) with ‘‘condition’’ (control and stress) as between-subjects factor (BDNF, cytokines, apoptosis, CORT) and “day” (1, 7, 21) and ‘‘time” (0 and 180) as within-subject repeated measures (CORT assessment only for the restraint stress). *Post hoc* comparisons were performed using the Tukey’s test. Statistical analysis was performed using Statview II (Abacus Concepts, CA, USA). Data are expressed as mean + SEM. A significance level of 0.05 was chosen.

3.3. RESULTS

3.3.1. Experiment I - Restraint stress

3.3.1.1. Corticosterone. Restraint stress was effective in challenging the HPA axis. In fact, RS subjects showed overall higher CORT levels (main effect of condition: $F(1,8) = 17.917$, $P = 0.0029$) compared to controls, particularly 180 minutes from the onset of stress (interaction between condition and time: $F(1,8) = 12.022$, $P = 0.0085$). Moreover, a blunted HPA axis response characterised RS subjects on day 7 (main effect of days $F(1,8) = 3.618$ $P = 0.0505$; see Fig. 1 A).

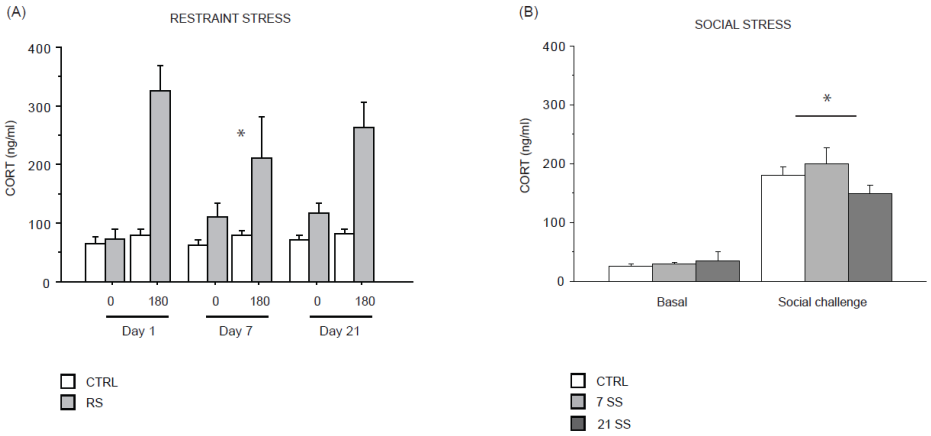

Figure 1. Effect of restraint stress on CORT secretion in mice. All subjects undergoing RS showed a reduced response of the HPA axis on day 7 (A). Effect of social stress on CORT secretion in mice. The response to an acute challenge (represented by the Social Interaction Test) was effective in inducing an increase in CORT secretion in all groups, with no differences in relation to social stress exposure (B). Results are presented as mean + S.E.M. *$P < 0.05$.

3.3.1.2. BDNF. BDNF evaluation was performed on 4 mice in each group since values from some subjects (1 subject for each group) were found to be outliers

and were therefore discarded from the analysis (Grubbs' test performed by GraphPad Software).
A time-dependent effect of RS was found for hippocampal BDNF levels (see Fig. 2). In particular, *post hoc* comparisons show a decrease in BDNF levels following 21 days of RS compared to RS7 (main effect of condition $F(2,9) = 4.164$, $P = 0.0500$). The latter group did not differ from the CTRL subjects. However, it is worth noticing that the lack of difference between these two groups might be related to a reduced power of the statistical test (0.574) suggesting that this result suffers from a low number of experimental subjects (only 4 animals per experimental group) possibly masking other significant trends (RS7 vs CTRL).

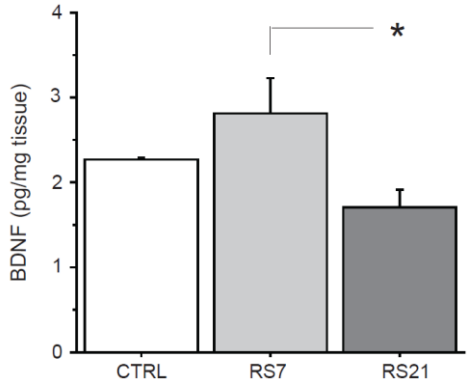

Figure 2. Effects of restraint stress on hippocampal BDNF levels. BDNF levels were decreased following a chronic 21 days restraint procedure compared to 7 days of repeated restraint. Data shown are mean + S.E.M. *$P < 0.05$.

3.3.1.3. Cytokine production. Following 7 days of restraint stress a tendency to increase was observed for levels of IL-6 ($F(2,12) = 3.480$; $P = 0.0643$, Fig. 3 A). This trend reached statistical significance when assessing TNF-α (main effect of condition: $F(2,12) = 5.558$; $P = 0.0196$, Fig. 3 B) that returned to basal levels after 21 days. By contrast, IL-10 increased only after 21 days of restraint (main effect of treatment: $F(2,12) = 5.345$; $P = 0.0219$, Fig. 3 C).

3.3.1.4. Splenocytes apoptosis. Splenocytes apoptosis slightly decreased after 21 days of restraint (main effect of condition: $F(2,12) = 8.597$; $P = 0.0048$, *post hoc* RS 7 days vs RS 21 days $P < 0.01$; see Fig. 3 D). No difference was found between CTRL and RS7.

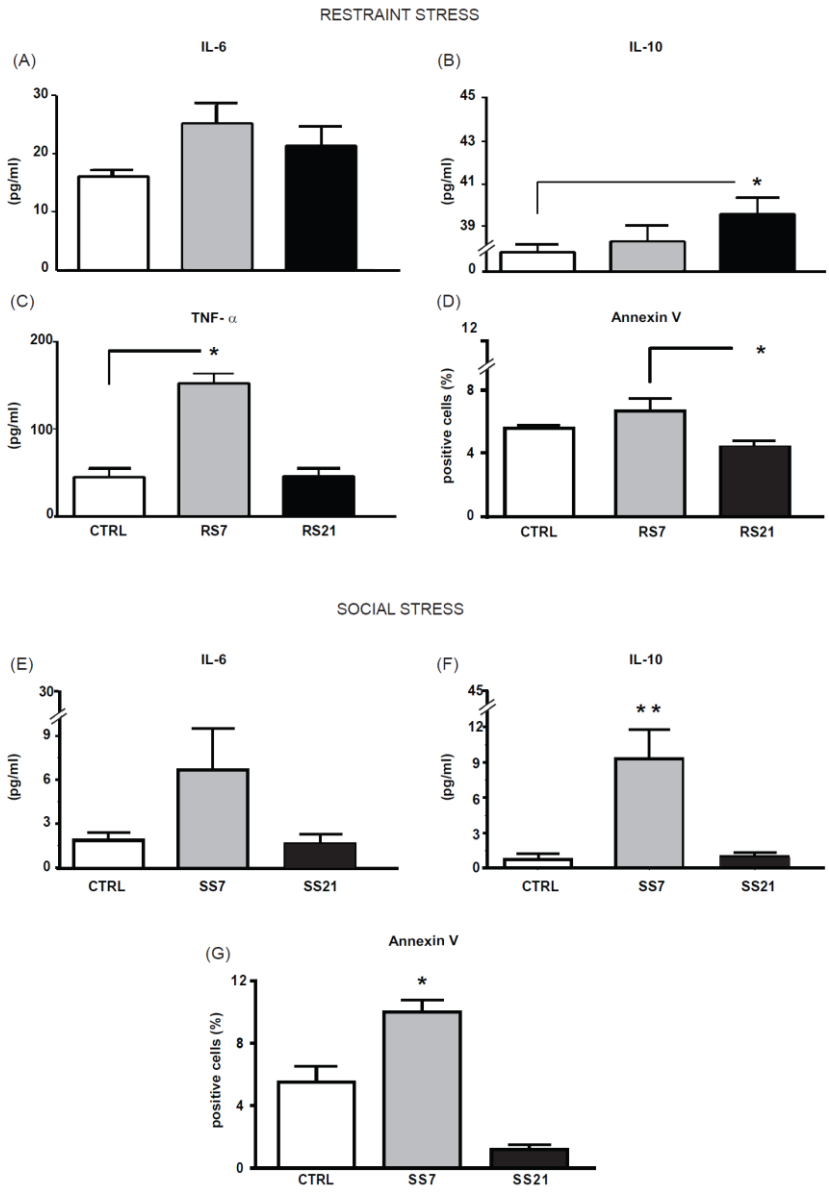

Figure 3. Effect of RS and SS on the immune system response. *Restraint stress procedure*. Following 7 days of restraint stress the pro-inflammatory cytokine TNF-α increases (**B**) while the increase in IL-6 during days 7 and 21 just missed statistical significance (**A**); by contrast the anti-inflammatory cytokine IL-10 increased following 21 days (**C**). The percentage of apoptotic splenocytes was found to be decreased following 21 days of stress (**D**). *Social stress procedure*. Levels of IL-10 were increased already after 7 days of the SS procedure (**F**). Splenocytes apoptosis was increased after 7 days of SS and decreased following 21 days (**G**). No difference is evident as for levels of IL-6 (**E**). Results are presented as mean + S.E.M.** $P < 0.01$, * $P < 0.05$

3.3.2. Experiment II - Social stress -

3.3.2.1. Corticosterone. The Social Interaction Test was effective in inducing the activation of the HPA axis in all groups, regardless of their stress history (effect of social challenge: $F(1,29) = 153{,}515$; $P < 0.0001$, Fig. 1 B).

3.3.2.2. BDNF. Social stress, per se, did not affect hippocampal BDNF levels (no main effect of condition: $F(2,12) = 2.015$ $P = 0.1759$, data not shown).

3.3.2.3. Cytokine production. Social condition did not affect significantly the production of IL-6, even if a slight increase after 7 days of social stress was observed, (effect of condition: $F(2,12) = 3.075$ $P = 0.0835$, see Fig. 3 E). By contrast, serum levels of IL-10 increased after 7 days of the social stress procedure (main effect of condition: $F(2,12) = 13.217$ $P = 0.0009$, see Fig. 3 F). Differences in serum TNF levels between control and social stressed mice appeared undetectable (data not shown).

3.3.2.4. Splenocytes apoptosis. Splenocytes apoptosis increased after 7 days of social stress and decreased following 21 days (main effect of treatment: $F(2,12) = 47.932$; $P < 0.0001$, see Fig. 3 G).

3.4. DISCUSSION

Data from this study show that chronic RS is a powerful stressor eliciting strong neuroendocrine and immune responses and that brief *vs.* prolonged exposure to this stress result in a differential activation of these systems in mice. In addition, we were able to identify a specific neuroendocrine-immune profile associated to specific changes in hippocampal BDNF levels. Results suggest a fine modulation of the crosstalk between central and peripheral pathways of adaptation and plasticity and that the length of stress exposure is crucial to determine its final outcome on health or disease.

Allostasis - or "stability through change" - is defined as any neural, neuroendocrine and immune activation leading to adaptation in the face of stressful challenges (McEwen, 2000). While in the short run activation of these systems is essential to the maintenance of homeostasis and survival yet, over longer time intervals, it imposes a cost - allostatic load - that can accelerate disease processes or participate to pathological changes associated, among others, to immunosuppression (McEwen, 2000). When we studied the characteristics of the diverse stressors applied for different lengths of times, we found that upon prolonged exposure to RS (21 days) an increase in the immunogenic/allostatic load was observed, mirrored by a peak in CORT levels

comparable to that observed on day 1. This was associated to a suppression of the immune system with decreased levels of the pro-inflammatory cytokine TNF-α and increased levels of the anti-inflammatory cytokine IL-10. In addition, a decrease in hippocampal BDNF levels was found, suggesting a reduction in the ability to cope with prolonged stress (brain plasticity).

Analyzing more in detail the effects of RS, we found that, following 7 days of this procedure, CORT elevation was significantly lower than on the first stimulation (day1), suggesting an habituation of the system to the chronic procedure, as previously shown (Bhatnagar et al., 2002). This effect, which appears to be mediated by limbic regions (Cirulli and Alleva, 2009), is likely to have consequences for the functioning of a number of GC-sensitive systems, including the immune system. Indeed, reduced CORT levels could disinhibit immune function, leading to a pro-inflammatory response, as suggested by the increase in the levels of TNF-α.

A number of evidence support the hypothesis that a moderate increase in the levels of pro-inflammatory cytokines, such as TNF- α might result in an overall 'priming effect' on the immune system, leading to better abilities to cope with further physiologic/stressful stimuli (Ehrke et al., 1998; Ehrke et al., 2000; van Horssen et al., 2006; Dhabhar, 2009; Watters and O'Connor, 2011). Worth noticing, the response to stress observed does not only involve peripheral targets, but also extends to central mediators. In fact, after 7 days of RS, hippocampal BDNF protein levels showed a trend towards an increase, possibly reflecting a neuroprotective mechanism. By contrast, after 21 days of RS, BDNF levels were found to be decreased and this was associated to an augmented anti-inflammatory response by the immune system with increased IL-10 levels and a return of TNF-α to basal. It must be emphasized that while acute changes in BDNF levels might represent a coping response to stressful events, and thus being beneficial, prolonged exposure to stressors and increased allostatic load would lead to detrimental effects as reduced BDNF signalling in the adult brain may be involved in the pathophysiology of psychiatric disorders (Altar, 1999; Castren, 2005; Sen et al., 2008).

A fine regulation of apoptosis might positively affect optimal immune function. This is achieved by maintaining lymphocyte homeostasis by a continuous removal of cells that have been activated once they have served their function. Therefore, inappropriate induction of such a mechanism could result in a variety of pathological effects such as autoimmune diseases, while the maintenance of physiologically regulated levels of apoptosis might exert a beneficial/protective effect (Avula et al., 2001). In this context, the observed decrease in apoptosis levels following 21 days of RS, suggests a long-term impairment of the immune system response.

Compared to RS, SS resulted in an overall lower response of the HPA axis as well as of the immune system. Differences were both quantitative and qualitative. In particular, no change in TNF- α could be detected, while an earlier increase in IL-10 was observed compared to RS, suggesting an

anticipated anti-inflammatory reaction in response to this specific stressor. Differently from RS data, splenic apoptosis increased after 7 days of SS, suggesting that it might represent a reliable early stress-sensitive physiological marker.

Taken together, data from this study clearly indicate a differential role of psychophysical vs. social and of brief vs. prolonged stress on neuroendocrine and immune function suggesting that the quality and the extent of the stress period are crucial in determining individual neuroendocrine-immune responses to external challenges. In addition, and more intriguingly, all these peripheral responses were associated to specific changes in hippocampal BDNF levels. We hypothesize that this neurotrophin might represent a key modulator of neuro-immuno-endocrine pathways, playing a pivotal role in the orchestration/maintenance of the brain and peripheral plasticity leading to optimal coping strategies to stressful events (Cirulli and Alleva, 2009). Future studies should employ pharmacological challenges aimed at investigating such interactions.

While this is a first attempt to mimic some of the qualitative and temporal features of "stress", studies are ongoing to extend the range of mediators analyzed and the peripheral targets, to evaluate more extensively the role of acute vs. chronic stressors on neuroendocrine and immune function. In addition a thorough characterization of the specific changes occurring in brain plasticity in other regions involved in neuroendocrine-immune integration will help elucidating the mechanisms underlying the beneficial/pathological effects of stress increasing the translational value of these studies.

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3.5. REFERENCES

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CHAPTER 4

4. SOCIAL ISOLATION PROMOTES BREAST CANCER PROGRESSION THROUGH A BDNF-NEUROENDOCRINE AXIS

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ABSTRACT

Breast cancer is the second leading cause of cancer death and the most common carcinoma among women. A wealth of data has been so far collected to indicate that stressful conditions might accelerate tumor onset and progression although the mechanisms are hard to tackle. We tested the hypothesis that psychological stress might trigger a Brain-derived Neurotrophic Factor (BDNF) - neuroendocrine cascade leading to cancer progression. To this aim, we assessed the effects of a prolonged social isolation (6 months) in a transgenic mouse model of breast cancer susceptibility (MMTVNeuTg). In addition, by the time mice started developing tumors (about 5 months), they were exposed to a further stressful challenge (restraint stress). Data indicate that in MMTVNeuTg mice social isolation accelerates disease onset, earlier detection of lumps in the mammary glands and tumor development being accompanied by significant histological modifications in the mammary fat pad. A higher rate of splenocyte apoptosis was also found, suggesting an impairment of immune function. In general, effects were magnified in those subjects experiencing both isolation and restraint stress and were accompanied by low BDNF expression in the hypothalamus as well as increased leptin and decreased adiponectin expression in peripheral fat tissue, suggesting the involvement of a neuroendocrine/metabolic cascade in tumor progression. From a behavioural point of view, MMTVNeuTg mice were characterised by greater anhedonia, a behavioural trait indicative of a depressive-like state, which was exacerbated by restraint stress. Overall, data indicate that social isolation stress plays a pivotal role in promoting disease onset and progression in this animal model and that BDNF and metabolic signals are mechanistically involved in translating stressful experiences into disease susceptibility.

Keywords: Breast cancer; Stress; BDNF; Leptin; Adiponectin; Cytokines; Fat; Depression

4.1. INTRODUCTION

Breast cancer is one of the most common cancers among women with greater than 1.300.000 cases and 450.000 deaths each year worldwide. Although most cancer research is mainly focused on molecular and cellular interactions, there is now clear evidence that psychological factors can influence disease progression. Stressful conditions, in particular, are a recognized risk factor for cancer in the clinic and are linked to breast cancer aggressiveness in animal models (Hermes et al., 2009; Volden et al., 2013). Lack of social support, a known psychosocial stressor, has been associated with pain, fatigue, lethargy and higher inflammation among cancer patients (Gagliardi et al., 2009; Hughes et al., 2014), while in mouse models of cancer, exposure to social isolation alters tumor and spleen macrophage populations and potentiates tumor growth and metastasis (Hermes et al., 2009; Williams et al., 2009; Madden et al., 2013; Volden et al., 2013). It is important to consider that 15-50% of breast cancer patients meet diagnostic criteria for anxiety and depression (Burgess et al., 2005; Gagliardi et al., 2009; Lutgendorf and Sood, 2011) and that the tumor itself can produce depressive symptoms, through the expression of cytokines and growth factors, generating a positive feedback loop between coping style and cancer progression (Sephton et al., 2009).

Stress begins in the brain with the perception and elaboration of an external event as stressful and affects the brain itself, as well as the rest of the body through plastic changes, leading to adaptation. The connection between central stress response pathways and peripheral targets involves the activation of a number of neurochemical and/or inflammatory mediators that ultimately can affect cell function and/or survival including cytokines and growth factors (Hayley et al., 2005; Cirulli and Alleva, 2009; Moreno-Smith et al., 2011; Capoccia et al., 2013). Depressed metastatic breast cancer patients are often characterised by blunted cortisol awakening responses reflecting a physiologic profile associated with chronic stress (Sephton et al., 2009). These endocrine changes have the potential to override the immune defense or act directly on the tumor microenvironment, affecting cancer progression. Evidence in animal models suggests that prolonged exposure to stress – with a persistent activation of the hypothalamic-pituitary-adrenal axis and the consequent suppressed activity of the immune system, mediated by glucocorticoid (GCs) hormones, can render subjects more susceptible to disease, including neoplastic processes (Cavigelli et al., 2008).

In addition to classic neuroendocrine factors, BDNF has been recently recognized as playing an important role in integrating neural, immune and endocrine responses to stress (Cirulli and Alleva, 2009; Capoccia et al., 2013). This neurotrophin is implicated in the pathophysiology of the nervous system, including the etiopathogenesis of depression, while in the periphery it regulates metabolic responses to stress (Cirulli and Alleva, 2009) and has been recently involved in tumor development and progression (Cirulli and Alleva, 2009; Yang

et al., 2012; Liu et al., 2014). Indeed, recent data indicate that increased expression of hypothalamic BDNF, resulting from environmental enrichment, activates sympathetic innervation of white adipose tissue, leading to important changes in circulating levels of adipokines, ultimately resulting in tumor suppression (Cao et al., 2010; Cao and During, 2012; Liu et al., 2014). The crosstalk between brain and peripheral targets could also be mediated by other stress-responsive genes, also playing a role in metabolic pathways, such as neuropeptide Y (NPY), agouti-related peptide (AgRP), serum and glucocorticoid-regulated protein kinase 1 (SGK1) and insulin receptor (InsR) (Hou et al., 2011; Anacker et al., 2013; Baver et al., 2014).

While the effects of environmental enrichment – and the role of BDNF – on tumor suppression have been previously documented (Cao et al., 2010; Cao and During, 2012), much less research has been dedicated to investigate whether an opposite condition, such as social isolation, might instead promote cancer progression. Social isolation has been previously shown as a relevant stressor capable to promote breast tumor growth in epidemiological studies (Gagliardi et al., 2009; Hughes et al., 2014) and it has been employed to examine potential long-lasting effects on behavioural and biological responses to stress in mice (Williams et al., 2009; Ma et al., 2011; Berry et al., 2012). However, none of these studies has taken into account the role of BDNF and of related metabolic signals in the effects of stress on tumor biology.

In this paper we tested the effects of social isolation on tumor progression in a transgenic mouse model of breast cancer. The MMTVNeuTg mice specifically overexpress ErbB2 (Her2/Neu), a member of the ErbB family of receptor tyrosine kinases (RTKs), which is overexpressed in advanced breast cancers with an especially poor clinical prognosis (Slamon et al., 1987; Perou et al., 2000; Ursini-Siegel et al., 2007).

The hypothesis underlying this work is that stress would act on the hypothalamus, activating neuroendocrine pathways, thwarting immune function and modifying the expression of genes involved in the response to stress, including BDNF, affecting the production of circulating cytokines and adipose tissue-derived adipokines, promoting tumor progression (Berry et al., 2012; Capoccia et al., 2013; Wosiski-Kuhn et al., 2014). Often an acute stressful event can be superimposed upon a long-term vulnerability. Thus we assessed whether a 21-day restraint stress procedure, experienced by the time tumor growth was well established, would worsen the effects of a long-term social isolation. Depressive symptoms often accompany breast cancer patients as a result from situational fear related to diagnosis and prognosis or may be directly related to the effects of the tumor and Lamkin and co-workers have recently shown an increase in endophenotypes of depression following tumor implantation (Lamkin et al., 2011). Thus a further aim of this study was to assess whether changes in tumor biology, resulting from stress exposure, would also be accompanied by an increase in endophenotypes of depression, previously validated in animal models, such as anhedonia.

4.2. MATERIALS AND METHODS

4.2.1. Animals

Experimental subjects were ErbB-2(Neu)TgMMTV-ErbB-2 (FVB background) and FVB mice purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) via Charles River (Calco, Italy). Upon arrival, all animals were housed in the same room provided by air conditioning (temperature 21 ± 1°C, relative humidity 60 ± 10%), in transparent Plexiglas cages (29 cm × 12 cm × 14 cm), under a reversed 12/12 h light/dark cycle with lights off from 0800 to 2000 h. Pellet food (standard diet Altromin-R, Rieper, Italy) and tap water were continuously available. More in detail, MMTVNeuTg subjects develop hyperplasia at 5 months and focal adenocarcinoma and lung metastases at 7 months. Mice were used to fulfill the criteria of: i) late latency of tumor occurrence to allow chronic stress treatment and ii) extensively characterised tumor model with respect to morphological and histological features. Subjects in this experiment were all females, 36 MMTVNeuTg and 40 FVB (wild type – WT-, control group). Half of them were socially isolated at weaning to model long-term stress; to test the effect of a further stressful challenge experienced at adulthood half of them underwent restraint stress (RS) by the time they started developing tumors (5 months) (n = 9 MMTVNeuTg group-housed control; n = 10 MMTVNeuTg group-housed RS; n = 8 MMTVNeuTg isolated control; n = 9 MMTVNeuTg isolated RS; n = 10 FVB group-housed control; n = 10 FVB group-housed RS; n = 10 FVB isolated control; n = 10 FVB isolated RS). At the end of stress procedures, all mice were sacrificed, hypothalamus and adipose tissue were dissected out in order to assess, respectively, BDNF, SgK1, AgRP, InsR, Npy and leptin and adiponectin gene expression. Lymphocyte apoptosis was assessed in the spleen. In order to assess "basal" physiological responses, before tumor development, a dedicated batch of 2-month-old females was divided into unhandled controls (n = 10 MMTVNeuTg; n = 4 FVB) and acute RS (n = 8 MMTVNeuTg; n = 7 FVB) undergoing a single stressful challenge for 3 hours. Unhandled controls were sacrificed one week following acclimation in the animal facility while, on the same day, RS subjects were sacrificed 1 hr after the end of the stress procedure. Hypothalamic BDNF gene expression, adipose tissue leptin and adiponectin gene expression and splenocytes apoptosis were assessed.

Animal handling and experimental procedures were performed in accordance with the EC guidelines (Directive 86/609/EEC) and with the Italian legislation on animal experimentation (Decreto L.vo 116/92).

4.2.2. Experimental procedures

4.2.2.1. Assessment of breast lumps and tumor masses. We assessed the effects of a prolonged social isolation (6 months) starting at weaning in MMTVNeuTg mice. In order to test the effect of further stressful challenges experienced at

adulthood, experimental subjects underwent restraint stress by the time they started developing tumors (5 months). We performed the assessment of lumps and tumor masses in the ten mammary glands of each mouse through palpation performed once a week from the age of 16 weeks until week 26 (Fig. 1). Breast lumps and tumor masses were then calculated as follows:

$$\left(\frac{\text{Tot n. breast lumps/tumor masses for each group}}{\text{n. mice for each group x 10}} \right) \text{X } 10$$

To control for the handling procedure due to palpation, all mice were palpated, including FVB.

4.2.2.2. Restraint stress. Each mouse was introduced in a conical 50mL falcon tube, provided with holes for breathing, adjusted on a laboratory bench with tape to prevent rolling. The stress was administered each day for 3 consecutive hrs/day at random times in order to prevent habituation to the procedure. Blood samples were collected by tail nick on days 1 and 21 at 0 (basal) 180 and 240 min from the onset of stress to assess changes in CORT levels.
For the acute procedure in 2-month-old females HPA axis activity was evaluated only on day 1 before and immediately after 3 hours of contention. On these days the procedure was always administered at a fixed times in order to take into account circadian rhythm, that is, from 1700 to 2000.

4.2.2.3. Estrous cycle monitoring. Since natural fluctuations in sex hormones have enormous influences on neuroendocrine function and tumor growth, the oestral cycle of the experimental subjects was assessed. In particular, at the end of RS (day 1 for acute challenge and days 1 and 21 for chronic procedure) vaginal secretions were collected, transferred on a glass slide and a trace of methylene blue was added to increase contrast and bring out the nuclei. Oestrus cycle was determined by examining the proportion and morphology of leukocytes and epithelial cells present in the vaginal smear of mice: proestrus (80–100% intact, live epithelial cells), estrus (100% cornified epithelia), metestrus (50% cornified epithelia and 50% leukocytes), or diestrus (80–100% leukocytes).

4.2.2.4. Saccharin consumption – Anhedonia. Mice have strong preference for sweet solutions such as those containing sucrose or saccharin and the intake of these compounds are considered to be due to their hedonic properties (Cryan and Holmes, 2005; Branchi et al., 2013). Eighteen-week-old MMTVNeuTg mice were habituated to a saccharin solution for three days. After this period, experimental subjects underwent 10 days of familiarization and each cage was

provided with two bottles, one containing fresh tap-water and one containing 0.1% of saccharin solution. Bottles were daily weighed in order to monitor liquid consumption and switched to balance the effect of side preference in drinking behaviour, which has been reported to be of importance for the correct evaluation of saccharin preference (Strekalova et al., 2004; Branchi et al., 2013). Following the establishment of the preference for saccharin solution, half of the experimental subjects underwent chronic restraint stress (21 days) and the bottles were daily weighed in order to monitor liquid consumption. For isolated subjects saccharin preference was evaluated through the single mouse consumption, while for group housed, the average of the mice was considered. Saccharin preference was then calculated as follows:

$$\%\ \text{saccharin preference} = \frac{\text{Saccharin solution intake x 100}}{\text{Water intake} + \text{Saccharin solution intake}}$$

Saccharin consumption was derived from bottles' weight. Body weight of experimental subjects was registered before and after familiarization, and on days 7, 14 and 21 from restraint stress.

4.2.2.5. Radioimmunoassay (RIA) for corticosterone determination. Blood samples (20 µl, approximate volume) were collected individually in potassium EDTA coated tubes (1.6 mg EDTA/ml blood, Sarstedt, Germany). All samples were kept on ice and later centrifuged at 3000 rpm for 15 min at +4 °C. Blood plasma was transferred to Eppendorf tubes for CORT determination and stored at -20 °C until further analysis. CORT was measured using a commercially available radioimmunoassay (RIA) kit containing 125iodine labelled CORT; 5 µl of plasma were sufficient to carry out CORT measurement. Sensitivity of the assay was 0.125 mg/dl, inter- and intra-assay variation was less than 10 and 5%, respectively (MP Biomedicals Inc., CA, USA). Vials were counted for 2 min in a gamma-scintillation counter (Packard Minaxi Gamma counter, Series 5000).

4.2.2.6. Quantitative real-time reverse transcription-PCR (RT-PCR). Total RNA was extracted from frozen hypotalamic and subcutaneous white adipose tissues with Trizol (Invitrogen, Carlsbad, CA) and the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. Quantity and quality were assessed by Nanodrop ND-1000 spectrophotometer using OD260 for calculation of the concentration and the ratios 260/280 and 260/230 for assessing the purity of the samples. After cDNA synthesis using Superscript III (Invitrogen), Real-time RT–PCR was performed by the TaqMan technology, using the ABI PRISM 7700 DNA Sequence Detection System (Applied Biosystems, Foster City, CA). TaqMan reactions were carried out in 96-well plates using cDNA, TaqMan universal PCR mastermix, preoptimized, and preformulated TaqMan gene expressions assays including specific primers

and fluorescent probes for mouse, and water to a final volume of 25µl according to the manufacturer's instructions. Commercial ready-to-use primers/probe mixes (Assays on Demand Products, Applied Biosystems) are listed: Adipoq #Mm00456425_m1; Lep #Mm00434759_m1; Insr #Mm01211875_m1; Bdnf # Mm 04230607_s1; Sgk1 #Mm 00441387_g1; Agrp #Mm 00475829_g1; Lepr #Mm 00440181_m1, Npy #Mm 03048253_m1; Eef2 #Mm 01171435_gh; GAPDH #Mm99999915_g1. Eef2 and GAPDH were used as the internal control for efficiency of RT-PCR and subsequent normalization for hypothalamus an adipose tissue respectively. The Δct values were used for statistical analysis.

4.2.2.7. Splenocytes Apoptosis. Spleens were gently removed and suspended in ice-cold culture RPMI-1640 medium (GIBCO BRL, Grand Island, NY). Splenocytes were isolated from mice spleen by flushing 5mL of RPMI-1640 medium into spleen by needle and syringe. Cells were then centrifugated at 1200 rpm in order to remove cellular debris and treated as previously described (Capoccia et al. 2013). In this study, we analyzed specifically "early apoptosis" characterised by nuclear changes observed first, in contrast to the changes seen in the later stages of apoptosis and then in the necrosis, which usually begin with cell membrane damage (Wyllie et al., 1980; Perandones et al., 1993). Acquisition was performed on a FACSCalibur cytometer (BD Immunocytometry Systems) and 50.000 events per sample were run. Data were analyzed using the Cell Quest Pro (BD Immunocytometry Systems) software.

4.2.2.8. Cytokines Determination. Trunk blood was collected individually in anticoagulant-free vials allowed to clot for 20 minutes at room temperature and then centrifuged at 3000 rpm for 15 min at +4 °C. Serum was then stored at -80 °C until cytokines determination was carried out. Quantitative evaluation of TNF-*α*, IL-6, and IL-10 in sera from trunk blood of stressed and control mice, was determined by ELISA kits (R&D Systems, Inc., Minneapolis, USA) according to the manufacturer's instructions. Briefly, standards, controls, and sera were placed into the wells and incubated 2 h at room temperature. After washing 5 times, the enzyme-linked polyclonal antibody specific for mouse cytokines was added to the wells and then, after washing, the substrate solution was added. The enzyme reaction was read at 450nm (correction wavelength set at 570 nm). The samples values were read off the standard value.

4.2.2.9. Histological analysis. The hematoxylin and eosin stain (H&E) was performed on Formalin Fixed Paraffin Embedded (FFPE) tissue sections from 6-months-old subjects (n=5 for each experimental group). Slides (5µm thick) were deparaffinized, hydrated through graded alcohols and H&E staining was performed according to standard protocols.

4.2.2.10. Statistical Analysis. Data were analyzed using parametric analysis of variance (ANOVA) with "social condition" (group-housed and isolation) and "treatment" (control and restraint stress) as between-subjects factor (breast lumps, tumor masses, genes, apoptosis, CORT) and "day" (1, 7, 14 and 21) and "time course" (0, 180 and 240) as within-subject repeated measures (CORT assessment only for the restraint stress; breast lumps and tumor masses assessment, saccharin consumption and body weight). Since transgenic mice developed tumors at 6 months of age, we analyzed separately WT and transgenic mice for hypothalamic and adipose tissue gene expression and for cytokine levels. A linear multiple regression model was used to assess the effect of estrous cycle on corticosterone levels, with genotype (WT = 0; MMTVNeuTg = 1), social condition (group-housed = 0; isolation = 1), day (1, 21), and estrous phase (estrous = 0; proestrous = 0-1, phase 1; post-estrous (diestrous + metaestrous) = 0-1, phase 2) as dependent variables and CORT level at different time course (0, 180 and 240) as independent variable. Post hoc comparisons were performed using the Tukey's test. Statistical analysis was performed using Statview II (Abacus Concepts, CA, USA). Data are expressed as mean + SEM. A significance level of 0.05 was chosen.

4.3. RESULTS

4.3.1. Assessment of breast lumps and tumor masses

Nineteen weeks of social isolation resulted in a greater number of breast lumps compared to group-housed controls (interaction between social condition and weeks of age $F(4,136) = 5.266$, $p = 0.0006$). This effect was magnified over time (main effect of social condition $F(1,32) = 9.008$, $P = 0.0052$; effect of weeks of age $F(2,64) = 14.474$, $p < 0.0001$) and when RS was superimposed upon isolation (interaction among RS and weeks of age $F(2, 64) = 5.707$, $p = 0.0052$, Fig. 1A, upper panel). Social deprivation accelerated onset of breast cancer: at 20 weeks of age we found that 33% of the isolated mice had developed at least one tumor, while no group-housed subject did (main effect of social condition $F(1, 34) = 3.845$, $p = 0.0581$; interaction between social condition and weeks of age $F(4, 136) = 3.845$, $p = 0.0054$), although no difference in tumor masses was found between isolated and group-housed mice after 21 weeks of age, when restraint stress procedure was applied (no interaction among social condition, restraint stress and weeks of age $F(2, 64) = 0.387$, $p = 0.6807$) (Fig. 1B, upper panel). No breast lumps were found in stressed FVB mice.

4.3.2. Histological analysis

Histological examination of mammary glands in the transgenic mice indicated that prolonged exposure to social isolation induced white adipose tissue (WAT) "browning", adipocytes being characterised by multilocular small lipid drops (Fig. 1C, panel d), compared to control FVB subjects, which, by contrast showed WAT made of cells showing a single (unilocular) large lipid drop (Fig. 1C, panel a). The effect of social isolation was amplified by restraint stress, which was able to induce a milder response when imposed alone (Fig. 1C, panels e and c respectively).

4.3.3. Hypothalamic gene expression in response to stress

At 2 months of age, we tested hypothalamic BDNF gene expression in response to an acute restraint stress to test the system well before MMTVNeuTg subjects developed any tumor. At this age no difference in BDNF expression was found as a result of transgenosis and, as expected, expression of this neurotrophin was significantly reduced following stress, (main effect of acute restraint stress $F(1,12) = 5.510$, $p = 0.0369$, data not shown). When subjects were tested at 6 months of age, a time when tumor progression becomes manifest, MMTVNeuTg mice were characterised by lower basal BDNF levels in the hypothalamus and were found to be hyporesponsive upon a stressful challenge (restraint stress) compared to wild types (interaction between genotype and restraint stress $F(1,30) = 7.345$, $p = 0.0110$).

Interestingly, we found that both social isolation (main effect of social condition $F(1,16) = 5.593$, $p = 0.0310$) and restraint stress reduced hypothalamic BDNF expression (main effect of restraint stress $F(1,16) = 7.532$, $p = 0.0144$) in MMTVNeuTg subjects (Fig. 2A). At this age, gene expression for a number of genes involved in the response to stress (serum/glucocorticoid regulated kinase (SgK1), agouti related peptide (AgRP), Insulin receptor (InsR) and Neuropeptide Y (Npy) was measured. Regardless of genotype, prolonged social isolation decreased hypothalamic expression of these genes (main effect of social condition $F(1,32) = 4.053$; 7.728; $p = 0.0526$; 0.0090 respectively for AgRP and InsR). Following restraint stress these genes were activated, rather than suppressed, as in the case of BDNF (interaction between social condition and restraint stress $F(1,32) = 85.640$; 43.926; 34.110; 40.733, $p < 0.0001$ respectively for SgK1, AgRP, InsR and Npy genes, suggesting a different regulatory mechanism (Fig. 2B).

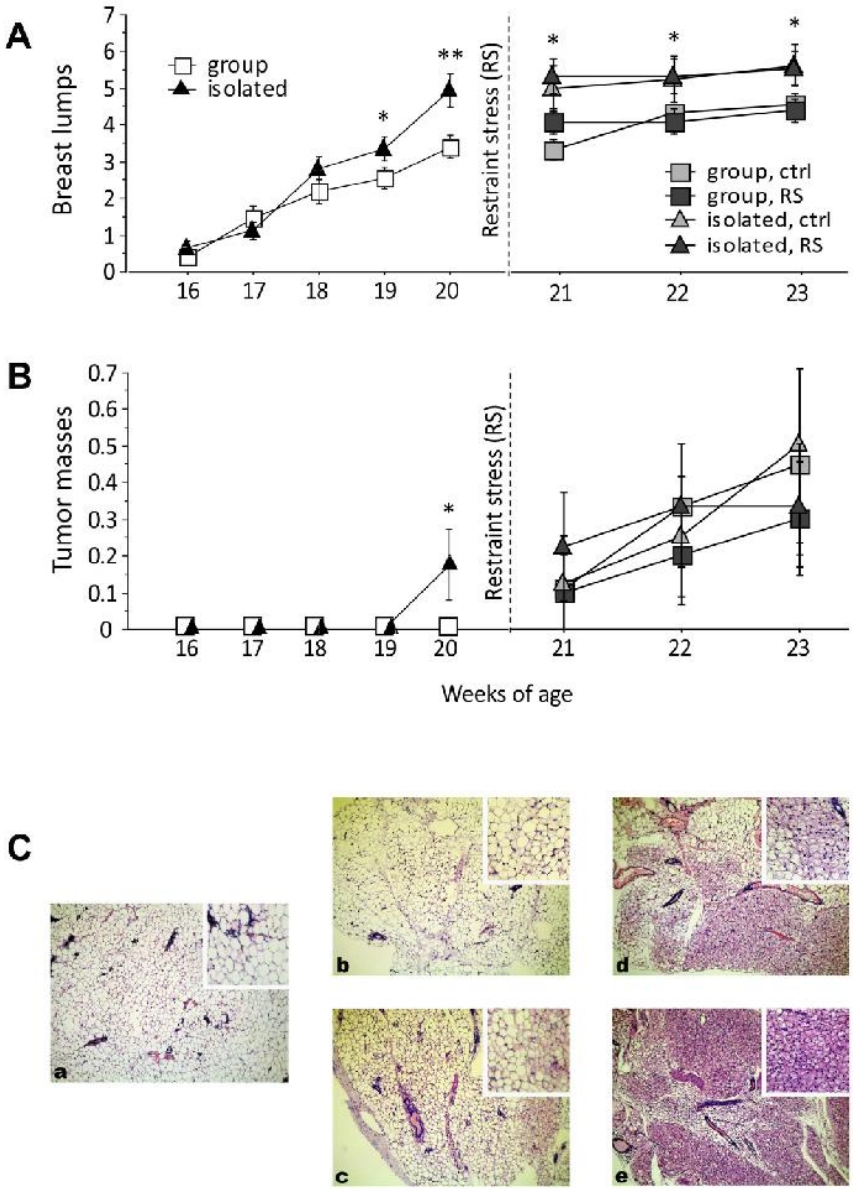

Figure.1. Breast lumps (A) and tumor masses (B) in the MMTVNeuTg mice mammary gland assessed through palpation. Isolation stress increased breast lumps and promoted tumor onset. At 20 weeks of age we found that 33% of the isolated mice had developed at least one tumour, while no group-housed subject did. Data show mean + S.E.M. *p<0.05. **(C) H&E staining of mammary glands sections.** Brown adipocytes in WAT were found in MMTVNeuTg following chronic isolation (panel d) compared to FVB (panel a) and group housed MMTVNeuTg (panel b). The effect of social isolation was amplified by restraint stress (panel e), which was able to induce a milder response when imposed alone (panel c). All specimens are shown under 100-fold magnification. The boxed areas are enlarged images with 200-fold magnification.

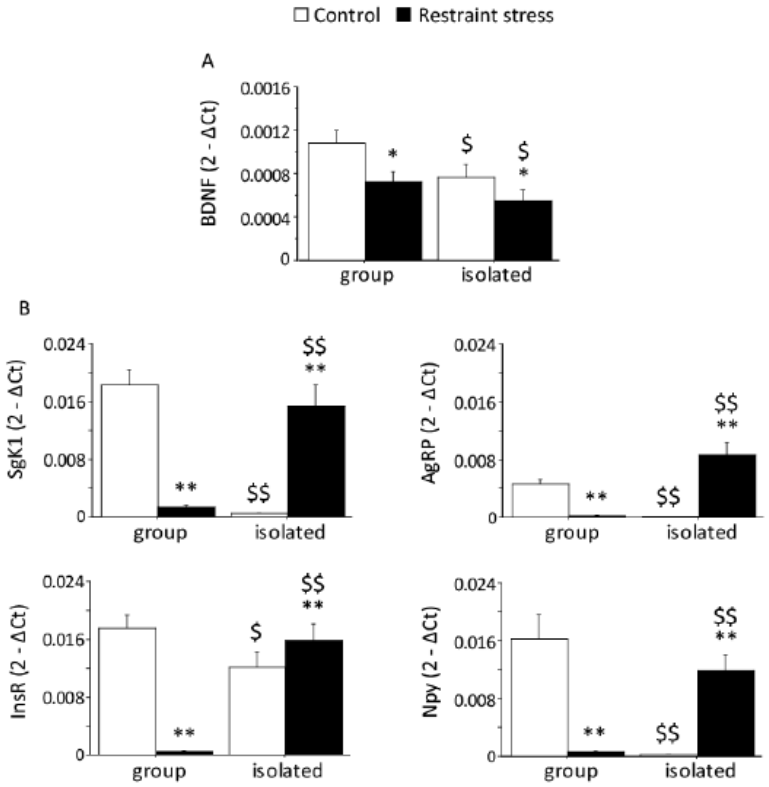

Figure 2. Gene expression in the hypothalamus of MMTVNeuTg mice through RT-PCR. Both social isolation and restraint stress reduced BDNF m-RNA levels (A). In addition, prolonged social isolation decreased expression of Sgk1, Agrp, InsR and NPY, while restraint stress activated these genes (B). Data show mean + S.E.M. (n = 5 for each experimental group). *p < 0.05, **p < 0.01 main effect of the social condition, $p< 0.05, $$p< 0.01 main effect of stress.

4.3.4. Gene expression in adipose tissue

At 2 months of age, acute restraint stress decreased leptin gene expression (main effect of acute restraint stress $F(1,12) = 67.330$, $p < 0.0001$) in all subjects and adiponectin only in MMTVNeuTg mice (main effect of genotype $F(1,12) = 10.308$, $p = 0.0075$; interaction between genotype and acute restraint stress $F(1,12) = 7.920$, $p = 0.0156$, Fig. 3A). These responses changed drastically when transgenic mice developed tumor and chronic stressors were applied. More in detail, at 6 months of age, regardless of genotype, social isolation increased leptin gene expression in adipose tissue (main effect of social condition $F(1,32) = 15.361$, $p = 0.0004$, data not shown). Interestingly, the same procedure affected the adiponectin gene expression differently in MMTVNeuTg and FVB mice, isolated transgenic mice showing lower levels of the adipokine when restraint stress was superimposed (main effect of genotype

F(1,32) = 7.016, p = 0.0124; interaction between social condition and restraint stress F(1,32) = 24.496, p < 0.0001).
We found that leptin and adiponectin had different roles in mediating stress reactions in MMTVNeuTg mice. More in detail, leptin expression was increased significantly by social isolation but not by the stressful challenge (main effect of social condition F(1,16) = 5.262, p = 0.0357). However, adiponectin expression in fat increased following 21 days of restraint while this same stress was able to revert the effect of social isolation, reporting levels of this chemokine to those of controls (interaction between social condition and restraint stress F(1,16) = 41.511, p < 0.0001, Fig. 3B). Interestingly, social isolation decreased InsR expression (main effect of social condition F(1,16) = 7.264, p = 0.0159) possibly promoting insulin resistance (Fig. 3B).

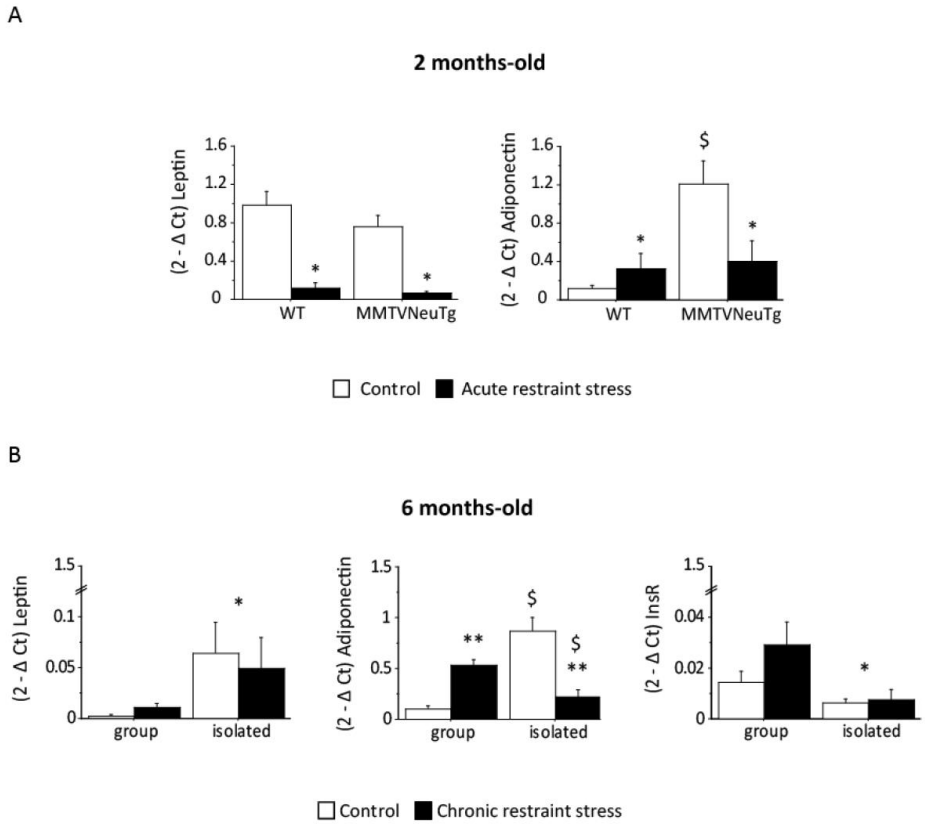

Figure 3. Gene expression in the adipose tissue through RT-PCR. (A) Acute restraint stress decreased leptin m-RNA levels in all subjects and adiponectin only in MMTVNeuTg mice (2 months of age). *p < 0.05, main effect of RS, $p< 0.05 main effect of genotype. (B) Gene expression in MMTVNeuTg mice. Six month-old isolated transgenic mice were characterised by increased expression of leptin and decreased expression of adiponectin (after restraint exposure) and InsR. *p < 0.05, **p < 0.01 main effect of social condition, $p< 0.05 main effect of RS. Data show mean + S.E.M. (n = 5 for each experimental group).

4.3.5. Neuroendocrine response

HPA axis activity was found to differ in MMTVNeuTg females at 2-months of age, at a time when these subjects have not developed tumors yet. At this age, the transgenic animals showed increased CORT levels after 180 min of restraint compared to WT, with evidence of negative feedback after 240 min, at a time when CORT levels in WT subjects were still rising (interaction between genotype and time course $F(1,28) = 19.922$, $p < 0.0001$, Fig. 4). This profile was no longer evident at 6 months of age. MMTVNeuTg group-housed subjects showed lower HPA axis activation on the first day of restraint stress, an effect reverted by social isolation (interaction between genotype and social isolation $F(1,35) = 9.340$, $p = 0.0043$) (Fig. 4).

The response of the HPA axis to chronic restraint stress was affected by social isolation and genetic background, MMTVNeuTg mice were more resilient to changes in corticosterone levels, suggesting an inability to mount a coping response to stressful challenges after 21 days of restraint, compared to FVB mice (interaction among genotype, social condition and days $F(2,66) = 5.084$, $p = 0.0309$, data not shown).

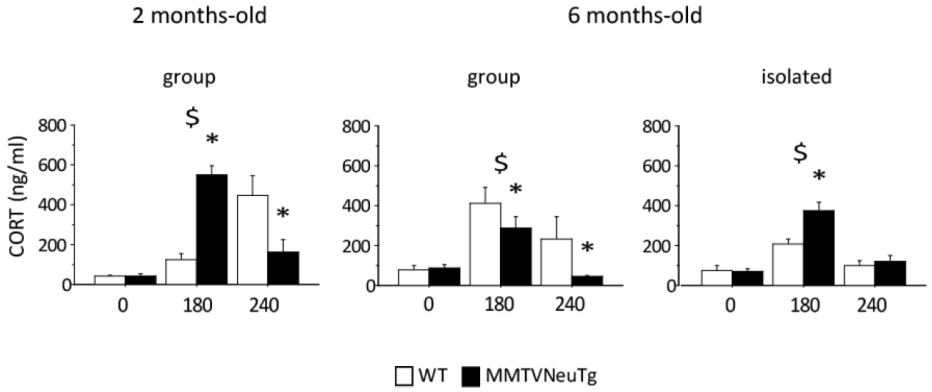

Figure 4. Neuroendocrine response. At 2-months of age MMTVNeuTg females showed increased CORT levels after 180 min of restraint compared to WT, with evidence of negative feedback after 240 min. At 6-months of age social isolation increases corticosterone levels in the transgenic mice after the stressful challenge. Data show mean + S.E.M. (n = 8 group 2 months-old; n = 10 group 6 months-old; n = 8 isolated 6 months-old). *$p < 0.05$ main effect of genotype , \$$p < 0.05$ main effect of the time course: 180 vs. both 0 and 240.

4.3.6. Estrous cycle

Mammary glands change during estrous cycle and there is evidence linking the increase in estrogen exposure throughout life with an increase in breast cancer risk (Velie et al., 2005; MacLennan et al., 2011). We thus tested estrous cycle to discern whether effects of stress on tumor progression might depend upon

changes in sex hormones. An interaction between tumor susceptibility and stress exposure was found, transgenic mice moving to the proestrous phase (MMTVNeuTg group housed: 10% on day 1 and 40% on day 21 of restraint; MMTVNeuTg isolated: 44.4% on day 1 and 100% on day 21 of restraint) thus indicating that sex hormones are not likely to mediate the effects on stress on tumor progression. By contrast, isolated WT mice were found more often more in estrous phase compared to other subjects (50% and 40% on day 1 and 21 of restraint stress respectively). In addition, no correlation between stress and sex hormones was found (regression coefficient = 33.614; 80.482; 40.294, SE = 20.318; 71.040; 53.724, $p = 0.1027$; 0.2612; 0.4558 respectively at 0, 180 and 240 minutes from the chronic restraint procedure on day 21).

4.3.7. Cytokine Production

MMTVNeuTg and WT subjects did not differ for the overall levels of cytokines (main effect of genotype $F(1,33) = 0.448$; $F(1,54) = 1.189$; $F(1,53) = 0.642$, $p = 0.5078$; 0.2803; 0.4267 respectively forIL-6, IL-10 and TNF-α). However, serum cytokine determination is complex and cytokines resulted undetectable for some mice (above all wild type subjects). In the MMTVNeuTg mice, TNF-α decreased upon isolation after stressful challenge exposure, while IL-6 production increased (interaction between social condition and restraint stress $F(1,20) = 5.420$; $F(1,17) = 4.696$; $p = 0.0305$; 0.0447 respectively for TNF-α (Fig. 5A) and IL-6 (Fig. 5B)), while the anti-inflammatory cytokine IL-10 increased after prolonged social isolation (main effect of social condition $F(1,26) = 4.679$, $p = 0.0399$, Fig. 5C).

4.3.8. Splenocytes Apoptosis

The immune system response was evaluated also analyzing splenic lymphocytes apoptosis, a marker of cellular injury and reduced immune functions. At 2-months of age higher levels of splenocyte apoptosis were found in MMTVNeuTg mice compared to WT (main effect of genotype $F(1,15) = 4.993$, $p = 0.0411$) suggesting that tumor susceptibility was already reflected in a reduced immune response. Moreover this profile was inverted after an acute challenge (interaction between genotype and treatment $F(1,15) = 20.654$, $p = 0.0004$, data not shown). The impairment of immune response in transgenic mice was still present at adulthood, MMTVNeuTg mice showing greater splenocyte apoptosis compared to WT subjects, an effect amplified by social isolation and restraint stress exposure (main effect of genotype $F(1,67) = 150.700$, $p < 0.0001$; interaction among genotype, social condition and restraint stress $F(1,67) = 13.033$, $p = 0.0006$, Fig. 5D). Interestingly, regardless of genotype, stressful conditions increased the percentage of Annexin V positive cells (main effect of social condition $F(1,67) = 41.423$, $p < 0.0001$; main effect of restraint stress $F(1,67) = 24.292$, $p < 0.0001$).

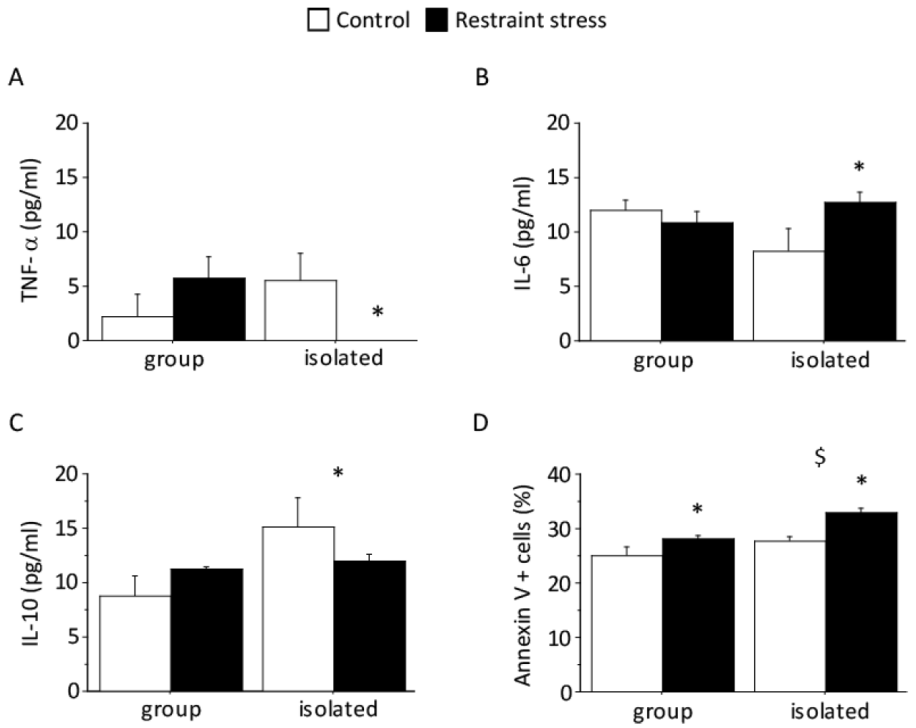

Figure 5. Immune response in MMTVNeuTg mice. Isolated transgenic mice were characterised by increased levels of IL-10 (C). After restraint stress IL-6 levels were increased (B), while levels of TNF-α were decreased (A). In addition, MMTVNeuTg mice showed a higher rate of splenocyte apoptosis after stressful exposure (D). Data show mean + S.E.M. (n = 5 group control, 6 group restraint stress, 5 isolated control, 5 isolated restraint stress for IL-6; n = 4 group control, 5 group restraint stress, 6 isolated control, 6 isolated restraint stress for TNF-α; n = 9 group control, 7 group restraint stress, 7 isolated control, 7 isolated restraint stress for IL-10; n = 9 group control, 10 group restraint stress, 8 isolated control, 8 isolated restraint stress for splenocyte apoptosis). *p < 0.05, $p< 0.05, main effect of the social condition.

4.3.9. Anhedonia and body weight

MMTVNeuTg mice were characterised by an anhedonic profile, already during the familiarization phase, showing lower levels of saccharin consumption compared to WT (main effect of genotype $F(1,72) = 4.067$, $p = 0.0474$). This effect was also present during the restraint stress exposure (main effect of genotype $F(1,68) = 24.111$, $p < 0.0001$; interaction between restraint stress and repeated measure $F(2,136) = 5.486$, $p = 0.0051$; interaction among genotype, social condition and repeated measure $F(2,136) = 2.927$, $p = 0.0569$, Fig.6).Worth noticing, all isolated mice exhibited a greater preference for the saccharin solution compared to group-housed (main effect of social condition $F(1,72) = 23.870$, $p < 0.0001$) confirming that prolonged social isolation

influences response to stimuli associated with reward as previously shown (Berry et al., 2012).
Body weight was influenced by genotype and stress, MMTVNeuTg and isolated subjects showing lower body weight compared to FVB group-housed mice during familiarization phase (main effect of genotype $F(1,72) = 17.090$, $p < 0.0001$; main effect of social condition $F(1,72) = 12.465$, $p = 0.0007$; interaction between genotype and social condition $F(1,72) = 5.391$) (data not shown). These data were confirmed over time, MMTVNeuTg group-housed mice showing a significant decrease of body weight after restraint stress procedure (main effect of restraint stress $F(1,68) = 36.468$, $p < 0.0001$; interaction among genotype, social condition, restraint stress and time course $F(2,136) = 3.485$, $p = 0.0334$, data not shown).

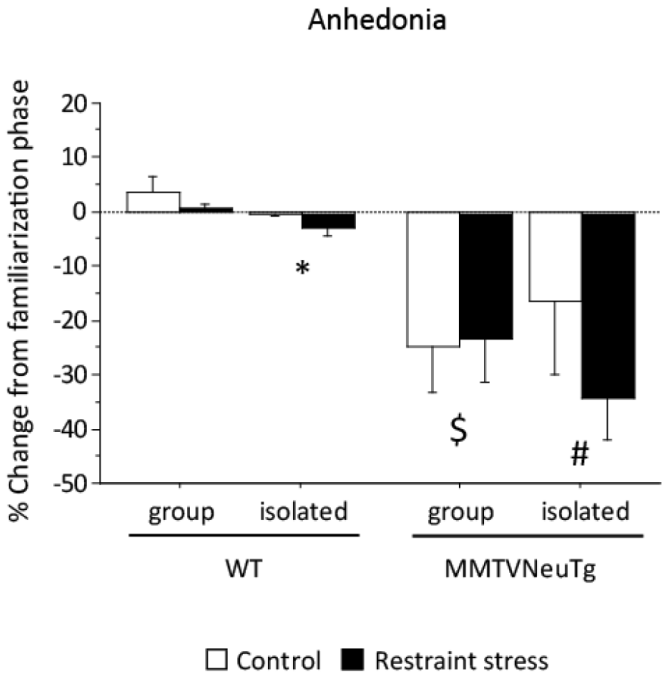

Figure 6. Anhedonia. MMTVNeuTg mice were characterised by an anhedonic profile showing lower levels of saccharine consumption compared to WT and this effect was amplified by stressful exposure. Data show mean + S.E.M. (n = 10 FVB for each experimental group; n = 9 MMTVNeu Tg group control, 10 group restraint stress, 8 isolated control, 9 isolated restraint stress). *$p < 0.05$ WT isolated vs WT group housed, $$p < 0.05$ MMTVNeuTg group vs WT group, #$p < 0.05$ MMTVNeuTg isolated vs WT isolated.

4.4. DISCUSSION

Data from this study reveal that social isolation accelerates onset of breast cancer in MMTVNeuTg mice with earlier detection of lumps in the mammary

glands and earlier tumor development, accompanied by browning of the mammary fat pad, compared to controls. These effects were magnified in those subjects experiencing a further stress (restraint stress) and were accompanied by increased HPA axis activity, low levels of BDNF expression in the hypothalamus, increased cytokines levels, as well as increased expression of leptin and decreased expression of adiponectin in peripheral fat tissue.

The MMTVNeuTg mouse model used was chosen for this study because of its late latency of tumor occurrence, which allows studying the effects of a chronic stress treatment, such as social isolation. Subjects were isolated starting at weaning and up to a time when mice started to develop tumors in the mammary glands. Our data indicate that already by week 19, social isolation was able to affect mammary glands structure, MMTVNeuTg isolated subjects being characterised by a greater number of breast lumps, compared to group-housed controls. In addition, an early onset of breast tumors was found at 20 weeks of age, isolated mice having developed, on average, at least one tumor by this time, compared to group-housed subjects, who developed none. The effect of social isolation on breast lumps was still present after 21 weeks of age and up to 23 weeks, although this was not paralleled by a significant increase in tumor masses. The histological analysis of mammary glands indicated that isolated transgenic mice display small, multilocular adipocytes that are reminiscent of brown adipose tissue (BAT). This kind of fat is exclusively detected in the mouse early in mammary gland development, particularly during stages of ductal growth, and is associated with the synthesis and secretion of angiogenic and growth factors, resulting in markedly increased vascular density and, therefore, involved in cancer development (Jones et al., 2011; Cao et al., 2014). Recently, it has been reported that a switch from white to brown fat is found in cancer-associated cachexia and is linked to greater tissue energy expenditure (Petruzzelli et al., 2014). The higher presence of BAT in stressed and isolated mice indicates that these subjects have a more metabolically active tissue, a process which is likely to fuel the tumor, and which could be at the base of the greater detection of breast lumps through palpation. It must be emphasized here that, given the peculiarity of the transgenic model, it is likely that a ceiling effect at some point occurs so that it is hard to detect significant differences in tumor number. Nonetheless, a more metabolically active tissue could lead to an earlier onset of cachexia in these animals, a hypothesis that will need to be tested with subsequent studies (Petruzzelli et al., 2014).

These effects of social isolation are in agreement with previous reports indicating an increased effect of social isolation on mammary tumors (Williams et al., 2009; Volden et al., 2013). However, differently from these studies, which were mainly concentrated on the tumor microenvironment, we attempted to tackle the cascade of events starting with the hypothalamus - an area of the brain that is critical in the regulation of both energy balance and neuroendocrine-immune interaction through the HPA axis – assessing changes in the expression of genes known to be involved in the neuroendocrine response

to stress, and with demonstrated effects on cancer progression, such as BDNF (Cirulli and Alleva, 2009; Yang et al., 2012; Liu et al., 2014). The work of Cao and collaborators (Cao et al., 2010; Cao and During, 2012) has very effectively shown how the maintenance of high levels of this BDNF in the hypothalamus can be a preventive factor for tumor growth. Given the numerous data documenting a negative regulation of the BDNF gene by stress-induced changes in glucocorticoid hormones (Barbany and Persson, 1992) we hypothesized that a prolonged exposure to a social stressor might act as a risk factor for tumor onset and progression in this genetically modified mouse model. Our data indeed show low levels of hypothalamic BDNF expression as characterising isolated mice, thus supporting a role for this neurotrophin in the above-described changes in the mammary glands tissue found in these mice. Accompanying changes in BDNF expression, we found decreased hypothalamic expression of a number of genes involved in the response to stress and in metabolic regulations (SgK1, AgRP, InsR and Npy), as expected given the role of the hypothalamus as a sensor of metabolic signals. Interestingly, when a further stress was applied for 21 days, we found that BDNF levels were still low, while the other stress-responsive genes were re-activated, suggesting a selective regulatory mechanism underlying BDNF transcriptional regulation.

Hypothalamic BDNF plays an important role in stress adaptation ultimately affecting endocrine and metabolic responses. Recent data indicate that BDNF can affect sympathoneural innervation of peripheral fat tissue, maintaining an optimal level of adipokines production, ultimately influencing tumor growth. Thus, the decreased expression of this neurotrophin following chronic social stress, could affect this peripheral target, promoting the disease (Cirulli and Alleva, 2009; Cao and During, 2012). Leptin, mainly secreted by the adipose tissue exerts pro-angiogenic, pro-inflammatory, mitotic and anti-apoptotic actions inducing breast cancer development (Grossmann and Cleary, 2012; Khan et al., 2013). By contrast, adiponectin represses tumor proliferation in breast cancer cells, low blood concentrations of adiponectin being associated with high incidence and poor prognosis of breast cancer (Jarde et al., 2011). In this study, we found that isolated subjects showed increased leptin and decreased adiponectin gene expression in peripheral adipose tissue, both promoting tumor progression. Both leptin and adiponectin expression were increased by social isolation, but a further stressful challenge, was then able to reduce adiponectin levels, thus creating a permissive environment for tumor progression. The finding of higher levels of adiponectin gene expression characterising transgenic mice at two months of age, allows hypothesizing a protective role of this hormone in response to transgenosis.

Adipokines have been linked to both inflammation (Kwon and Pessin, 2013) and cancer progression (Grossmann and Cleary, 2012). At the same time, cytokines have been reported to be associated with breast cancer development (Nicolini et al., 2006) and we found that TNF-α decreased in isolated MMTVNeuTg mice after stressful challenge exposure, while IL-6 production

increased. These results are in line with the antitumor properties of TNF-α, which is a multifunctional cytokine, playing a key role in apoptosis and cell survival as well as in inflammation and immunity (van Horssen et al., 2006). Indeed, our data reveal that chronic social isolation affects the production of this cytokine, promoting disease. As for IL-6, there is some evidence that elevated serum levels of this cytokine are significant indicators of poor prognosis and favor tumor growth (Nakashima et al., 2000; Petruzzelli et al., 2014). In addition, also the immunosuppressive cytokine IL-10 increased after prolonged social isolation suggesting a response of the immune system aimed at restoring homeostasis following stress (Capoccia et al., 2013). Complementing these data is the greater splenocyte apoptosis in MMTVNeuTg mice, an effect amplified by social isolation and restraint stress exposure. Interestingly, we found increased levels of splenocyte apoptosis in transgenic subjects already at two months of age and this result might be related to the susceptibility of the mouse model.

Thus overall, increased leptin and decreased adiponectin levels in the adipose tissue, together with increased levels of IL-6 could contribute to promote an insulin-resistant inflammatory state involved in the promotion and progression of cancer. Furthermore, increased levels of cytokines, such as IL-6, as well as increased NPY expression are also likely to contribute to promote depressive symptoms as it is has been previously shown that these are increased and correlate with depression severity in patients (Dantzer et al., 2008; Maes et al., 2014). Our data indicate increased expression of anhedonia in MMTVNeuTg mice (Lamkin et al., 2011) both in the familiarization phase and during the restraint stress exposure suggesting the potential role of a stressful challenge to precipitate depression symptoms.

In summary, results from this study indicate that the macro-environment, here exemplified by lack of social interactions, can affect physiological responses and breast cancer onset and progression at multiple levels. In particular, social deprivation and stressful events can amplify the consequences of a genetic susceptibility accelerating and precipitating the disease, also through modifications of the mammary fat pad tissue. One important factor that deserves attention, and which has not been specifically tested in this study, is the role of the sympathetic nervous system in the regulations described, as beta blockers have been shown to reduce the effects of stress on tumor growth (Hasegawa and Saiki, 2002), sympathetic innervation of white adipose tissue being regulated by hypothalamic BDNF (Cao et al., 2010). Studies are currently in progress to add this piece to the puzzle.

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CHAPTER 5

5. GENERAL DISCUSSION

Within a species, the ability to cope with environmental stressful challenges largely determines individual survival in their natural habitat. Coping with stress involves the activation of a number of physiological and behavioural responses that are functional to maintaining homeostasis. Since human and mouse physiology largely overlaps, at least as for the stress-system responses, murine models of stress are widely used, in a translational approach, to dissect out how physiological and behavioural responses can be finely modulated as a result of different stressful conditions. Indeed exposure to stressful events can be an important risk factor for a number of pathological conditions, including neoplasia, although the mechanisms underlying the ultimate outcome of stress are poorly understood. In fact, factors such as the duration (acute vs. chronic) of stressful events as well as the time of exposure to GC, relative to the activation and time course of the immune response, might differently impact health outcome and the directionality of these effects is still a matter of intense investigation.

Thus **the objective** of the studies described in this thesis was to explore the relationship among brain activation and neuroendocrine and immune system changes in response to different environmental challenges. **To this aim** both central (BDNF levels) and peripheral (CORT, cytokines, splenic apoptosis, leptin and adiponectin levels) responses, as well as endophenotype of depression, were analyzed in wild type and transgenic mice following different stressors, applied for different time lengths.

5.1. MAIN FINDINGS

5.1.1. Social isolation promotes anxiety and depression-like behaviour

In a first study we investigated the effect of housing conditions on the behavioural response. Since mice are social animals and live in social groups, both the disruption of social hierarchy as well as social isolation represent two ethologically relevant stressors.

Chronic stress is a triggering factor for several psychiatric disorders. Interestingly, in rodents, social stress appears to be a good model to induce both anxiety and depression-like behaviours (Pothion et al., 2004; Mutlu et al., 2009) leading to reduced locomotor activity, reduced food and water intake and decreased responsiveness to reward stimuli (Griffiths et al., 1992), all behavioural changes resambling those observed in human clinical condition of depression. Our analyses on the behavioural characterisation shown in **Chapter 2** are in line with these studies and indicate a crucial role for social isolation as a relevant condition leading to increased anxiety and depressive-like behaviours,

accompanied by higher levels of CORT and reduced brain BDNF levels, all traits indicative of a disrupted emotional state.

When emotional behaviours were assessed in the Open Field, isolated mice showed an anxious phenotype, spending more time in the periphery and showing a higher self-grooming frequency. As for the typical test capable to elicit anxiety, the Elevated Plus Maze test, the same experimental subjects spent more time in the "safe" closed arms of the maze and were characterised by reduced locomotor activity and by a lower latency and higher frequency of stretched-attend posture, a behaviour indicative of risk assessment. In addition, in the Forced Swim Test, isolated subjects showed the highest floating duration and the lowest struggling frequency, which are commonly interpreted as measures of depressive-like behaviour (Porsolt et al., 1977). However, in terms of anhedonic profile, isolated mice exhibited a greater preference for a sucrose solution compared to the other experimental groups. These data are in line with many studies reporting a higher response to stimuli associated with reward in isolated rodents (Jones et al., 1990; Consorti et al., 1992; Coudereau et al., 1999). It must be taken into account that, in **Chapter 2**, group-housed mice were exposed to a single-housing condition for 5 h in order to score individual sucrose consumption. Several studies have shown that social experiences are intrinsically valuable, even when there is no clear opportunity for the approaching individual to benefit (Panksepp and Lahvis, 2007; Trezza et al., 2010). Our results are in line with these data showing that only those subjects that were acutely (5 h) separated from the social group developed anhedonia (D'Andrea et al., 2010). Following this line of reasoning, we can deduce that individually housing mice leads to a change in sucrose preference which can override the effects of other treatments that could be superimposed on the same subject (Haenisch et al., 2009).

In agreement with behavioural data, when we investigated the effects of a social challenge on HPA axis activity, we found isolated subjects to be characterised by the highest levels of CORT, adrenal/body weight ratio confirming an increased neuroendocrine and emotional profile in response to social stimuli (Gavrilovic and Dronjak, 2005; Ulrich-Lai et al., 2006). Worth noticing, adrenal enlargement, in association with increased glucocorticoids levels, has been found in many patients suffering from major depression (Rubin et al., 1987; Nemeroff et al., 1992).

Interestingly, in our study anxiety and depression-like behaviours were related to reduced BDNF levels in limbic regions of isolated subjects, a result that confirms an association between neuroendocrine dysregulations, endophenotypes of depression and reduced neuroplasticity (Duman and Monteggia, 2006; Schule et al., 2006; Castren et al., 2007). Overall, these results represent further evidence that social isolation in mice have deleterious effects on health and can be used to model psychopathological condition induced or precipitated by isolation and lack of social support in human beings (Bartolomucci et al., 2003).

5.1.2. Nature and length of stress exposure are crucial to determine the final outcome on health or disease

In the short run stressful experiences result into neuronal, neuroendocrine and immune activation which are necessary for adaptation (McEwen, 2000, 2007; Dhabhar, 2009). However, over longer time laps, stress can lead to a wide variety of behavioural, cognitive, physiological, and neuronal changes that can promote vulnerability to diseases (McEwen, 2000). In **Chapter 3** we studied the characteristics of the diverse stressors applied for different lengths of times, and we found a fine modulation of the crosstalk between central and peripheral pathways of adaptation and plasticity, the length of stress exposure being crucial to determine its final outcome on health or disease. More in detail, upon prolonged exposure to restraint stress (21 days), an increase in the immunogenic/allostatic load was observed, mirrored by a peak in CORT levels comparable to that observed on the first day of stress (day 1, acute response). Interestingly, this response was associated to a suppression of the immune system with decreased levels of the proinflammatory cytokine TNF- α and increased levels of the anti-inflammatory cytokine IL-10. In addition, a decrease in hippocampal BDNF levels was found, suggesting a reduction in the ability to cope with prolonged stress (brain plasticity). By contrast, following 7 days of the same procedure, we found that CORT elevation was significantly lower than on the first stimulation (day 1) and this was associated to proinflammatory and coping responses, as suggested by the increase in the levels of TNF-α and in the central BDNF protein. Thus, our data clarify the mechanisms through which stress can differentially affect an organism, identifying a specific neuroendocrine-immune profile associated to specific changes in hippocampal BDNF levels. In particular, the central response after 7 days of restraint stress is likely to reflect a neuroprotective mechanism in response to HPA axis activation. By contrast, after 21 days of the same procedure, BDNF levels were decreased, an effect associated to an augmented anti-inflammatory response, as indicated by increased levels of IL-10. It must be emphasized that while acute changes in BDNF levels might represent a coping response to stressful events, and thus being beneficial, reduced BDNF signalling in response to prolonged exposure to stressors would have detrimental effects on brain plasticity (Altar, 1999; Sen et al., 2008; Cirulli and Alleva, 2009).

A fine regulation of apoptosis might positively affect optimal immune function. This is achieved by maintaining lymphocyte homeostasis through a continuous removal of cells that have been activated once they have served their function. Therefore, inappropriate induction of such a mechanism could result in a variety of pathological effects such as autoimmune diseases, while the maintenance of physiologically regulated levels of apoptosis might exert a beneficial/protective effect (Avula et al., 2001). In this context, the observed decrease in apoptosis levels following 21 days of restraint stress, suggests a long-term impairment of the immune system response.

In **Chapter 3** we extended our analysis to investigate the effects of stressors of different nature. In particular, we studied two experimental paradigms that can induce stress in mice: chronic disruption of the social hierarchy and restraint stress. From a behavioural perspective, restraint stress may model a naturally occurring event in the life of wild mice. In nature, mice live in underground burrows that may collapse, leaving a mouse trapped. Forced restraint may imitate the collapse of a burrow, triggering a "programmed" response specific for these situations. An appropriate response probably is an attempt to escape by digging a way out. After several unsuccessful attempts to escape, a mouse might give up and remain in its place, immobile (Avitsur et al., 2009). Social stress, by contrast, is based upon the highly territorial nature of rodents. An attacked mouse evaluates its opponent's strength and vigor based on its scent, behaviour, and size. Previous experiences in similar situations also would affect behaviour (Avitsur et al., 2009). These two stressors are especially different in that restraint stress is an uncontrollable and inescapable situation, while social stress allows some aspects of coping to be put in place, ultimately resulting in a milder stressor.

In this study, compared to restraint, social stress resulted in an overall lower response of the HPA axis, as well as of the immune system. Differences were both quantitative and qualitative. In particular, no change in TNF-α could be detected, while an earlier increase in IL-10 was observed compared to psychophysical stress, suggesting an anticipated anti-inflammatory reaction in response to this specific stressor. Differently from restraint stress data, splenic apoptosis increased after 7 days of SS, suggesting that it might represent a reliable early stress-sensitive physiological marker. Taken together, data from this study clearly indicate a differential role of psychophysical versus social and of brief versus prolonged stress on neuroendocrine and immune function, suggesting that the quality and the extent of the stress period are crucial in determining individual neuroendocrine-immune responses to external challenges.

5.1.3. Social deprivation and stressful events can amplify the consequences of a genetic susceptibility accelerating and precipitating breast cancer

Initial and progressive stages of carcinogenesis are affected by both genetic and environmental factors: stressful events, in particular, might impair immunosurveillance or cause persistent inflammation during critical life phases, affecting tumor progression. More in detail, the central nervous system and the immune system are known to be engaged in an intense bidirectional crosstalk which can be affected by stress and which involves multiple mediators (Hayley et al., 2005; Cirulli and Alleva, 2009; Moreno-Smith et al., 2011; Capoccia et al., 2013). Epidemiological studies indicate that a dysregulation of the HPA axis has been linked to breast cancer mortality, predicted by disrupted cortisol

diurnal rhythms in women with metastatic disease (Sephton et al., 2000). Interestingly, recent findings link high levels of primary tumor glucocorticoid receptor expression (and associated increased glucocorticoid-mediated gene expression) to more rapid estrogen-independent breast cancer progression. Furthermore, animal models of human breast cancer suggest that glucocorticoids inhibit tumor cell apoptosis (Volden and Conzen, 2013). In this context, loneliness and lack of social support, a known psychosocial stressor, has been associated with pain, fatigue, lethargy and higher inflammation among cancer patients (Gagliardi et al., 2009; Hughes et al., 2014), while in mouse models of cancer, exposure to social isolation alters tumor and spleen macrophage populations and potentiates tumor growth and metastasis (Hermes et al., 2009; Williams et al., 2009; Madden et al., 2013; Volden and Conzen, 2013). In **Chapter 4**, we investigated the effects of prolonged social isolation and restraint stress on a transgenic mouse model of breast cancer. Results indicate that in MMTVNeuTg mice social isolation accelerates disease onset, earlier detection of lumps in the mammary glands and tumor development, being accompanied by significant histological modifications in the mammary fat pad. More in detail, the histological analysis of mammary glands indicated that isolated transgenic mice display small, multilocular adipocytes that are reminiscent of brown adipose tissue. This kind of fat is exclusively detected in the mouse early in mammary gland development, particularly during stages of ductal growth, and is associated with the synthesis and secretion of angiogenic and growth factors, resulting in markedly increased vascular density and, therefore, involved in cancer development (Jones et al., 2011; Cao et al., 2014). Our data are in line with the recent discover that a switch from white to brown fat is found in cancer-associated cachexia and is linked to greater tissue energy expenditure (Petruzzelli et al., 2014). The higher presence of BAT in stressed and isolated mice indicates that these subjects have a more metabolically active tissue, a process that is likely to fuel the tumor, and which could favour the greater detection of breast lumps through palpation. It must be emphasized that, given the peculiarity of the transgenic model, it is likely that a ceiling effect at some point occurs so that it is hard to detect significant differences in tumor number. Nonetheless, a more metabolically active tissue could lead to an earlier onset of cachexia in these animals, a hypothesis that will need to be tested with subsequent studies (Petruzzelli et al., 2014).

Cao and colleagues have suggested a neurophysiologic mechanism through which increased expression of hypothalamic BDNF, resulting from environmental enrichment, activates sympathetic innervation of white adipose tissue, leading to important changes in circulating levels of adipokines, ultimately resulting in tumor suppression (Cao and During, 2012). By studying this mechanism in an opposite situation, that is social isolation, we were able to show how stress experiences might promote cancer progression in MMTVNeuTg transgenic mice (see **Chapter 4**). We found that subjects experiencing both isolation and restraint stress were characterised by low BDNF

expression in the hypothalamus as well as increased leptin and decreased adiponectin expression in peripheral fat tissue, suggesting the involvement of a neuroendocrine/metabolic cascade in tumor progression. Leptin exerts pro-angiogenic, pro-inflammatory, mitotic and anti-apoptotic actions inducing breast cancer development (Grossmann and Cleary, 2012; Khan et al., 2013). By contrast, adiponectin represses tumor proliferation in breast cancer cells, low blood concentrations of adiponectin being associated with high incidence and poor prognosis of breast cancer (Jarde et al., 2011). Interestingly, adipokines have been linked also to inflammation and we found that TNF-α decreased in isolated MMTVNeuTg mice after stressful challenge exposure, while IL-6 production increased. These results are in line with the antitumor properties of TNF-α, which is a multifunctional cytokine, playing a key role in apoptosis and cell survival as well as in inflammation and immunity (van Horssen et al., 2006). Indeed, our data reveal that chronic social isolation affects the production of this cytokine, promoting disease. As for IL-6, there is some evidence that elevated serum levels of this cytokine are important indicators of poor prognosis and can favor tumor growth (Nakashima et al., 2000; Petruzzelli et al., 2014). Complementing these data is the greater splenocyte apoptosis in MMTVNeuTg mice, an effect amplified by both stress procedures.

It must be taken into account that the tumor can produce cytokines and humoral factors that act on the brain to cause sickness behaviour. The decreased preference for sweet solutions represents a properly feature of depression (Dantzer et al., 2008; Lamkin et al., 2011) and in the **Chapter 4** we analyzed also this aspect finding increased expression of anhedonia in MMTVNeuTg mice both in the familiarization phase and during the restraint stress exposure, suggesting the potential role of a stressful challenge to precipitate depression symptoms.

5.1.4. BDNF is a key molecule capable to integrate behavioural, immune and endocrine responses to stress

The brain may be the missing link among emotions, stress, and disease. Results from this work show that the levels of the neurotrophin BDNF change according to behavioural phenotype (**Chapters 2 and 4**), neuroendocrine and immune responses (**Chapters 3 and 4**) and metabolic changes that can ultimately affect cancer progression (**Chapter 4**).

BDNF is produced by different cell types including immune cells, adipocytes, endocrine and endothelial cells and has a key position in integrating neural, immune and endocrine responses to stressors (**Chapter 3**). This neurotrophin is involved in the neuronal plasticity of brain structures underlying mood circuitry and we found that chronic stress decreases its levels in the limbic regions such as the frontal cortex, hippocampus, midbrain and hypothalamus (**Chapters 2, 3 and 4**) promoting depression like-behaviour. The hypothalamus, in particular, was the brain region showing the greater difference

in BDNF levels between socially isolated and group-housed mice. This piece of data is in agreement with previous reports suggesting a specific role of hypothalamic BDNF in regulating neuroendocrine responses to stress (Tapia-Arancibia et al., 2004). As for the midbrain, this area is central to antidepressant action as is characterised by high levels of serotonin, a neurotransmitter involved in mood disorders, which is directly related to BDNF function (Thoenen et al., 1991; Lindholm et al., 1994). This neurotrophin, because of its well recognized role in depression, could be a crucial factor involved both in the potentiation of neuroendocrine responses to stress, and in mood modifications which accompany exposure to stress. In the case of cancer, it must be taken into account that a diagnosis of cancer and its treatments are stressful events. Emotional distress and negative affect states are common after a cancer diagnosis and treatment and contribute to increased anxiety, depressed mood, social disruption and sleep and fatigue-associated disruption (Ganz et al., 2002; Stanton, 2006; Antoni, 2013) which could precipitate disease progression and promote recurrence. It is important to consider that the tumor itself can produce depressive symptoms, which might generate a positive feedback loop between coping style and cancer progression (Sephton et al., 2009). In this regard, BDNF may also play a role.

In **Chapters 3 and 4** we found that, following stress exposure, specific changes in central levels of BDNF were associated to the profile of cytokines production and to anhedonia (**Chapter 4**). Interestingly, inflammation can also linked to peripheral adipokines production and in **Chapter 4** low levels of hypothalamic BDNF were associated with increased leptin and decreased adiponectin gene expression in isolated transgenic mice. These results are in line with the recent description of BDNF as playing a main role in breast cancer cell growth, given its involvement in integrating neuroendocrine and metabolic responses to external challenges (Yang et al., 2012).

In summary, BDNF appears involved in most physiological changes that accompany stress perception and response: HPA axis activation (**Chapters 2, 3 and 4**), immune system function (**Chapters 3 and 4**), adipose metabolism (**Chapter 4**) and behavioural responses (**Chapter 2 and 4**) all regulating disease susceptibility. Thus, overall, our studies suggest that BDNF plays a central role in the orchestration of the brain and peripheral plasticity representing one of the principal mediators of this integrated response mechanism.

5.2. IMPACT AND PERSPECTIVES

The potentially negative impact of stressful life events on health and wellbeing is increasingly recognised as a significant public health issue. Indeed, decrements in health status and quality of life have been attributed to social factors such as poverty, early life adverse experiences, the presence or absence

of social support, employment status, the availability of food sources and the characteristics of the working environment (Marmot, 2005). Other studies have suggested that witnessing or experiencing violence, particularly intimate partner violence (Hegarty et al., 2004), political violence (Mollica et al., 1987; Eisenman et al., 2003) and child maltreatment (Felitti et al., 1998; Hegarty et al., 2004), may increase one's risk of developing depressive symptoms. Others have linked stressful events such as war and conflict, natural disasters, academic failure, injury, job loss, major financial crises, divorce, illness or death of a loved one to the experience of poor health (Schwarzer R. and Schulz U., 2002; Humphreys and Lee, 2009; Humphreys et al., 2012).

In summary, our data indicate that prolonged social isolation leads to long-term changes in HPA axis reactivity, in addition to reduced immune response and increased anxiety and depression like-behaviour in mouse models. It is of special interest that women are more at risk than men for depression and for most anxiety disorders. Studies in women specifically have suggested that social health issues and stressful life events are related to a range of physical and emotional health problems, and reduced health-related quality of life. These include increased sexual and reproductive health problems, chronic pain, somatic conditions, gastrointestinal disorders, suicidal ideation and risk-taking behaviours (Ellsberg et al., 2001). Interestingly, social isolation and related stress could contribute to increase susceptibility to disorders that are known to be characterised by a psychological component, such as human breast cancer (Gagliardi et al., 2009; Hughes et al., 2014).

5.2.1. Delivering biomarkers for early detection and prevention strategies for breast cancer

Breast cancer is the most common type of cancer in women throughout the world and remains a major health problem. The overall pattern of mortality for this cancer reveals high rates for Western, industrialized nations, particularly those of Northern Europe and North America, and lower rates for less industrialized and Asian nations. These differences in risk have been attributed to aspects thought to be profoundly involved in the aetiology of the disease such as dietary, cultural, and/or environmental factors. The risk of developing breast cancer is related to a number of factors including the events of reproductive life and lifestyle factors that modify endogenous levels of sex hormones.

In Italy approximately 48,000 new cases of breast cancer have been diagnosed in 2013 and early diagnosis and care allows save many women each year (AIRTUM, 2013). According to the National Health Plan, a major attention should be placed on interventions targeted to implement healthy life styles. While diet and exercise have been also found to play an important role in the aetiology of breast cancer, there are other important factors that should be considered as they might act as an incentive for inadequate life styles. Psychological stress, in particular, could favour bad eating habits or boost

cigarette smoking. Chronic levels of elevated cortisol characterising stress can weaken the ability of the immune system to fight disease, including not only breast cancer, but also high blood pressure, elevated blood glucose (linked with increased risk of weight gain and diabetes), and osteoarthritis. In addition, stressful life events could lead to a depressed state and influence cancer progression through alterations of immune functioning. Thus co-morbidity exists between stress-derived pathologies that might interact, leading to an amplification of effects with consequences on tumor progression or on recurring illness.

Data obtained from animal models might help identifying validated peripheral markers of stress and immune function related to breast cancer progression to allow rapid translation to the clinic and to study prospectively prognostic factors for development of depressive status of patients during chemotherapy. In particular, BDNF and pro-inflammatory cytokines stimulate multiple intracellular signalling cascades involved in the disruption of emotional state and in mood modifications known to characterise cancer patients. In this context, the increased levels of IL-6 and the decreased expression of BDNF could represent valid biomarkers for early detection of individuals more susceptibile to the negative consequences of stress.

In addition, targeting the neuroendocrine effector pathways in tumor cells (e.g. the use of GR antagonists and GR selective modulators) may benefit those cancer patients with a tumor microenvironment that could render them particularly susceptible to GC signalling effects. Therefore, to optimize patient care, cancer and behavioural researchers will must work together to understand the influence of glucocorticoid signalling in human cancer biology (Volden and Conzen, 2013).

Psychosocial interventions teach stress management skills (relaxation and coping strategies) to decrease distress and interpersonal skills to build social support (Antoni, 2013), improving the quality of life and helping to manage pain and other physical symptoms. In this context cognitive behavioural therapy could reduce tension, anxiety and distress through relaxation training, mindfulness, hypnosis, yoga and other techniques (Spiegel, 2008). Indeed, the professional figure of psycho-oncologist could be relevant to help patients during the management of disease and to identify frailty individuals most susceptible to adverse psychological outcomes and at high risk for relapse.

It must be taken into account that recent studies have shown that moderate physical activity can be beneficial for the brain and metabolic systems (Perseghin et al., 1996; Kramer et al., 2003; Barbour and Blumenthal, 2005). Voluntary physical activity has been shown to increase neurotrophin expression in cortex and hippocampal regions of the brain (Cotman and Berchtold, 2002), as well as to increase neurogenesis in the dentate gyrus in animals (van Praag et al., 2005). Moreover, increased neurogenesis in dentate gyrus has been linked to the actions of antidepressant drugs, providing a potential parallel with the antidepressant actions of physical activity (Olson et al., 2006). Increased

neurogenesis improves memory (Wiskott et al., 2006) and may contribute to greater cognitive flexibility (Karten et al., 2006; Wiskott et al., 2006). Since loneliness appears enhance cancer-related fatigue and physical disfunction, cognitive problems, anxiety and depression (Antoni, 2013; Mustian et al., 2013), the promotion of social groups dedicated to physical activity among cancer patients, could represent a valid therapeutic instrument. Importantly, cumulative evidence from social epidemiological studies has repeatedly demonstrated that different dimensions of social relationships can affect longevity and health via unique neurobehavioural pathways (Cohen, 2004; Cohen and Janicki-Deverts, 2009). One dimension of social relationships that has been most consistently linked to longevity and health is social integration, a multidimensional construct referring to an individual's effortful behavioural engagement in wide ranging social activities and relationships.

It must be taken into account that not only exercise, but also diet can play an important role in the aetiology of breast cancer. Psychological stress could favour bad eating habits leading to a shift to dysfunctional adipose tissue. In addition to its lipid-storing capacity, fat is a highly active endocrine and metabolic organ. Adipose tissue, which is made up of various cell types, such as adipocytes, pre-adipocytes, fibroblasts, macrophages, and blood vessels, produces numerous adipokines, such as leptin and adiponectin, that can stimulate or inhibit cell growth (van Kruijsdijk et al., 2009). In **Chapter 4**, we found that a mouse model of breast cancer, experiencing both isolation and restraint stress, was characterised by increased leptin and decreased adiponectin gene expression in peripheral adipose tissue, both promoting tumor progression.

Data from animal study could help to deliver biomarkers that can be used for early screening and prevention strategies: intervening early in the disease course could help the identification of patients with high risk of recurrence and ameliorate quality of life.

5.3. CONCLUSIONS

There is increasing evidence that stressful events can compromise the physiological response as well as behavioural strategies, promoting mood disorders and precipitating disease. The work presented in this thesis underlies the importance of considering the quality and timing of stressors in determining individual neuroendocrine-immune responses to external challenges.

While this is a first attempt to mimic some of the qualitative and temporal features of “stress,” our data clarify the mechanisms through which the length of stress can affect an organism, identifying a specific neuroendocrine-immune profile associated to specific changes in central mediators and behaviour. Stress affects peripheral targets acting through a mechanism that involves the HPA axis activity, cytokines production and metabolic response. In this context, the

neurotrophin BDNF appears the key molecule involved in all neurobiological changes that accompany stress appraisal.

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LIST OF PUBLICATIONS (2012-2014)

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Capoccia S., Berry A., Bellisario V., Raggi C., Barbati C., Ortona E., D'Urso T., Cecchetti S., Sestili P., Aricò E., Proietti E., Pelicci P.G. and Cirulli F. Social isolation promotes breast cancer progression through a BDNF-neuroendocrine axis. (*Cancer Prevention Research,* submitted).

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APPENDIX:
THESIS PUBLICATIONS