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Impact of CBG deficiency on emotional and cognitive processes

Gabriela Ferreira de Medeiros

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THESIS PRESENTED
TO OBTAIN THE DEGREE OF

**DOCTOR AT
BORDEAUX UNIVERSITY**

DOCTORAL SCHOOL OF HEALTH AND LIFE SCIENCES
SPECIALITY NEUROSCIENCES

By Gabriela FERREIRA DE MEDEIROS

**IMPACT OF CBG DEFICIENCY
ON EMOTIONAL AND COGNITIVE PROCESSES**

Under the supervision of: Dr. Marie-Pierre MOISAN

Defended on July 25th, 2016

Members of the jury:

Dr. BERACOCHEA, Daniel
Prof. ROOZENDAAL, Benno
Prof. RUIZ-GAYO, Mariano
Dr. CASTANON, Nathalie

Université de Bordeaux, France
Radboud University, the Netherlands
Universidad CEU San Pablo, Spain
Université de Bordeaux, France

Jury President
External Examiner
External Examiner
Invited Examiner

Title : Impact of CBG deficiency on emotional and cognitive processes

Abstract : The great diversity in the response to stress observed among individuals originates from their genetic background in interaction with environmental factors. Some responses can be less adaptive and increase the vulnerability to develop stress-associated disorders. CBG is a plasma glycoprotein that regulates the bioavailability of glucocorticoids, one of the main mediators of the stress response. Genetic studies pointed out variations in the gene coding for CBG as a major factor influencing the glucocorticoid response to stress. To better understand the mechanisms involved and the consequences on endocrine and behavioral responses to stress, our team has developed a mouse model of CBG deficiency. These mice present blunted glucocorticoid response to stress associated with increased despair-like behaviors. This thesis aimed at further exploring the physiological and behavioral alterations presented by the Cbg ko mice. We showed that the lower glucocorticoid levels observed in Cbg ko mice stems from their higher clearance from plasma. A study performed on Cbg ko female mice revealed that estrogens outpass CBG deficiency in inducing despair-like behavior. Additionally, we evidenced that CBG deficiency leads to lower behavioral and endocrine sensitivity to chronic stress, and we observed impairment of hippocampal-dependent long-term memory in these mice. Finally, we found that chronic stress combined to high-fat diet leads to alteration in glucose metabolism in CBG deficient animals. These findings reinforce the important role of CBG influencing the broad range of actions of glucocorticoids by modulating their levels and availability.

Keywords : transcortin, CBG, HPA axis, glucocorticoids, stress, memory consolidation, emotional behavior, western lifestyle.

Titre : L'impact de la déficience en CBG sur les processus émotionnels et cognitifs

Résumé : La grande diversité des réponses de stress observée entre individus a pour origine des facteurs génétiques en interaction avec des facteurs environnementaux. Certaines réponses peuvent être moins adaptées et accroître la vulnérabilité de l'individu aux divers troubles et pathologies liées au stress. La CBG est une glycoprotéine plasmatique impliquée dans la biodisponibilité des glucocorticoïdes, un des principaux médiateurs de la réponse au stress. Des études génétiques ont montré que des polymorphismes du gène codant la CBG ont un impact significatif sur la réponse des glucocorticoïdes au stress. Pour comprendre les mécanismes de l'impact de la CBG sur l'action des glucocorticoïdes et les conséquences sur les réponses endocriniennes et comportementales de stress, notre équipe a développé un modèle de souris déficiente pour le gène Cbg. Ces souris présentent une réponse diminuée des glucocorticoïdes au stress, associée à un niveau élevé de comportement émotionnel de type dépressif. Cette thèse a pour but d'explorer plus en profondeur les altérations physiologiques et comportementales des souris Cbg ko. Nous avons montré que le niveau plus faible de glucocorticoïdes observé chez la souris Cbg ko provient d'une élimination plasmatique plus importante. Une étude chez la souris Cbg ko femelles a montré que les estrogènes se surimposent à la déficience en CBG pour induire des comportements de type dépressif. Nous avons également démontré que la déficience en CBG conduit à une atténuation de la sensibilité comportementale et endocrinienne au stress chronique. Enfin, nous avons observé une détérioration de la mémoire long terme de ces souris. Par ailleurs, nous montrons que dans des conditions de stress chronique associé à un régime alimentaire déséquilibré le métabolisme du glucose était altéré chez les animaux déficients en CBG. Ces résultats renforcent l'importance du rôle de la CBG influençant l'ensemble des mécanismes d'actions des glucocorticoïdes par la modulation de leurs niveaux et de leur disponibilité.

Mots clés : transcortine, CBG, axe corticotrope, glucocorticoïdes, stress, comportement émotionnel, consolidation de la mémoire, style de vie occidental

Summary

	GENERAL INTRODUCTION	7
1	Stress	7
1.1	The concept of stress	7
1.1.1	The stress response as a complex system	7
1.1.2	Stress response: from adaptation to disease	11
1.2	HPA axis and the glucocorticoids	12
1.2.1	Regulation of the HPA axis	13
1.2.2	Negative feedback regulation of the HPA axis	16
1.2.3	Glucocorticoid synthesis	18
1.2.4	Glucocorticoid Metabolism	20
1.2.5	Glucocorticoid receptors and genomic responses	22
1.2.6	Non-genomic effects of glucocorticoids	23
1.2.7	Circadian and ultradian influences on the HPA axis	23
1.2.7.1	Effects of glucocorticoid pulsatility in the brain	26
1.2.8	The actions of glucocorticoids	27
1.2.8.1	Actions on growth and development	28
1.2.8.2	Actions on the immune and inflammatory responses	28
1.2.8.3	Actions on the metabolism	30
1.2.8.3.1	The impact of chronic stress on body weight	32
1.2.8.4	Actions on the central nervous system	34
1.2.8.4.1	Glucocorticoids and mood regulation	34
1.2.8.4.2	Glucocorticoids and memory	35
1.2.9	Variability in the HPA axis responses	36
1.2.9.1	Sex and gender	36
1.2.9.2	Genetic factors	38
1.2.10	Animal models of HPA axis alterations	39
1.2.10.1	CRH and CHR receptor	39
1.2.10.2	AVP and AVP receptor	41
1.2.10.3	ACTH receptor	42
1.2.10.4	Mineralocorticoid and glucocorticoid receptors	42
1.2.10.5	11-β-hydroxysteroid dehydrogenases	44
1.3	Corticosteroid binding globulin (CBG) or transcortin	51
1.3.1	Structure	51
1.3.2	Biosynthesis	52
1.3.3	Glycosylation	54
1.3.4	Clearance	55
1.3.5	Binding specificity	56
1.3.6	Binding dynamics	57
1.3.7	Interaction with cellular membranes	58
1.3.8	Variability of CBG levels	59
1.3.8.1	Variability among species	59
1.3.8.2	Physiological variability	59
1.3.8.3	Sexual dimorphism	60
1.3.8.4	Circadian variability	61
1.3.8.5	Changes in response to stress	62
1.3.8.6	CBG alterations during disease	63
1.3.8.7	Genetic variants and polymorphisms	66

1.3.8.7.1	In animal models	66
1.3.8.7.2	In humans	66
1.3.8.7.3	The Cbg ko mouse: a model of CBG dysfunction	70
	OBJECTIVES	73
	STUDY I - ROLE OF CORTICOSTEROID BINDING GLOBULIN IN EMOTIONAL REACTIVITY SEX DIFFERENCES IN MICE	76
	Introduction	76
	Article	78
	Complementary studies	90
	STUDY II – CHRONIC STRESS DOES NOT FURTHER EXACERBATE THE ABNORMAL PSYCHONEUROENDOCRINE PHENOTYPE OF CBG-DEFICIENT MALE MICE	95
	Introduction	95
	Article	97
	Complementary studies	103
	STUDY III – CBG DEFICIENCY SPECIFICALLY IMPAIRS HIPPOCAMPUS-DEPENDENT MEMORY CONSOLIDATION IN MALE MICE	111
	Article (under preparation)	111
	Complementary studies	121
	STUDY IV – BEHAVIORAL AND METABOLIC CONSEQUENCES OF CBG DEFICIENCY ON MALE MICE SUBMITTED TO HIGH-FAT DIET COMBINED TO CHRONIC MILD STRESS	122
	Introduction	122
	Article (under preparation)	124
	Complementary studies	158
	GENERAL DISCUSSION	195
	Conclusions	200
	REFERENCES	201

List of figures

Figure 1 – Main systems involved in the stress response.

Figure 2 – Time course of release and action of the stress response mediators.

Figure 3 – Negative impact of stress on physical and psychological health.

Figure 4 - Plasma corticosterone values of young male Wistar rats exposed to several different acute stressors,

Figure 5 – Negative feedback of the HPA axis.

Figure 6 - Glucocorticoid synthesis pathway.

Figure 7 – Principal routes of metabolism of cortisol.

Figure 8 – The two enzymes: 11 β -hydroxysteroid dehydrogenase 1 and 2 that interconvert active and inactive glucocorticoids.

Figure 9 – The circadian and ultradian dynamics of glucocorticoid secretion.

Figure 10 – The effect of glucocorticoid pulses and of an acute stressor on glucocorticoid receptors translocation to the nucleus and binding to DNA.

Figure 11 – Crystal structure of rat CBG.

Figure 12 – Quantitative analysis of Cbg mRNA by real-time PCR.

Figure 13 – Immobility scores displayed by male and female wild-type and Cbg ko mice in the forced swim test.

Figure 14 – Total, percentage of free and absolute free levels of corticosterone in the plasma of male and female wild-type and Cbg ko mice submitted to the tail suspension test.

Figure 15 – Free plasma corticosterone levels in the morning in wild-type and Cbg ko mice submitted to the social defeat stress protocol.

Figure 16 – Expression of genes from the HPA axis.

Figure 17 – Expression of genes involved in mood regulation and regulated by glucocorticoids and diet.

Figure 18 – Levels of monoamines measured in the frontal cortex by HPLC.

Figure 19 – The unpredictable chronic mild stress protocol.

Figure 20 – Weight and percentage of weight gained by wild-type and Cbg ko mice.

Figure 21 – Comparison of absolute weight and percentage of weight gained between wild-type animals from the western lifestyle experiments conducted in 2009 (original) and 2014 (replicate).

Figure 22 – Percentage of fat and lean mass of wild-type and Cbg ko mice after 21 weeks of diet.

Figure 23 – Percentage of brown, visceral and subcutaneous adipose tissue in wild-type and Cbg ko mice after 21 weeks of diet.

Figure 24 – Food intake throughout the 24 weeks of experiment.

Figure 25 – Oral glucose tolerance and insulin resistance tests performed in the wild-type and Cbg ko groups after 22 weeks of high-fat diet and one week after the end of the chronic stress.

Figure 26 – Performance of wild-type and Cbg ko mice in three depression-related behavioral tests.

Figure 27 – Percentage of distance travelled and of time spent in the center of the open field test by wild-type and Cbg ko mice.

Figure 28 – Composition and caloric content of standard chow, high-fat chow and very high-fat chow.

Figure 29 – Weight, percentage of weight gained and food intake of C57BL6/J mice submitted to either standard, high-fat or very high-fat diet, and housed over either spruce sawdust or corncob grit bedding.

Figure 30 – Percentage of body brown, visceral and subcutaneous adipose tissue in C57BL6/J mice submitted to either standard, high-fat or very high-fat diet, and housed over either spruce sawdust or corncob grit bedding.

Figure 31 – Plasma leptin levels, percentage of body fat and the correlation between both factors in C57BL6/J mice submitted to either standard, high-fat or very high-fat diet, and housed over either spruce sawdust or corncob grit bedding.

Figure 32 – Blood glucose after overnight fasting, plasma insulin levels, and blood glucose levels during the oral glucose tolerance and the insulin resistance tests in C57BL6/J mice submitted to either standard, high-fat or very high-fat diet, and housed over either spruce sawdust or corncob grit bedding.

Figure 33 – Total corticosterone levels in the morning and in the evening of C57BL6/J mice submitted to either standard, high-fat or very high-fat diet, and housed over either spruce sawdust or corncob grit bedding.

Figure 34 – The unpredictable chronic mild stress protocol.

Figure 35 – Weight and percentage of weight gained by wild-type and Cbg ko.

Figure 36 – Food intake throughout the 20 weeks of experiment for the unstressed groups and throughout the four weeks of UCMS for the stressed groups.

Figure 37 – Blood glucose after overnight fasting before the beginning of the experiment, after 14 weeks of very high-fat diet and after 4 weeks of unpredictable chronic mild stress.

Figure 38 – Percentage of fat and lean mass of wild-type and Cbg ko mice on weeks 0, 14 and 18.

Figure 39 – Oral glucose tolerance test performed in wild-type and Cbg ko groups after 18 weeks of very high-fat diet and one week after the end of the chronic stress.

Figure 40 – Performance of wild-type and Cbg ko mice in the tail suspension test and in the contextual fear conditioning.

Figure 41 – Percentage of body weight represented by brown adipose tissue, visceral adipose tissue and subcutaneous adipose tissue in wild-type and Cbg ko mice.

Figure 42 – Total corticosterone in the morning and evening on wild-type and Cbg ko mice.

List of tables

Table 1 – Genetic models of HPA axis alteration and their associated phenotypes.

Table 2 – CBG-bound endogenous steroids in human plasma.

Table 3 – Influence of various steroids *in vitro* on human CBG binding to cortisol.

Table 4 – Sample sizes of groups of animals submitted to the forced swim test (FST) and tail suspension test (TST) after being treated with imipramine (20 mg/kg) or saline solution.

Table 5 – Sequences of the primers used for gene expression analyses by RT-qPCR.

Table 6 – Mean body weight and mean percentage of weight gain from mice from the western lifestyle experiment replicate.

GENERAL INTRODUCTION

1. Stress

1.1 The concept of stress

All living organisms are exposed to endogenous and exogenous changes to which they have to adapt in order to maintain their bodies' dynamic equilibrium – or homeostasis. Although the concept of a harmonious internal balance associated to health has been described since ancient Greece, the term homeostasis was first proposed by Walter Cannon in the early 1900's (Johnson et al., 1992). In the classical stress concept, homeostasis is threatened (or perceived to be threatened) by certain physical and psychological events that are known as stressors. Stress can trigger physiological and behavioral responses that are aimed at reinstating homeostasis. This "stress response" is reflected in the rapid activation of several integrative mechanisms, such as the sympathetic nervous system, which leads to the release of noradrenalin (NA) from widely distributed synapses and adrenalin from the adrenal medulla, and the hypothalamic–pituitary–adrenal (HPA) axis, which promotes the secretion of glucocorticoids from the adrenal cortex. These mechanisms activate multiple physiological and/or behavioral traits simultaneously, while inhibiting several others, and thus mediate an integrated and appropriate response of the whole organism to a specific stimulus (McEwen, 2004, de Kloet et al., 2005, Angelier and Wingfield, 2013).

The adrenergic system was the first physiological system shown to be implicated in the stress response. Walter Cannon demonstrated in early 1900s that the sympathoadrenal system was responsible for coordinating the "fight or flight" response necessary to cope with external challenges. In the following decades, attention shifted from the sympathetic nervous system to the adrenal glands, notably through the work of Hans Hugo Bruno Selye – a pioneering Hungarian endocrinologist working in Canada – who also found that the adrenal cortex derived "corticoids" participate in the stress response, and named them glucocorticoids. Selye was also the one to borrow the term stress from physics, and defined it as "the non-specific neuroendocrine response of the body". All this has led to a more complete description of the HPA axis towards the end of the 20th century and culminated on the integrated stress system theory which is widely accepted nowadays (Johnson et al., 1992, Nicolaidis et al., 2015).

1.1.1 The stress response as a complex system

The process of adapting to challenges of daily life, as well as to major life stressors, to maintain homeostasis has been called allostasis. The biological mediators of allostasis, such as glucocorticoids and adrenalin, are released in response to stressors or to lifestyle factors such as diet, sleep, and exercise, promoting adaptation and being generally beneficial. Not only physical homeostatic disturbance but also purely psychological processes can disrupt homeostasis, triggering the stress response (McEwen, 2004, de Kloet et al., 2005).

Following disruption of homeostasis, the HPA axis and the sympathetic nervous system are activated. These constitute the essential elements mediating the stress response, leading towards homeostatic preservation. The stress system has both central and peripheral components (Figure 1). The central components of this system are mainly located in the hypothalamus and in the brainstem. They include the

parvocellular neurons of the paraventricular nuclei (PVN) of the hypothalamus that secrete corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), the CRH neurons of the paraventricular and parabrachial nuclei of the brainstem, and the *locus caeruleus* (LC) noradrenergic cell groups of the pons and medulla (the LC-NA system). CRH and AVP synergistically stimulate the secretion of adrenocorticotropic hormone (ACTH) by the corticotroph cells of the anterior pituitary, leading to adrenal steroidogenesis. The LC-NA/sympathetic systems are located in the brain stem, and can both activate and be activated by the amygdala, which – together with the hippocampus, anterior cingulate and prefrontal cortex (PFC) – promotes the attention focus on the perceived threat. Activation of the LC-NA system leads to release of NA from an extraordinarily dense network of neurons throughout the brain, resulting in enhanced arousal and vigilance. The peripheral components of the stress system are the HPA axis, together with the efferent sympathetic/adrenomedullary system, and components of the parasympathetic system (Chrousos and Gold, 1992, Nicolaides et al., 2015).

The part of the stress response under the command of the autonomous nervous system employs the well-known neurotransmitters acetylcholine and NA, as well as numerous neuropeptides, lipid mediators of inflammation, adenosine triphosphate and nitric oxide. As previously mentioned, the sympathetic system plays an important role through the adrenal medulla in the “fight or flight” reaction, by secreting adrenalin and NA into the general circulation. The activity of enzymes that regulate catecholamines’ biosynthesis is also stimulated by the stress response. The parasympathetic system, on the other hand, regulates the sympathetic functions by antagonizing (increasing its activity) or assisting them (withdrawing its activity) (Johnson et al., 1992, Nicolaides et al., 2015).

Regarding the HPA axis response to stress, its activation culminates in the secretion of glucocorticoids – mainly cortisol in humans and many mammals, and corticosterone in most rodents – from the adrenal cortex into the general circulation. Circulating corticosteroids act on a variety of tissues, basically mobilizing energy stores through an increase in catabolic activity – such as lipolysis and proteolysis – and a suppressing of energy-costing processes – such as reproductive function. They also influence a number of adaptive behaviors. All these responses to acute elevations in glucocorticoids that occur following stressors are thought to be beneficial in the short term, as they favor the fight or flight response (Chrousos, 2009, Handa and Weiser, 2014).

The stress response dynamics consists of two waves of serial hormonal secretion that will induce hormonal changes which will then be translated into cellular end-effects. The first wave begins within seconds after the stressor is perceived and involves: (i) increased release of adrenalin and NA from the sympathetic nervous system; (ii) secretion of hypothalamic CRH into the hypophysial portal system and subsequent enhanced release of ACTH; (iii) decreased release of hypothalamic gonadotropin-releasing hormone (GnRH) followed by decreased secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary, and (iv) enhanced secretion of pituitary-derived prolactin and growth hormone (GH), and increased release of glycogen from the pancreas. The effects of monoamines and peptides involved in the first wave are usually fast and mediated by G protein–coupled receptors and rapid second messenger pathways. A second wave occurs later in time, usually between 1 and 2 hours after the onset of the stressor, and involves the secretion of glucocorticoid hormones. Despite some fast non-genomic effects, the effects of this second wave occur mainly through the activation of the GR receptor and corticotropin

releasing hormone receptor 2 (CRHR2) signaling, and inhibit the lingering effects of the first wave of stress-induced actions. The effects of this second wave usually need more time – from at least 20 min to hours and even days – to complete the appropriate molecular scenario (Figure 2) (Joels and Baram, 2009, Nicolaides et al., 2015).

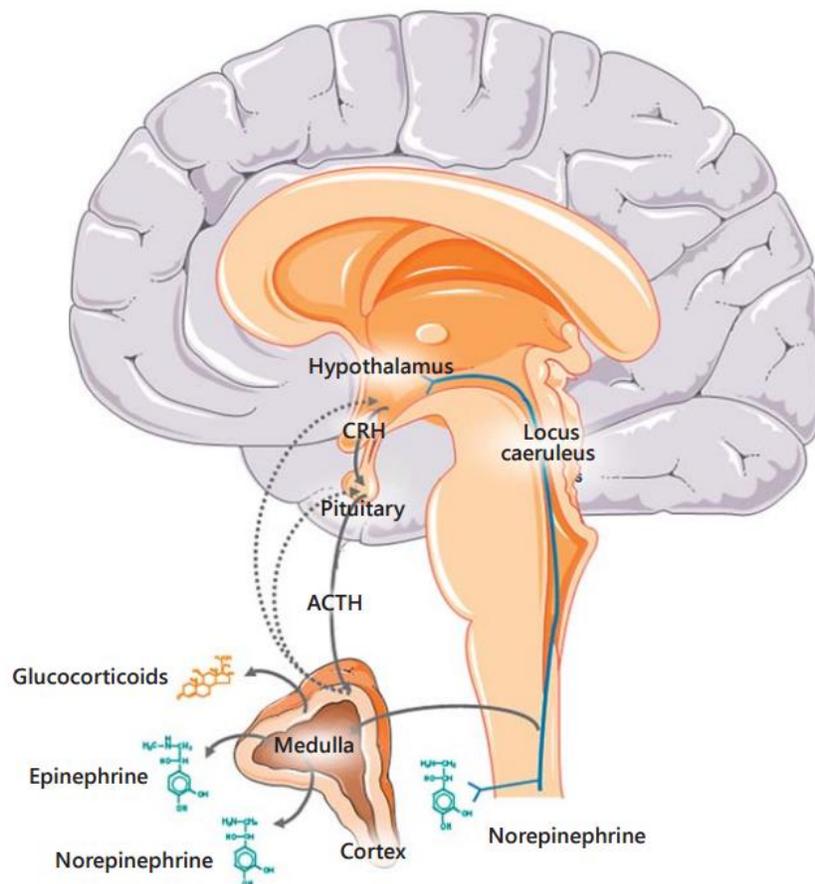


Figure 1 – Main systems involved in the stress response (from (Nicolaides et al., 2015)).

The central and peripheral components of the stress system have many sites of interaction with other systems at multiple levels. CRH neurons and the LC/NE system seem to participate in a positive, reverberatory feedback loop so that activation of one system tends to activate the other as well. In addition to interactions between the two central components of the stress system themselves, the stress system activates the mesocortical and the mesolimbic dopaminergic reward systems, while it also receives inhibitory input from the latter systems. The stress system also activates the central nucleus of the amygdala, which participates in emotional processing and which, upon activation, also stimulates the stress system, forming a positive regulatory feedback loop with it. The PVN of hypothalamus communicates with the arcuate nucleus through CRH neurons that induce the release of α -melanocyte-stimulating hormone and β -endorphin from neurons in the arcuate nucleus. These two molecules are both derived from the same precursor polypeptide – pro-opiomelanocortin (POMC) – which is also the precursor of ACTH. Increases in the secretion of α -melanocyte-stimulating hormone and β -endorphin inhibit the activity of the CRH and LC/NE systems.

Moreover, the two components of the stress system appear to respond to many neurochemical modulators, such as serotonin and acetylcholine, which are acutely

stimulatory, and γ -aminobutyric acid (GABA) and benzodiazepines, which are inhibitory to CRH neurons and the LC/NE system. Neuropeptide Y (NPY), the most potent orexigenic factor known, stimulates the secretion of CRH and inhibits the LC-NA system. This may be of particular relevance to changes in stress system activity in states of dysregulation of food intake. Interestingly, glucocorticoids stimulate hypothalamic NPY gene expression while they inhibit both the PVN CRH and LC/NE-sympathetic systems (Chrousos and Gold, 1992, Chrousos, 1998, 2009, Nicolaidis et al., 2015).

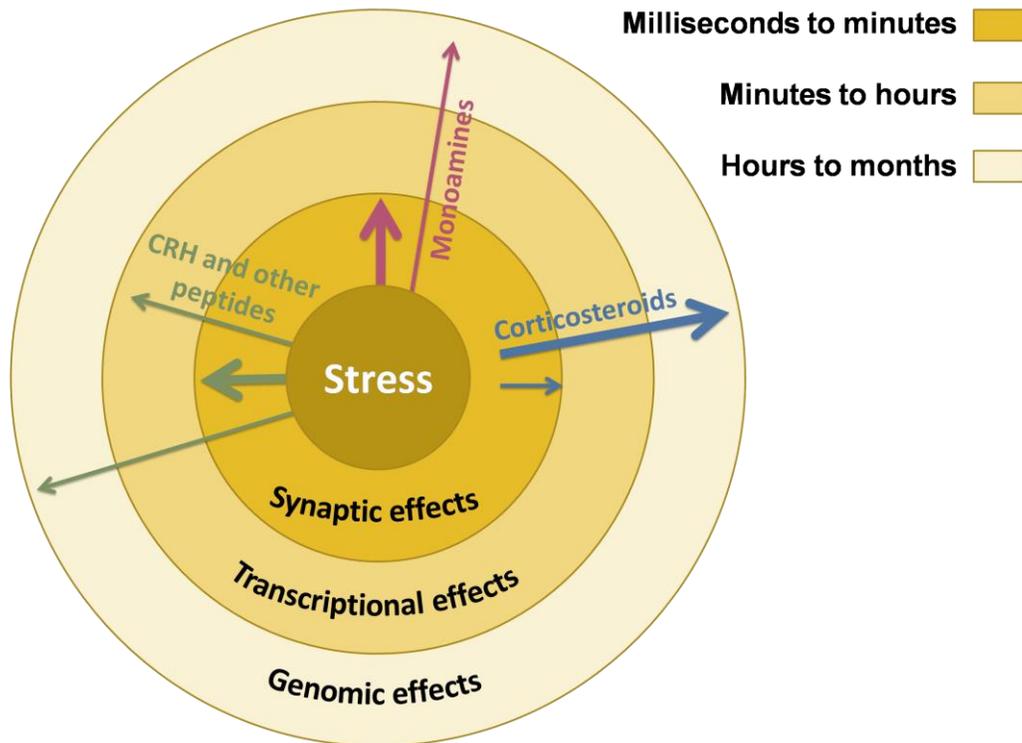


Figure 2 – Time course of release and action of the stress response mediators. Shortly after a stressful event, the secretion of monoamines – including noradrenaline, dopamine and serotonin – is promptly increased. Their actions are fast and hardly outlast the duration of stressor exposure. Many are the neuropeptides that mediate the effects of stress, such as vasopressin, orexin, ghrelin, dynorphin, oxytocin and neuropeptide Y. Their time of release and the duration of their effects also vary greatly. Corticosteroids are naturally secreted in a pulsatile and circadian fashion, but stress triggers the release of a large burst of corticosteroids that superimposes on these rhythms. Levels of glucocorticoids tend to peak between 30 min and 1 hour after the onset of the stressor, and despite having some fast actions, most of their effects are a result of their roles as transcription factors (adapted from (Joels and Baram, 2009)).

Different stress mediator receptors are often expressed in “hot spots” of the brain, such as the PFC, the amygdala, the hippocampus and the neurotransmitter synthesis areas (as the dorsal raphe nucleus and the LC, for example). This facilitates the interplay between the different systems during the stress response. These hot spots are strategic hubs that connect networks involved in different aspects of the stress response. For example, neurons in the basolateral amygdala (BLA), which are affected by NA, dopamine, CRH and corticosteroids, are instrumental for sympathetic and neuroendocrine activation (through their output to the central amygdala), for fine-tuning of arousal (through reciprocal connections with the LC) and for the processing of

emotional and contextual aspects of stress (through the PFC and hippocampus). A second hot spot is the hippocampus, where individual neurons can express receptors for corticosteroids, neuropeptides, monoamines (including serotonin and NA) and opioids, enabling the fine-tuning of neuronal firing in response to a range of signals. Thus, although each stress mediator has a characteristic and unique spatial domain, their functional contribution to the stress response is subject to integration with that of other mediators, through convergence on interconnected networks (Joels and Baram, 2009).

1.1.2 Stress response: from adaptation to disease

The interaction between homeostasis, stressors and allostasis can have three potential outcomes: first, the match may be perfect and the organism returns to its basal homeostasis; second, the match may also be perfect, but the organism gains from the experience and a new, improved homeostatic capacity is attained; and third, the adaptive response may be inappropriate (insufficient, inadequate, excessive and/or prolonged) and the organism fails to cope with the new situation. When coping fails, various events occur that result in a long-lasting state of distress, which is reflected in aberrant HPA axis activity, leading to physical and behavioral consequences (de Kloet et al., 2005, Chrousos, 2009).

The previously introduced term allostasis allows differentiating between the systems that are essential for life – homeostasis – and those that maintain these systems in balance – allostasis. When the systems involved in allostasis are active in a sustained manner, this is referred to as an allostatic state. An allostatic state results from an imbalance of the primary allostatic mediators, reflecting in an excessive production of some and an inadequate production of others. Some examples of allostatic states are hypertension, an altered cortisol rhythm in major depression or after chronic sleep deprivation, or chronic elevation of inflammatory cytokines and low cortisol in chronic fatigue syndrome. These states can be sustained for limited periods of time if enough energy is available to fuel homeostatic mechanisms (McEwen, 2004).

As already mentioned, the stress system not only responds to stressors on demand but also has a basal circadian activity. An appropriate basal activity, as well as an adequate response to stressors – quantitatively and temporally – is essential for both physical and psychological wellbeing. Regarding its activity in response to stressors, flaws in switching off the stress system after the stimulus is over, in turning it on adequately during the stimulus, overusing it by many, long-lasting or repeated stimuli may induce cumulative changes that lead to a wear-and-tear of the body and brain. These cumulative changes are called allostatic load, and the pathophysiology that can be triggered by these changes are called allostatic overload. The terms allostasis and allostatic overload allow for a more restricted and precise definition of the overused term “stress”, and provide a view of how the essential protective and adaptive effects of allostasis can lead to physical and psychological negative consequences when mismanaged or overused (McEwen, 2004, Chrousos, 2009, McEwen et al., 2015).

Some of the conditions that may be triggered by acute stress include allergic manifestations (asthma, eczema, urticaria), angiokinetic phenomena (migraines, hypertensive and hypotensive attacks), different types of pain (headaches, abdominal, pelvic, lower back pain), gastrointestinal symptoms (pain, indigestion, diarrhea, constipation) and psychological manifestations (panic attacks, psychotic episodes). The pathogenesis of acute stress induced disorders can be attributed to increased secretion and effects of the major stress mediators in the context of a vulnerable background.

Thus, acute allergic attacks may be activated by CRH-induced degranulation of mast cells in the vulnerable organ, such as the lungs or the skin, causing asthma or eczema, respectively. Similarly, migraines could be caused by the same process taking place in meningeal blood vessels, inducing local vasodilatation and increased permeability of the blood-brain barrier. Panic or psychotic attacks could also be triggered by CRH bursts in the central amygdala, activating a fear response. And finally, hypertensive or hypotensive attacks could be caused by excessive sympathetic or parasympathetic system outflow, respectively (Chrousos, 2009).

Chronic stress, on the other hand, may cause physical, behavioral and neuropsychiatric manifestations, contributing to the development of chronic stress-related disorders. Some of these manifestations are mainly linked to a hyperactivity of the stress response system, such as Cushing's syndrome, melancholic depression, anorexia nervosa, obsessive-compulsive disorder, panic disorder, alcoholism, diabetes mellitus, metabolic syndrome and hyperthyroidism. Among the conditions predominantly associated to a hypoactivity of the system, are atypical/seasonal depression, chronic fatigue syndrome, fibromyalgia, adult post-traumatic stress disorder, hypothyroidism, rheumatoid arthritis, asthma and eczema. Just as for acute stress related disorders, the pathogenesis of chronic stress-related disorders can also be explained by excessive – and, in the case of chronic stress, sustained – secretion, and effects of the major mediators of stress and sickness syndromes, which influence the activities of multiple homeostatic systems. A weak or insufficient stress response might also lead to damaging effects on health, since stress mediators – notably glucocorticoids hormones – are known to act by suppressing the activity of several body systems, such as the immune and inflammatory responses. Therefore, a malfunctioning of the stress response could culminate in an excessive activation of other pathways. These disorders thus represent chronic, maladaptive effects of two physiological processes whose mediators are meant to be secreted in a quantity- and time-limited fashion, but have gone awry (Chrousos, 2009).

The development and severity of the conditions associated with an inadequate stress response depend on the genetic, epigenetic and constitutional vulnerability or resilience of the individual to stress, on their exposure to stressors during critical periods of development, on the presence of concurrent adverse or protective environmental factors, and on the timing, magnitude and duration of stress, as illustrated on Figure 3. Indeed, several of the previously listed conditions associated to stress are part of the so called multifactorial polygenic disorders, such as obesity, metabolic syndrome, type 2 diabetes mellitus, hypertension, autoimmunity, allergy, anxiety, insomnia, depression, pain and fatigue syndromes (Chrousos, 2009, Joels and Baram, 2009).

1.2 HPA axis and the glucocorticoids

The HPA axis is the major neuroendocrine system in mammals and, as previously mentioned, its activation culminates in the secretion of glucocorticoids from the adrenal glands. This axis is highly conserved through evolution, with its components being present in early vertebrates. Glucocorticoid hormones are vital and regulate many physiological functions, including growth, glucose, fat, and protein metabolism. They also exert anti-inflammatory and immunosuppressive actions, and can affect mood and cognitive functions (Spiga et al., 2014).

1.2.1 Regulation of the HPA axis

Several endogenous and exogenous factors, such as stress, light, food, sleep, exercise and inflammation can have an impact on the activity of the HPA axis. Exercise represents a physical stress that challenges homeostasis and, therefore, activates the HPA axis, leading to an elevation of cortisol. However, sustained physical conditioning in highly trained athletes is associated with a decreased HPA response to exercise. Interestingly, these highly trained athletes also exhibit a mild but chronic hypercortisolism at basal conditions, which may be an adaptive change to chronic exercise (Mastorakos et al., 2005). Assuming that the stress response is a neuroendocrine mechanism that occurs to prepare the individual to perform a physical action, then physical activity should be the natural means to prevent the consequences of stress. Indeed, accumulating evidence corroborate the beneficial effects of regular exercise in preventing or ameliorating the metabolic and psychological consequences of chronic stress. These benefits are thought to derive from the reduction in the sensitivity to stress, and also from peripheral actions influencing metabolic functions (Tsatsoulis and Fountoulakis, 2006).

The activity of the HPA axis may also impact and be impacted by diet. For example, several studies have used removal of endogenous corticosteroids by adrenalectomy to show that, in the absence of glucocorticoids, feeding and body weight gain are reduced, at the same time that sensitivity to leptin and insulin are increased. Systemic – but not central – administration of glucocorticoids is able to reverse the feeding effects. Interestingly, sucrose ingestion is also capable of restoring food intake and many metabolic deficits following adrenalectomy. Sucrose and lard ingestion have also been shown to decrease CRH messenger ribonucleic acid (mRNA) in the central amygdala, suggesting that ingestion of palatable foods reduces the activity in the central stress response network, perhaps reducing the feeling of stressors (Green et al., 1992, Chavez et al., 1997, Zakrzewska et al., 1997, la Fleur, 2006). Negative energy balance can also be seen as a stressor, threatening the physiological homeostasis. In lean animals and humans, this negative balance has been shown to consistently activate the HPA axis, with emerging evidence of similar changes in overweight and obese people during lifestyle interventions for weight loss (Sainsbury and Zhang, 2012, Seimon et al., 2013).

For being secreted in daily cycles and act on widespread targets throughout the organism, glucocorticoid hormones are obvious candidates to mediate the temporal communication between the suprachiasmatic nucleus (SCN) of the hypothalamus central clock and the rest of the brain and body, regulating circadian rhythms. It has been suggested that, under stable lighting conditions, circulating glucocorticoids reinforce resistance of the circadian timing system to variations of the photoperiod. This would explain why abrupt surges of glucocorticoid concentrations in blood during stress exposure do not interfere in the organism's circadian rhythms (Sage et al., 2004). Despite the fact that lights on during nighttime may be interpreted as a stressor, glucocorticoids are not critical for the manifestation of changes in metabolism associated with nocturnal illumination. Nighttime light exposure has been inconsistently reported to elevate, reduce, or not affect glucocorticoid secretion, both in animal and human studies (Fonken and Nelson, 2014).

Sleep, in particular deep sleep, has an inhibitory influence on the HPA axis, whereas activation of the HPA axis or administration of glucocorticoids can lead to arousal and sleeplessness. In addition, induced sleep disruption is associated with

significant increases of plasma cortisol levels. Furthermore, mean 24-hour plasma cortisol levels are significantly higher in subjects with a shorter total sleep time than those with a longer total sleep time, and insomnia – the most common sleep disorder – is associated with a 24-hour increase of ACTH and cortisol secretion. Sleep deprivation represents a stress to the organism and should be associated with activation of the stress system. However, several studies that have assessed the effects of one night sleep deprivation on the HPA axis found that cortisol secretion either is not affected or is minimally affected following prolonged wakefulness (Moldofsky et al., 1989, Spath-Schwalbe et al., 1991, Steiger and Holsboer, 1997, Brun et al., 1998, Vgontzas and Chrousos, 2002).

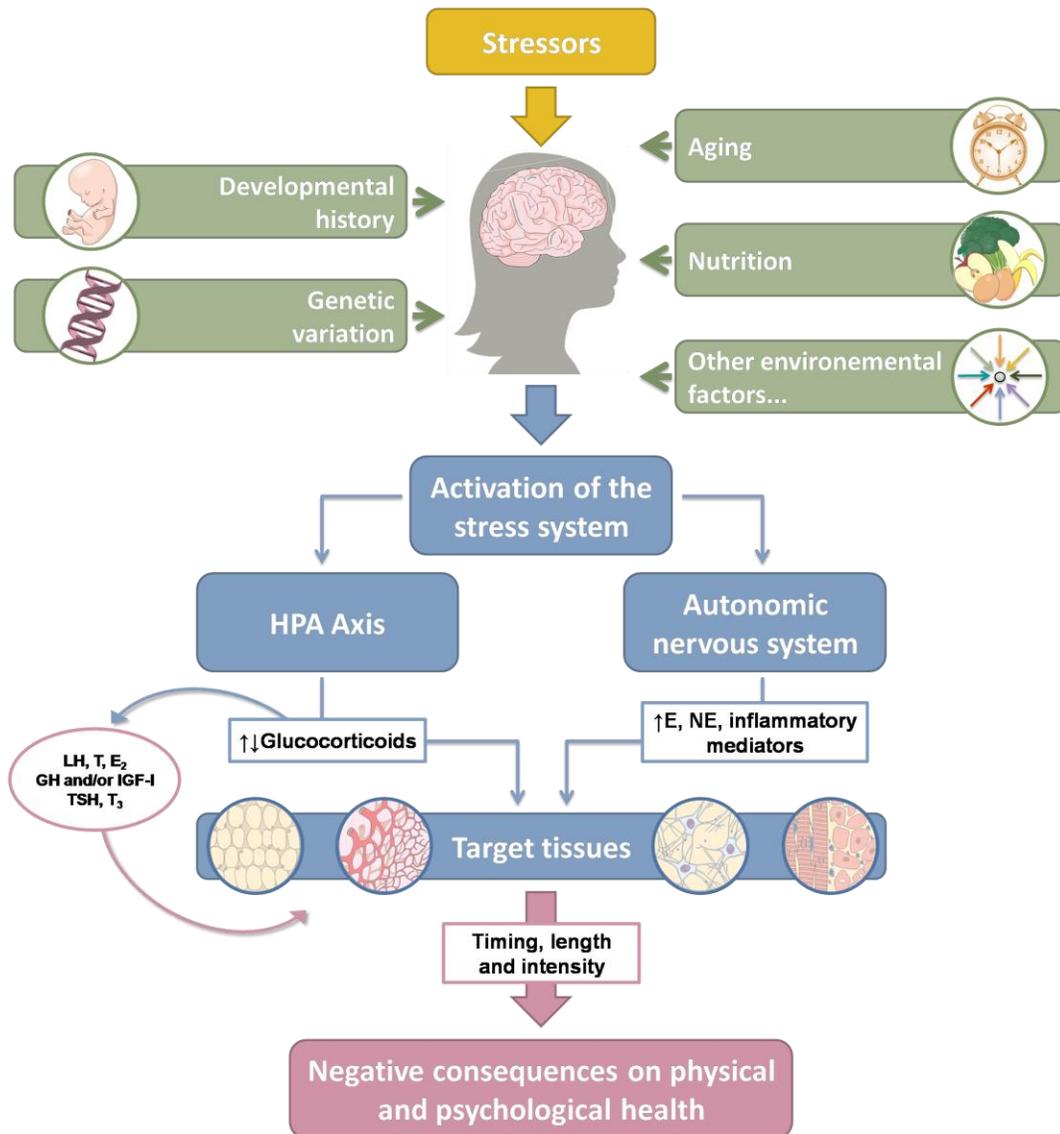


Figure 3 – Negative impact of stress on physical and psychological health. The response of the stress system to a stressor is modulated by several individual factors, such as developmental history, genetic constitution, aging and other environmental factors, such as nutrition and sex. Once activated, the main systems implicated in the stress response – the HPA axis and the autonomic nervous system – will release stress response mediators that will exert direct and indirect effects in the different body tissues. This individually shaped stress response might lead to several adverse outcomes depending on its timing, length and intensity (adapted from (Chrousos, 2009)).

Stress is one of the most well-known factors influencing HPA axis activities. Acute stress exposure activates the HPA axis resulting in the secretion of CRH and AVP from the PVN of the hypothalamus. CRH and AVP bind corticotropin releasing hormone receptor 1 (CRHR1) and arginine vasopressin receptor 1B (AVPR1B) receptors, respectively, located on corticotroph cells in the pituitary, and induce the release of ACTH. ACTH in turn stimulates the biosynthesis and release of glucocorticoids by the adrenal cortex. The level of glucocorticoids secreted may vary according to the type, intensity and length of the stressor, as illustrated in Figure 4. Ultimately, glucocorticoids bind to their receptors in various tissues including the tissues regulating the HPA axis, thereby reducing the secretion of CRH and ACTH. This negative feedback mechanism is essential for keeping the balance of the HPA axis activity during basal conditions and in response to stress (Djordjevic et al., 2003, Handa and Weiser, 2014).

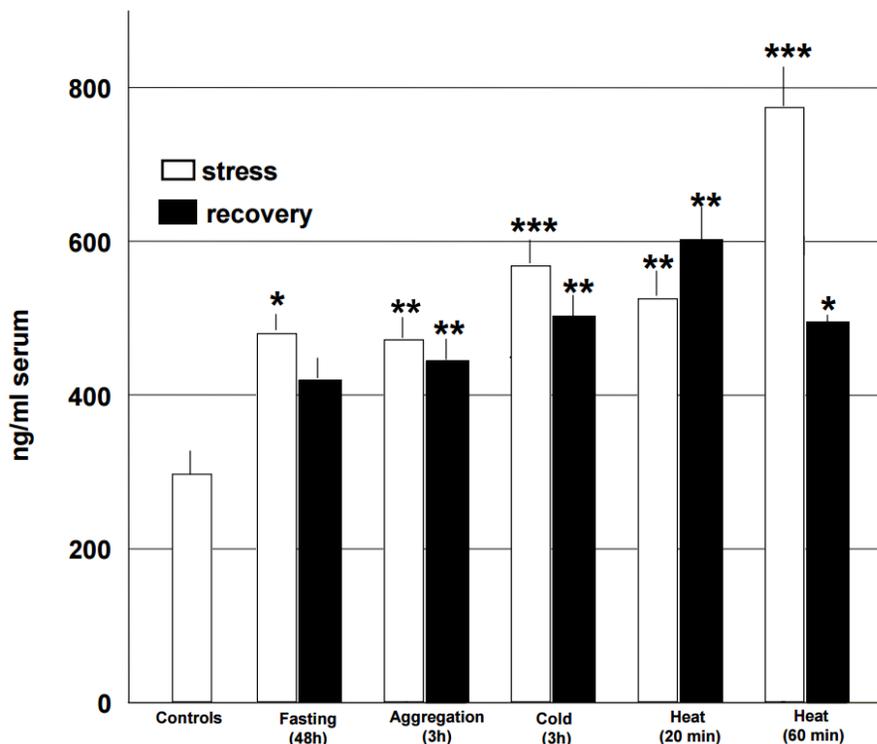


Figure 4 - Plasma corticosterone values of young male Wistar rats exposed to several different acute stressors: 48 hours fasting followed by a 48 hours stress recovery period; aggregation of 12 rats per cage followed by a 3 hours stress recovery period; exposure to 6°C cold for 3 hours followed by a 3 hours stress recovery period; and exposure to 38°C heat for either 20 or 60 min followed by a 20 or 60 min stress recovery period. (*), (), and (***) represent significant difference ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) from the control group (one-way ANOVA) (from (Djordjevic et al., 2003)).**

CRH is the primary regulator of ACTH secretion by the anterior pituitary gland. Nonetheless, AVP and oxytocin have been shown to co-localize with CRH in a few PVN neuronal populations and to be co-released with CRH, which supports their roles as co-secretagogues. AVP and oxytocin have also been shown to stimulate ACTH secretion even in the absence of CRH, and to inhibit HPA responses when applied directly to the PVN. Recently, other regulatory factors expressed by PVN neurons have been shown to modulate CRH responses, such as the endocannabinoids. These endogenous arachidonate-like lipids are ligands for cannabinoid receptors (CB1 and CB2) that are

expressed in the brain. Studies have demonstrated that the CB1 receptor – which activation causes the suppression of neurotransmitter release to the synapse – is involved in modulating HPA axis function (Handa and Weiser, 2014).

1.2.2 Negative feedback regulation of the HPA axis

The HPA axis is governed by a closed-loop negative feedback system typical of most neuroendocrine axes (Figure 5). Glucocorticoid-dependent negative feedback control is essential for the termination of the stress response and reduces the risk of deleterious high amplitude oscillations in circulating levels of glucocorticoids. Normal HPA function is dependent on a dose and duration dependent glucocorticoid-mediated negative feedback. Negative feedback can inhibit the axis by acting at the level of the CRH and AVP neurons of the PVN, through corticotrophs of the anterior pituitary, or indirectly through brain regions containing glucocorticoid receptors that project to the PVN. The influence of corticosteroids on negative feedback depends on the type of ligand (cortisol, corticosterone, synthetic glucocorticoids...) and on the site of action.

Negative feedback of the HPA axis occurs in several time domains. Fast feedback can occur within seconds to minutes, does not depend on protein synthesis and is mediated at the cell membrane level. Evidence suggests that this fast mechanism involve the endocannabinoid system. The delayed feedback, on the other hand, occurs in the minutes to hours time frame and is driven by changes in gene expression through classical actions of glucocorticoid receptors (GR and MR) (Hill and Tasker, 2012, Handa and Weiser, 2014).

Since circulating glucocorticoids can bind either GR or MR, both have been implicated in the negative feedback regulation of the HPA axis, where differential sensitivity to a common ligand appears to allow selective actions. MR has a particularly high affinity for endogenous glucocorticoids and these receptors are predominantly bound under baseline levels of corticosterone. Evidence suggests that MR receptors – particularly those located in the hippocampus – regulate HPA axis activity during the non-stressed state. On the other hand, the relatively lower affinity of GR for endogenous glucocorticoids is thought to direct its actions toward negative feedback following stressors. The GR appears to be largely unoccupied during basal glucocorticoid conditions, but becomes rapidly occupied when glucocorticoid levels increase following stress. This way, GR activation allows the return of HPA activity to baseline following high amplitude peaks of corticosteroids. Similarly to MR, the negative feedback effects of GR on HPA axis following a stressor seems to be mediated by receptors located in the hippocampus, despite the also high expression of this receptor in the PVN and in the anterior pituitary gland. Thus, the ratio of MR to GR may be important for regulating HPA reactivity as well as stress related behaviors (Handa and Weiser, 2014).

Given the traditional view that glucocorticoids work by changing gene transcription, it is surprising to how fast the negative feedback can occur. Powerful glucocorticoid inhibition of the HPA axis occurs within minutes, a time-frame far too fast to be mediated by genomic effect. Thus, rapid feedback must be mediated by non-genomic actions, perhaps working at or near the cell membrane. The mechanism underlying fast feedback remained enigmatic until only recently, when electrophysiology studies suggested that glucocorticoids bind membrane receptors on PVN CRH neurons, eliciting an intracellular cascade that mobilizes the synthesis of endocannabinoids. Endocannabinoid release then causes presynaptic inhibition of glutamate release, which reduces the neural activity of parvocellular neurons (Di et al., 2003). Interestingly,

despite these non-genomic effects being proposed to be mediated by membrane-bound receptors, they have recently been shown to still be dependent on the nuclear GR on a study using conditional GR knockout mice with genetic deletion of GR in the PVN and supraoptic nucleus. Nuclear GR would therefore be responsible for transducing the rapid steroid response at the membrane, or be a critical component in the signaling cascade, or regulate a critical component of the signaling cascade of a distinct membrane receptor (Jacobson and Sapolsky, 1991, Herman et al., 2012, Nahar et al., 2015).

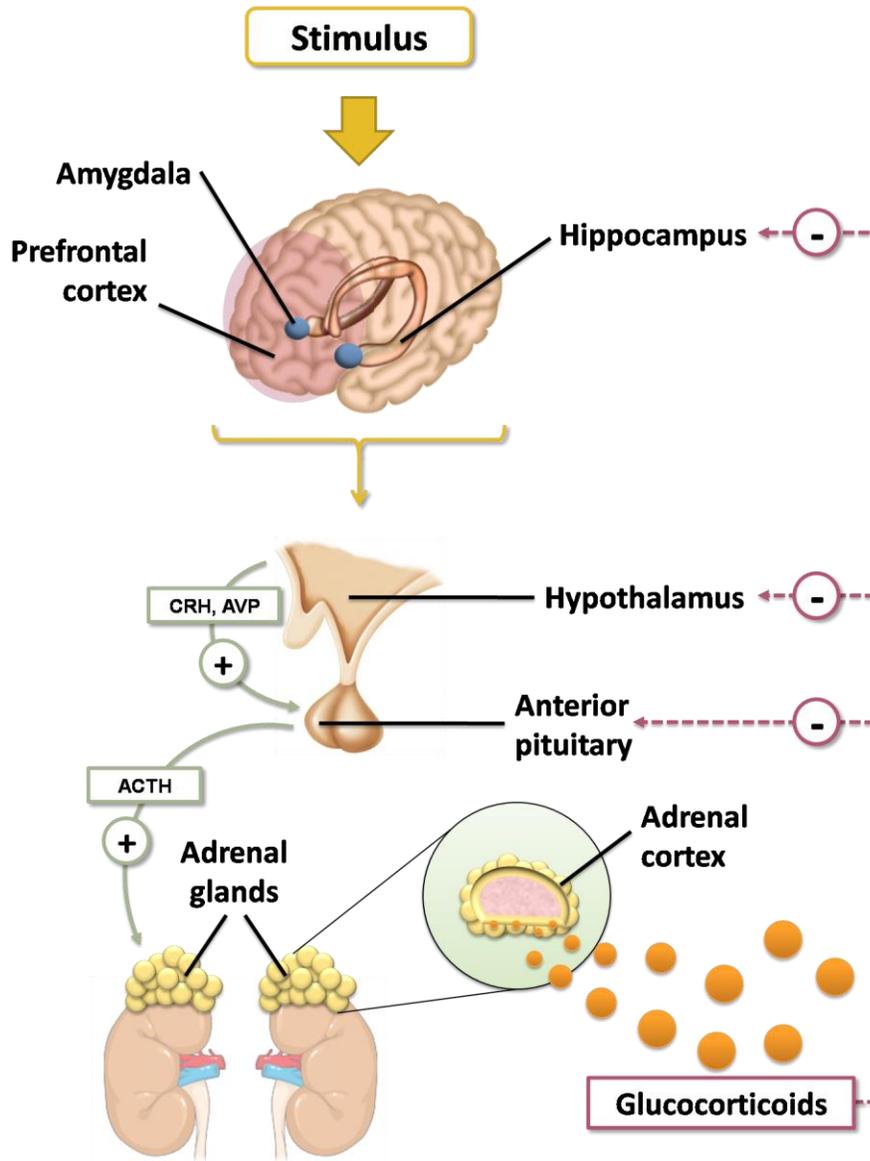


Figure 5 – Negative feedback of the HPA axis. Different environmental stimuli are processed by the limbic system, which activates the paraventricular nucleus of the hypothalamus triggering the release of corticotropin releasing hormone (CRH) and vasopressin (AVP). CRH and AVP bind to receptors located in the pituitary and induce the synthesis of adrenocorticotropic hormone (ACTH), which stimulates the biosynthesis and release of glucocorticoids by the adrenal cortex. Glucocorticoids can exert a negative feedback by acting directly at the level of the hypothalamus or of the pituitary, or indirectly through brain regions containing glucocorticoid receptors, notably the hippocampus.

A rich literature attributes feedback control of the HPA axis stress responses to limbic system structures, including the hippocampus and the medial prefrontal cortex. Electrical stimulation of the hippocampus is sufficient to reduce circulating glucocorticoid levels, and lesion studies indicate that destruction of the hippocampus prolongs HPA axis responses to acute stressors of a psychogenic nature, consistent with an inhibitory effect of hippocampal activation on the HPA axis (Herman et al., 1998, Herman et al., 2003). Inhibition of the stress responses of the HPA axis is also observed following chemical stimulation of the prelimbic division of the PFC where, similarly to the hippocampus, lesions delay the shut-off of HPA axis responses to psychogenic stress (Diorio et al., 1993, Figueiredo et al., 2003, Jones et al., 2011). The timing of response prolongation following either PFC or hippocampal damage is 1-2 h after stress, suggesting a probable genomic mechanism. Since both hippocampus and PFC are regions rich in GR, the inhibition of stress responses by these regions is probably mediated by glucocorticoid feedback. The role of the GR in limbic feedback regulation of the HPA axis was addressed in a series of studies using mice bearing deletion of the GR in the cerebral cortex, hippocampus and BLA, but with intact GR expression in regions such as the central and medial amygdaloid nuclei and the PVN (as well as in all peripheral tissues). Stress testing of these mice revealed they have delayed shut-off of HPA axis responses to psychogenic (but not systemic) stressors, and have impaired dexamethasone suppression of the circadian corticosterone peak. These studies provided evidence for limbic forebrain GR control of negative feedback inhibition of the HPA axis, and suggested that forebrain GR feedback is stressor-specific, probably being only active when the stressful stimulus actively engages the brain structure in question (Boyle et al., 2005, Furay et al., 2008).

1.2.3 Glucocorticoid synthesis

Glucocorticoid hormones are lipophilic and therefore cannot be simply stored in vesicles – as it happens for some other hormones such as CRH and ACTH – but they need to be newly synthesized in response to ACTH. Glucocorticoid steroidogenesis takes place in the zona fasciculata of the adrenal cortex. It is activated when ACTH binds to its specific cell surface G protein coupled receptor, the melanocortin type-2 receptor (MC2R). This binding induces conformational changes in the MC2R that activate adenylyl cyclase, leading to an increase in intracellular levels of cyclic adenosine monophosphate (cAMP). This intracellular increase in cAMP activates downstream signaling pathways, including the protein kinase A (PKA) pathway. Activation of PKA induces acute synthesis of glucocorticoids via both genomic and non-genomic mechanisms. The rate-limiting step in glucocorticoid synthesis is the mobilization of cholesterol and its transfer inside the mitochondria. This process is mediated by the activity of the steroidogenic acute regulatory protein (StAR). Activation of the PKA signaling pathway activates nuclear transcription factors, such as the cAMP response element (CRE) binding protein (CREB), which in turn induce transcriptional activation of a number of steroidogenic genes, including StAR. In parallel to StAR transcription, PKA also induces posttranslational modification of StAR, resulting in phosphorylation at a specific site that activates StAR and increases its ability to transport cholesterol across the mitochondrial membrane. Within the mitochondria, cholesterol is subjected to enzymatic modifications by a cascade of steroidogenic regulators, which ultimately lead to glucocorticoid synthesis (Figure 6) (Spiga et al., 2014, Spiga and Lightman, 2015).

Once released in the human or rodent bloodstream, about 80 to 85% of glucocorticoids bind with high affinity and specificity to transcortin, or corticosteroid binding globulin (CBG – a plasma glycoprotein which will be further described on page 51), while more or less 10% bind with limited specificity and low affinity to albumin, and only around 5% are found in their free and active form (Breuner and Orchinik, 2002, Hammond, 2016).

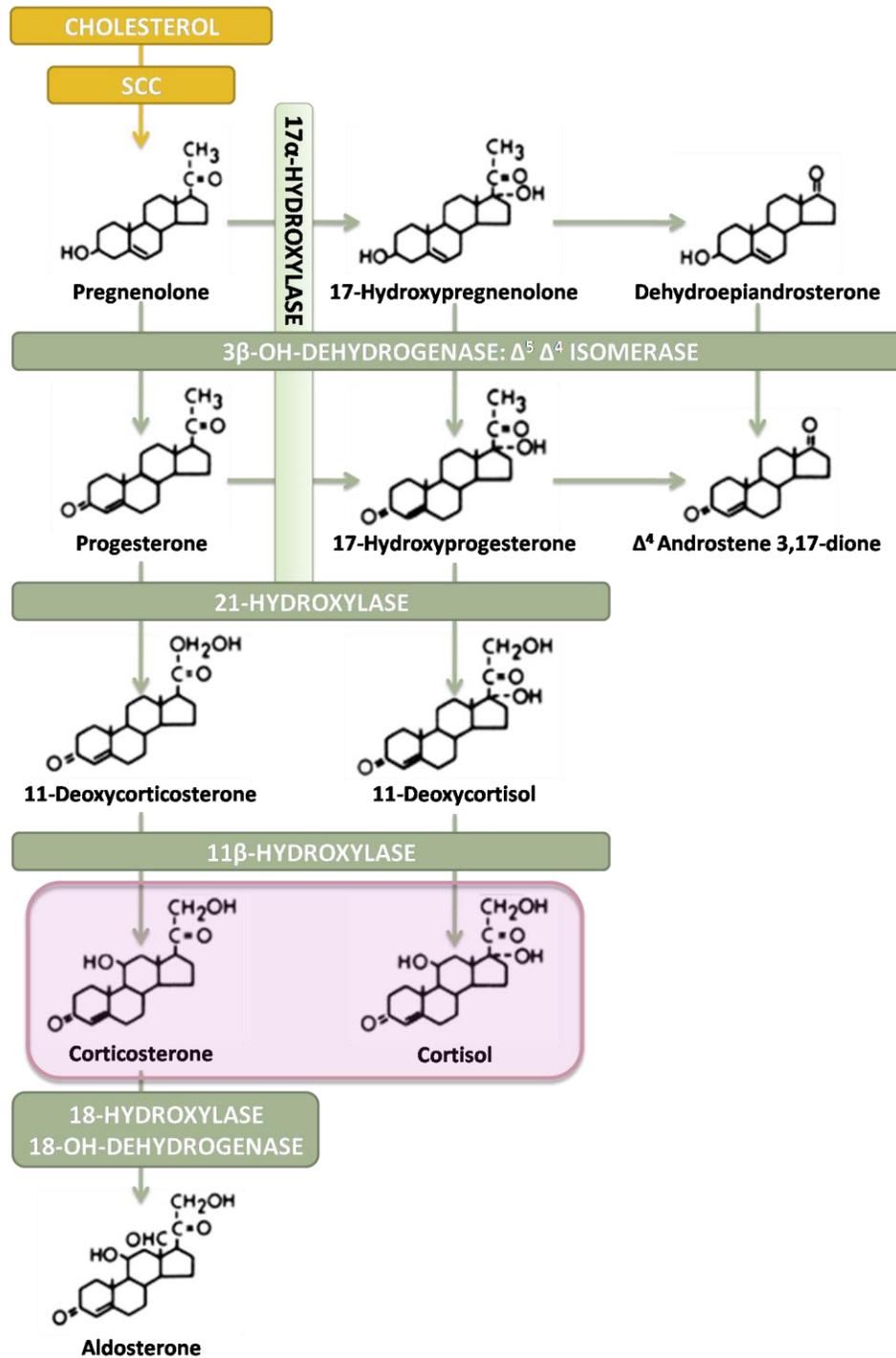


Figure 6 - Glucocorticoid synthesis pathway. The adrenal steroids, glucocorticoids (cortisol and corticosterone) and mineralocorticoids (aldosterone), are synthesized through a series of enzymatic reactions from cholesterol molecules (adapted from (Murray et al., 2009)).

1.2.4 Glucocorticoid Metabolism

Among the enzymes directly metabolizing cortisol are two short-chain dehydrogenase/reductase (SDR) enzymes (11 β -hydroxysteroid dehydrogenase 1 (HSD11B1) and 2 (HSD11B2)), the A-ring reductases (5 α - and 5 β -reductases, 3 α -hydroxysteroid dehydrogenases), 6 β -hydroxylase and 20 α - and 5 β -reductases. Corticosterone is subject to an analogous metabolism (Figure 7).

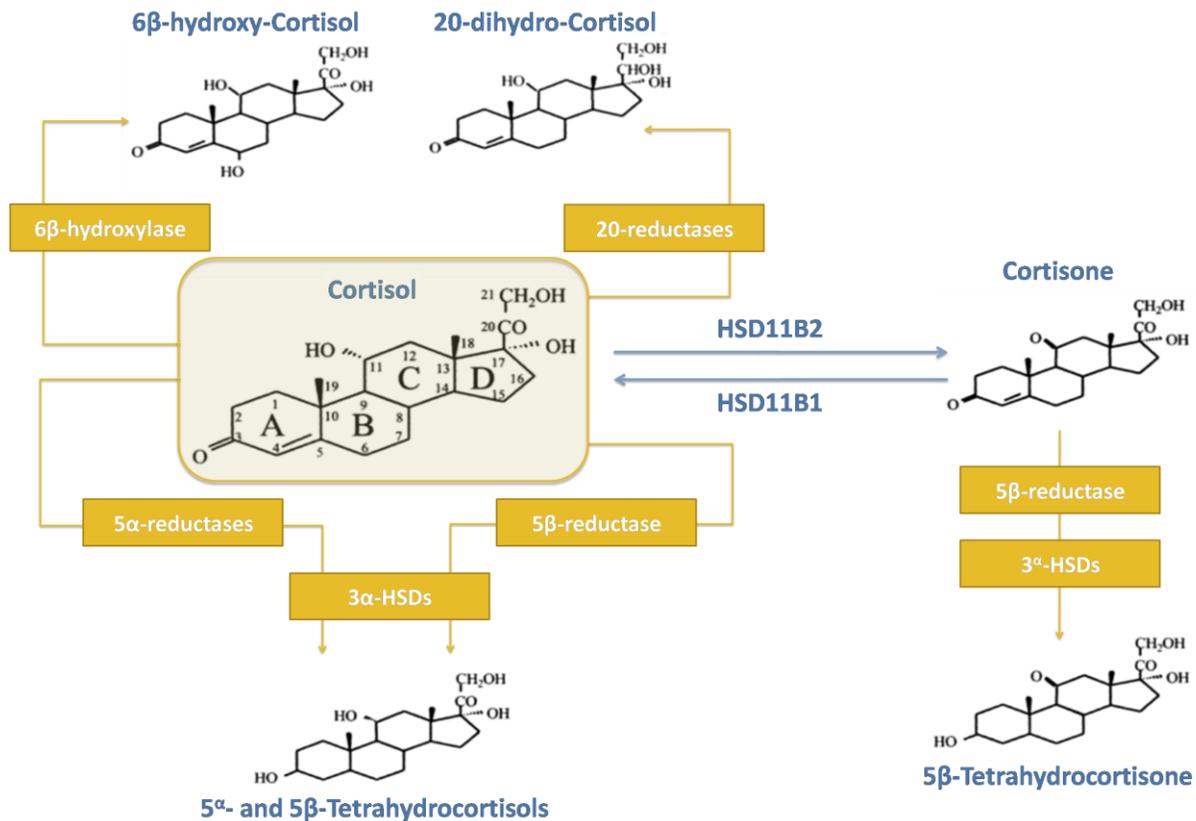


Figure 7 – Principal routes of metabolism of cortisol (adapted from (Andrew and Walker, 2004)).

During evolution, several mechanisms appeared that allowed a fine-tuned regulation of glucocorticoid signaling and downstream effects. Among these mechanisms are the enzymes HSD11B1 and HSD11B2, that interconvert active (cortisol or corticosterone) and inactive glucocorticoids (cortisone or 11-dehydrocorticosterone) (Figure 8). HSD11B2 is an endoplasmic reticulum membrane protein and acts exclusively as an oxidase on endogenous glucocorticoids, thereby playing an important role in the tissue-specific inactivation of glucocorticoids. It utilizes oxidized nicotinamide adenine dinucleotide (NAD⁺) as a cofactor and is expressed in mineralocorticoid target tissues such as kidney, colon and sweat glands where it serves to protect the MR from excessive activation by glucocorticoids, thereby rendering access of aldosterone to its receptor. HSD11B2 is also expressed in the placenta to protect the fetus from high maternal glucocorticoids. HSD11B1 is also an integral membrane enzyme of the endoplasmic reticulum, with a luminal orientation of its active site. It is mostly expressed in key metabolic tissues, such as the liver, adipose tissue, and central nervous system. Its functions *in vivo* are predominantly as a reductase, and its activity is dependent on the availability of hexose-6-phosphates and reduced nicotinamide adenine dinucleotide

(NADPH). It has an important role in the regeneration of active glucocorticoids and in the prolongation of their biological half-life (Odermatt and Klusonova, 2015).

The production of the active endogenous 11β -hydroxyglucocorticoids is tightly controlled by the HPA axis. As previously mentioned, in both humans and rodents, only about 5% of circulating glucocorticoids are found in their free and active form. The inactive form – cortisone and 11-dehydrocorticosterone – is approximately 50% bound to plasma proteins (to albumin and, mostly, to CBG), and a higher fraction is available to peripheral target cells expressing HSD11B1. The active glucocorticoids generated by HSD11B1 may then enhance the local GR-dependent effects on gene expression (Odermatt and Klusonova, 2015).

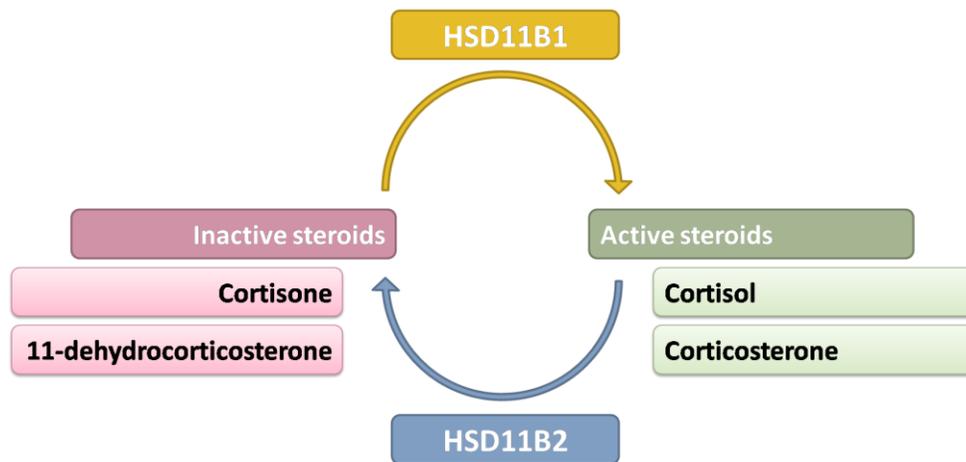


Figure 7 – The two enzymes: 11β -hydroxysteroid dehydrogenase 1 (HSD11B1) and 2 (HSD11B2) that interconvert active (cortisol or corticosterone) and inactive (cortisone or 11-dehydrocorticosterone) glucocorticoids.

5α - and 5β -reductases are A-ring reductases which catalyze the transformation of cortisol into 5α - and 5β -dihydrocortisol, respectively. These dihydro-metabolites are then subject to rapid metabolism in the liver by 3α -hydroxysteroid dehydrogenases. This last reaction is not rate limiting, and most cortisol metabolites are excreted in the form of 5α - and 5β -tetrahydrocortisol. 5α -reduction is catalyzed by two isozymes from different genes. 5α -reductase type 1 is expressed in liver, non-genital skin, brain and some cells in the adipose tissue. 5α -reductase type 2 is expressed in liver, specific areas of the developing brain, prostate and genital skin. The relative contribution of each isozyme to metabolism of cortisol is uncertain in humans, but both purified enzymes can catalyze 5α -reduction of glucocorticoids *in vitro*. These enzymes can also use androgens, progesterone and aldosterone as substrates, but the resulting 5α -metabolite products of these other classes of steroids are known to be biologically active. It has only recently been shown that 5α -reduced glucocorticoids may have an activity, notably binding and activating GR receptors. Despite their lower potency when compared to the original steroids, they may play an important role in regulating GR activation locally in the tissues where they are expressed. 5β -reductase is a low affinity enzyme highly expressed in the liver which can metabolize bile acid intermediates and a range of pregnane type steroids, such as glucocorticoids, mineralocorticoids, androgens and progestogens. Some reports have suggested that, similarly to 5α -reductase, a second 5β -reductase existed, but this has not yet been corroborated. 5β -reduced corticosterone has been reported to activate pregnane-X-receptor, a nuclear receptor implicated in the

metabolism of a wide range of endogenous and exogenous compounds, but the physiological implications of this activation is still uncertain (Andrew and Walker, 2004).

Other minor metabolic routes for cortisol exist besides HSD11B1 and HSD11B2 and the A-ring reductases. The cytochrome P450 microsomal enzyme 6 β -hydroxylase and 20-reductases (20 α - and 20 β -HSDs) are expressed mainly in the liver, and are responsible for less than 10% of cortisol metabolites (Andrew and Walker, 2004).

1.2.5 Glucocorticoid receptors and genomic responses

It is widely accepted that glucocorticoids exert most of their effects genomically. According to the classic genomic theory of action, glucocorticoids bind to glucocorticoid receptors (MR or GR) located in the cytoplasm of cells from different target tissues. GR and MR differ in their affinity for glucocorticoids. The affinity of the endogenous hormones corticosterone and cortisol for MR is approximately 10-fold higher than that for GR. Therefore, variations in circulating levels of glucocorticoids lead to shifts in the balance between MR and GR occupation, and thus activity. The high-affinity MR is activated at very low concentrations of glucocorticoids, whereas the lower affinity GR is only activated upon high concentrations of glucocorticoids. In basal physiological states, when glucocorticoid concentrations are low, MR is activated and localized in the nucleus, whereas GR remains latent in the cytoplasm. Once glucocorticoid concentrations increase above a threshold level, for example during the circadian peak or following stress exposure, GR translocates into the nucleus where it exerts its genomic effects (Spiga et al., 2014).

MR and GR belong to the superfamily of nuclear receptors. When inactive, they reside in the cytoplasmic compartment, stabilized by chaperone molecules. Upon glucocorticoid binding, the chaperone-receptor complex dissociates and enables the ligand-receptor complex to translocate to the nucleus, where it modulates the transcriptional activity of glucocorticoids responsive genes. This positive or negative modulation of the expression of glucocorticoids target genes happens either through the binding of the complex to specific sequences – glucocorticoid-response elements (GREs) – in the promoter region of target genes, or through protein-protein interactions with other transcription factors, such as nuclear factor- κ B (NF- κ B), activator protein-1 (AP-1), and several signal transducers and activators of transcription (STATs) (Jiang et al., 2014, Spiga et al., 2014).

MR and GR are differently distributed throughout the body. While GR is ubiquitously expressed in the periphery and in the brain, the distribution of MR is more restricted to specific organs such as the kidney and the heart. In the brain, the distribution of these receptors is also not uniform. GR is almost ubiquitously expressed in neurons and glial cells, with particularly high density in the hippocampus and in parvocellular neurons of the PVN. In contrast, MR localization is restricted to the hippocampus, PFC and amygdala, structures highly involved in learning, memory, and emotional behavior. Both receptors are abundantly expressed in areas regulating the HPA axis function, including a number of limbic structures that regulate the activity of the PVN, such as the hippocampus and amygdala, as well as in the anterior pituitary. In addition to the overlap in GR and MR expression within hippocampus and cortex, GR and MR can be co-expressed within individual cells. Interestingly, brain regions or cells that express both receptor types are capable of mediating varied responses to the endogenous glucocorticoid, according to the occupancy level of each type of receptor (van Eekelen et al., 1991, Handa and Weiser, 2014, Spiga et al., 2014).

1.2.6 Non-genomic effects of glucocorticoids

One of the most important actions of glucocorticoids is to enhance the response to stress and to protect the organism from specific challenges to homeostasis. Almost any type of stress, whether physical or psychogenic, causes a rapid and marked increase in ACTH secretion by the anterior pituitary gland, followed within minutes by greatly increased adrenocortical secretion of glucocorticoids. Even though it is well known that glucocorticoid secretion increases significantly in stressful situations, and that this enhances tolerance to stress, it is believed traditionally that glucocorticoids exert these effects genomically. Non-genomic effects of glucocorticoids are characterized as short latency and insensitive to inhibitors of deoxyribonucleic acid (DNA) transcription and protein synthesis compared with genomic effects, which require at least 30 min and up to several hours or days to take effect. Thus, we can infer that the non-genomic effects of glucocorticoids play an important role in the early phase of acute stress. In fact, non-genomic effects of glucocorticoids have already been described for a series of processes, such as the inhibition of inflammatory factors and the inflammatory response, the maintenance of blood pressure by potentiating the effect of catecholamines and the elevation of blood glucose concentrations. All these phenomena were described to take place in less than 20 min after the administration of glucocorticoids (Jiang et al., 2014).

Although some studies indicate that nuclear MR and GR receptors can initiate second messenger signaling or interact with other cellular signaling components, there is growing body of evidence to suggest that these rapid actions are mediated by specific receptors localized at the plasma membrane. Membrane-impermeable glucocorticoid conjugates are able to trigger these rapid actions, which directly supports the membrane-initiated signaling. These effects seem to be mediated by G protein coupled membrane-associated receptors, with a pharmacological profile different from the well-known intracellular receptors. The inhibitory activity of glucocorticoids on the HPA axis is thought to be mediated by these membrane receptors, given the rapidity of this effect at both the level of the pituitary and the brain (Spiga et al., 2014).

One interesting example of an effect mediated by membrane receptors is the rapid and non-genomic effects of acute stress on memory retrieval. An acute stress (electric footshock) induces a corticosterone rise in the dorsal hippocampus and memory retrieval impairment in a spontaneous delayed alternation task. Bilateral injections of either corticosterone or a corticosterone-bovine serum albumin (BSA) complex (impermeable to the plasma membrane) in the dorsal hippocampus before memory retrieval produced impairments similar to those resulting from acute stress. Furthermore, bilateral intra-hippocampal injection of RU-28318 (an MR antagonist) but not of RU-38486 (a GR antagonist) totally blocked the memory retrieval deficit induced by the corticosterone–BSA complex administration. This demonstrates that the membrane receptors mediating the rapid and non-genomic effects of acute stress on memory retrieval are of MR type (Dorey et al., 2011).

1.2.7 Circadian and ultradian influences on the HPA axis

The term circadian comes from the Latin *circa diem*, meaning “around a day”. Circadian rhythms refer to biological oscillations within a period of approximately 24 hours. These rhythms are highly conserved among living organisms, and are characteristic of a number of biochemical, physiological, endocrine, and behavioral

functions, allowing the organism to anticipate and prepare for predictable environmental changes, such as food availability, predator risk, and the likelihood of reproductive success. The result is an optimization of energy expenditure by an optimal coordination of physiological processes (Kalsbeek et al., 2012, Spiga et al., 2014).

In mammals, circadian rhythms are generated and synchronized by a central clock in the SCN of the ventral hypothalamus. The activity of the SCN is entrained by the light input received from the retina, and this allows the organisms' physiology to be entrained to solar time. The molecular mechanism regulating the circadian activity of each single cell within the SCN consists of an oscillating transcriptional network of a series of "clock genes", regulated by a network of positive and negative transcriptional, translational and posttranslational feedback loops, resulting in the rhythmic expression of their protein products. Signaling between the SCN and the periphery involves both hormonal and neuronal mechanisms. Rhythmic activity of the SCN is translated into circadian patterns of gene expression in target tissues, which in turn determine circadian rhythms in the physiological function of that specific organ. In rodents, lesions in the SCN result in a loss of physiological circadian rhythmicity, including body temperature, locomotor activity, and hormone secretion, even when the 24 hours light-dark cycle is maintained, and the circadian rhythm of locomotor activity can be reinstated in arrhythmic animals by transplantation of an intact SCN (Stephan and Zucker, 1972, Abe et al., 1979, Ralph et al., 1990, Buijs et al., 1993). In addition to the central master clock in the SCN, clock genes are also expressed rhythmically in peripheral tissues, including liver, kidney, skeletal muscle, lung, and adrenals. However, although they share the same molecular dynamics of clock genes, only the SCN can independently generate and maintain its own circadian rhythms, while peripheral clocks require SCN-dependent drive to maintain synchronicity with the light-dark cycle (Reppert and Weaver, 2002, Welsh et al., 2004, Levi and Schibler, 2007, Spiga et al., 2014).

The HPA axis operates under a circadian timing mechanism that is both diurnal and ultradian in nature. The circadian rhythm of the HPA axis is primarily regulated by the central pacemaker in the SCN. The SCN regulates the circadian rhythm of glucocorticoids in part by modulating the release of CRH from neurons in the PVN, and in part via the autonomic nervous system, through a multisynaptic neural pathway from the SCN to the adrenal gland. Additionally, there is evidence of an intra-adrenal circadian pacemaker that can regulate glucocorticoid synthesis independently from the SCN (Oster et al., 2006, Son et al., 2008). Evidence for the involvement of adrenal clock genes in circadian glucocorticoid synthesis has been shown *in vivo* using mice defective in components of the circadian clock system. In fact, studies in clock gene knockout mice have shown that disruption of the circadian clock in these animals is associated with a disruption of the HPA circadian rhythm. The circadian rhythm of clock genes in the adrenals was also shown to be independent of circadian rhythmicity of ACTH, corroborating the important role of clock genes in the adrenals in regulating circadian rhythms of glucocorticoid synthesis (Spiga et al., 2014).

Basal levels of corticosteroids fluctuate throughout the day, with peak levels attained around the waking and active period (daytime in humans and nighttime in nocturnal animals such as rodents). It is thought that the increased levels of glucocorticoids at waking gear the body up for the impending activity phase, and act to enable foraging behavior by increasing the amount of available energy. The nadir in corticosterone secretion is found on the opposite period of the peak – that is, nighttime in humans and daytime in nocturnal animals. At its peak, the diurnal elevations of corticosterone can reach levels that are 5 to 10 times that found at its nadir. ACTH

shows a similar, but lower amplitude rhythm throughout the day that can vary also by up to several folds when measured from trough to peak. It is thought that this dampened rhythm of ACTH, when compared to corticosterone, is partly due to a rhythm in adrenal sensitivity to ACTH. The peak of the circadian rhythm is triggered by the combination of a reduction in inhibitory tone from the SCN through its projections to the PVN and an increase in adrenal sensitivity to ACTH driven by autonomic inputs, thereby enhancing corticosterone secretion (Lightman and Conway-Campbell, 2010, Kalsbeek et al., 2012, Handa and Weiser, 2014).

The circadian rhythm tracks an underlying ultradian activity of the HPA axis, so that the apparent peak and trough of circadian activity actually reflects changes in the amplitude of the ultradian pulses (Figure 9). The interpulse interval of these pulses is of approximately 1 hour. These ultradian secretory patterns do not appear to be dependent on the circadian pacemaker since pulsatile secretion is maintained in the absence of circadian cues. The exact origin of ultradian rhythmicity in the HPA axis is not known, although it is thought to be dependent upon hypothalamic CRH, which activates a pituitary–adrenal network responsible for the oscillations. Corresponding pulses of CRH are not necessary for the ultradian pattern and elevated levels of CRH appear to disrupt the network. Thus, unlike other neuroendocrine systems, it is the level, not the pattern of CRH secretion that sets the pulsatile dynamics of glucocorticoid secretion. Interestingly, GR has been shown to have an extremely rapid association and dissociation from DNA following activation, and its exit from the nucleus reflects the timing of the 1 hour pulse of corticosteroid secretion, whereas the same pattern is not observed for MR (Figure 10). Thus, this MR and GR difference further provides evidence for different patterns of MR and GR binding to sites on DNA and allows multiple cell specific responses (Lightman and Conway-Campbell, 2010, Conway-Campbell et al., 2012, Waite et al., 2012, Handa and Weiser, 2014).

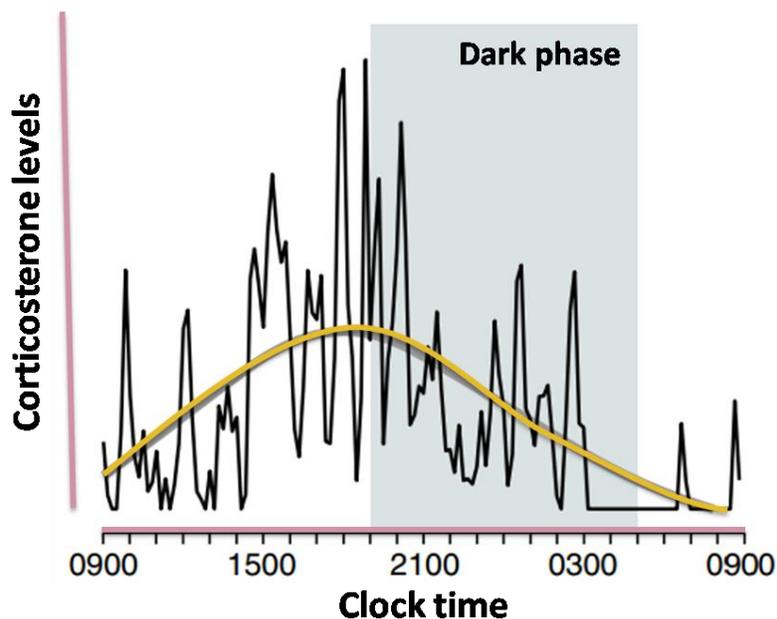


Figure 9 – Under basal (unstressed) conditions, the dynamics of glucocorticoid secretion is characterized by both a circadian (yellow line) and an approximately hourly ultradian rhythm (black line). In nocturnal rodents (as laboratory mice and rats), peak levels of corticosterone occur in the beginning of the active evening phase (Adapted from (Waite et al., 2012)).

1.2.7.1 Effects of glucocorticoid pulsatility in the brain

As only 5% of plasma glucocorticoids are found in their free and active form (while 95% are bound to carrier proteins), it is important to assess whether levels of free steroid also show an ultradian rhythmicity. A study performed by Droste and colleagues (Droste et al., 2008) showed that the discrete corticosterone pulses that can be detected in circulating blood are also present in the hippocampus, an area in which both GR and MR are abundantly expressed and known to regulate neural functions related to memory and learning processes. Oscillating levels of corticosterone provide a remarkably sensitive signaling system that is rapidly responsive to changes in plasma corticosterone levels, allowing optimal interactions between endogenous glucocorticoid activity and responsive brain nuclei. Pulses of corticosterone were shown to cause a rapid and transient nuclear translocation of GR in hippocampal *cornu ammonis 1* (CA1) neurons, with successive “waves” of GR activation and DNA binding. These pulses of GR activation result in cyclical recruitment of GR to a GRE in the promoter of *Per1* (period circadian clock 1, a clock gene). Each glucocorticoid pulse induces a burst of hippocampal *Per1* transcription but, interestingly, the accumulation of the mature mRNA transcript reaches a constant level during exposure to pulsatile corticosterone. This indicates that ultradian activity with pulses at hourly intervals is optimized to maintain a constant expression of *Per1*. Within 2 hours of the final pulse of corticosterone, levels of *Per1* mRNA return to baseline, indicating that this transcript has a short half-life. Thus, oscillating levels of corticosterone provide a system in which perturbations of pulse frequency or pulse duration can have rapid effects on *Per1* mRNA levels in the hippocampus. These findings provide mechanisms through which ultradian rhythmicity of glucocorticoid levels could modulate cognitive function. One interesting example of the importance of brain glucocorticoid oscillations is the work of Liston and colleagues (Liston et al., 2013), which shows that tightly regulated circadian glucocorticoid oscillations are important for learning-dependent synaptic formation and maintenance. They demonstrate that circadian glucocorticoid peaks promote postsynaptic dendritic spine formation in the mouse cortex after a motor skill learning task, while troughs are required for stabilizing newly formed spines which are important for long-term memory retention. They have also shown that the rapid spine formation associated to learning takes place through a non-transcriptional mechanism, and that chronic and excessive exposure to glucocorticoids eliminates these new spines, besides disrupting previously acquired memories.

Functional interactions between glucocorticoid pulsatility and the stress response have been known for more than a decade. Recent studies have showed that acute stress reactivity seems to be facilitated when the stressor is applied during the rising phase of a corticosterone pulse compared to the falling phase. The display of risk assessment behavior, as well as c-Fos activation in the amygdala are also increased during the rising phase of the pulse, suggesting that this brain region – which is important in the regulation of emotional responses – is responsive to rapid changes in circulating glucocorticoid levels (Windle et al., 1998). In humans, deregulation of the pulsatile pattern of glucocorticoid secretion is associated with various disease states, in particular with an increased incidence of depression and stress-related diseases (Lightman and Conway-Campbell, 2010, Spiga et al., 2015).

Treatment of inflammatory disorders with constant high levels of anti-inflammatory glucocorticoid analogues has been associated with long-term, continuous GR activation, abnormal patterns of GR-DNA interaction and unwanted side effects.

Furthermore, deregulation of the ultradian glucocorticoid rhythm (from pulsatile to constant by pellet implantation) has recently been shown to influence gene transcription in the brain of rats in response to a glucocorticoid challenge, and to alter the electrophysiological properties of the hippocampus. Evidence show that the effects of glucocorticoid ultradian pulses are also brain region-specific. It has been observed in adrenalectomized animals receiving exogenous glucocorticoids in a pulsatile pattern that some genes are rapidly and robustly induced in several brain regions after a single pulse of corticosterone, while other genes require two or three pulses before transcription can proceed. This effect is brain region-specific, and this specificity is likely to allow differential physiological induction of specific genes in different brain regions under basal conditions. Furthermore, it is also likely that a different genomic response occurs under conditions of stress, during which the basal pulses are replaced by a prolonged glucocorticoid surge (Lightman and Conway-Campbell, 2010).

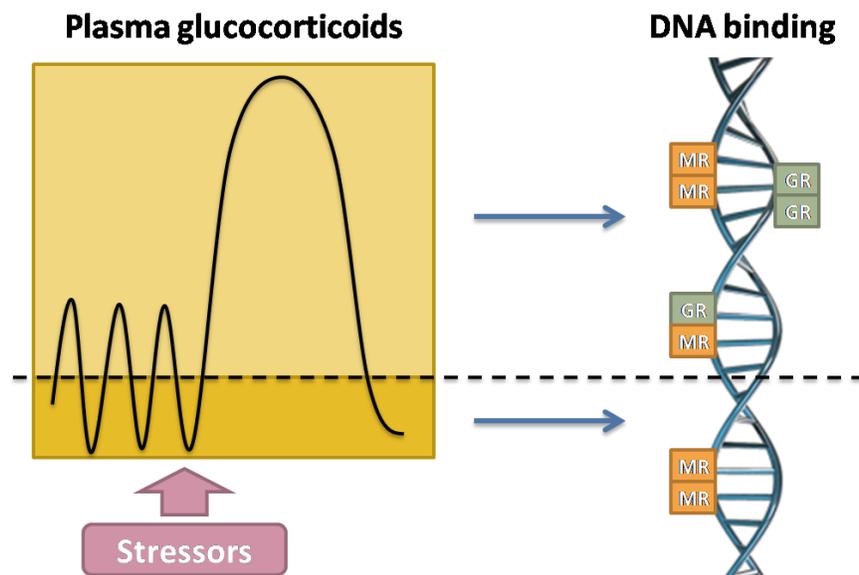


Figure 10 – The effect of glucocorticoid pulses and of an acute stressor on glucocorticoid receptors translocation to the nucleus and binding to DNA. At nadir levels of corticosterone, only mineralocorticoid receptor (MR) is found bound to DNA, while during peak and stress levels both glucocorticoid receptor (GR) and MR are found bound to DNA (adapted from (Lightman, 2008)).

1.2.8 The actions of glucocorticoids

Glucocorticoids are essential to adaptation by controlling energy supply through stimulation of glycolysis, gluconeogenesis and lipolysis, as well as inducing proteolysis to supply amino acids as substrates for gluconeogenesis. In addition, glucocorticoids modulate the immune system activity and the synthesis and action of a number of hormones. These hormones can affect memory acquisition and consolidation, besides also impacting the function of other neuroendocrine systems, such as central control of metabolic activity, feeding behaviour, growth and reproductive activity. Those actions are a result of either their direct activity in neurons or of the modulation of neuropeptides' and neurotransmitters' synthesis, secretion and activity (Uchoa et al., 2014).

1.2.8.1 Actions on growth and development

Glucocorticoids are major players involved in development and growth, by coordinating the expression of specific sets of genes involved in metabolic homeostasis. Effects on growth and growth hormone can be distinguished, and differentially influenced by glucocorticoids. Growth, in terms of height and weight, is a complex physiological process in which several growth factors appear to be involved in a coordinated fashion, among which the GH is one of the major contributors. On the other hand, GH is not only a growth factor, but also has a distinct role as a major hormone involved in regulation of the whole body metabolism. We will focus this brief discussion on GH, a prominent metabolic factor that is targeted by glucocorticoids. Glucocorticoids are thought to be essential for differentiation and maturation of GH-secreting cells. Also, a fully functional HPA axis seems to be required for the establishment of somatotroph cells - the GH-producing cells of the anterior pituitary - before birth, and glucocorticoids continue to be required for maintenance of GH production after birth (Vakili and Cattini, 2012, Mazziotti and Giustina, 2013).

Glucocorticoids are traditionally seen as negative modulators of GH and, more specifically, growth. Thus, it is believed that excessive amounts of glucocorticoids, endogenously or exogenously, are accompanied by impaired growth. However, conflicting data and theories are found in the literature about the role of glucocorticoids in the regulation of GH production. Glucocorticoids act at the level of the somatotrophs, the GH-producing cells of the anterior pituitary. There they regulate GH gene expression both directly – through GREs, and indirectly – via their interaction with other transcription factors (Vakili and Cattini, 2012). Glucocorticoids have been described to exert dual effects on GH production: while short term exposure to glucocorticoids seems to increase GH production, long-term exposure seems to decrease it. Both *in vivo* and *in vitro* studies support this hypothesis that glucocorticoids can be both stimulators and inhibitors of GH secretion, with the final biologic effect depending on their concentrations and on the duration of exposure (Vakili and Cattini, 2012, Mazziotti and Giustina, 2013).

1.2.8.2 Actions on the immune and inflammatory responses

Both natural and synthetic glucocorticoids are widely prescribed drugs the treatment of inflammatory disorders since the discovery of their anti-inflammatory actions. Due to the almost ubiquitous GR expression, glucocorticoids can affect nearly every cell of the immune system. They can target specific cell populations to combat hyperactivation of the immune system or systemic infections, at both cellular and transcriptional levels. Although glucocorticoid actions are typically described as anti-inflammatory, studies suggest that glucocorticoids can also have pro-inflammatory effects, and that the type of exposure to glucocorticoids and the basal state of the immune system are important factors influencing the effects of glucocorticoids (Busillo and Cidlowski, 2013, Cruz-Topete and Cidlowski, 2015).

Chronic exposure to glucocorticoids is immunosuppressive, and this anti-inflammatory action takes place via a complex interplay between GR-mediated transcriptional regulation and signal transduction within target tissues. Acute exposure, on the other hand, enhances the peripheral immune response. This positive regulation of components of the immune response suggests that glucocorticoids might be important in preparing the immune system for a quick and efficient response to pathogens, being this glucocorticoid-mediated initial pro-inflammatory activity perhaps

required for a functional immune system. Nevertheless, the mechanisms by which this same hormone directs opposite responses towards the immune system are not well understood (Cruz-Topete and Cidlowski, 2015).

Specifically in the brain, Nadeau and Rivest showed that, while mice do not usually present any neurodegeneration sign following the administration of bacterial lipopolysaccharide (LPS), mice previously injected with the GR antagonist RU486 before the intracerebral LPS infusion exhibit a rapid and severe neurodegeneration. This phenomenon was shown to be essentially caused by increased tumor necrosis factor alpha (TNF) activity, since inhibiting the biological activity of TNF in the central nervous system suppressed this neurotoxic effect of LPS. Furthermore, the inflammatory response lasted longer in the brain of animals that received the GR antagonist. These data provide evidence that endogenous glucocorticoids protect the brain from the detrimental consequences of the innate immune response activation and the resulting overproduction of TNF (Nadeau and Rivest, 2003).

The cross-sensitization between the neuroinflammatory consequences of stress and pro-inflammatory immune activation has been long known. Acute and chronic stress have been found to sensitize the neuroinflammatory response to both peripheral and central immunologic challenges (Johnson et al., 2003, de Pablos et al., 2006, Espinosa-Oliva et al., 2011). Several studies have also shown that stress modulates the immunophenotype of microglia, as indicated by up-regulation of several activation markers, and suggest that stress may shift the neuroimmune microenvironment towards a pro-inflammatory immunophenotype (de Pablos et al., 2006, Nair and Bonneau, 2006, Wohleb et al., 2011). The specific action of glucocorticoids on the stress-induced priming of the neuroinflammatory response, and the central role of microglia in mediating these responses have been recently demonstrated through elegant approaches combining *in vivo* and *ex-vivo* studies (Frank et al., 2007, Frank et al., 2014). Even more recently, the time of the day was pointed out as a novel regulator of the stress-induced neuroinflammatory state. In this study, rats which were exposed to a stressor during the light (inactive) phase showed the expected enhanced neuroinflammatory response to a subsequent LPS challenge, while those exposed during the dark phase did not show neuroinflammatory priming. These *in vivo* findings were mirrored by *ex vivo* microglial inflammatory state, as only microglia isolated from rats stressed during the light phase showed enhanced LPS-elicited activation, and pro-inflammatory cytokine induction was not significantly amplified in rats stressed during the dark phase (Fonken et al., 2016).

Impaired HPA responsiveness has been associated with several autoimmune diseases, both in animal models and in humans, including rheumatoid arthritis, Crohn's disease, colitis, inflammatory bowel disease, multiple sclerosis (and its animal equivalent autoimmune encephalomyelitis), asthma, dermatitis and other inflammatory conditions. Other conditions which are linked to an enhanced inflammatory state have also been shown to be associated to HPA axis disturbances, such as chronic fatigue syndrome, fibromyalgia, depression and post-traumatic stress disorder (PTSD). The enhanced susceptibility to inflammatory and autoimmune diseases can be related to decreased HPA axis activity and associated hypocortisolism, or to glucocorticoid resistance resulting from impairments in local factors affecting glucocorticoid availability and function, including alterations in glucocorticoid receptors. On the other hand, enhanced inflammation and hypercortisolism that typically characterize stress-related illnesses, such as depression, metabolic syndrome and cardiovascular disease, may be

related to increased HPA axis activity due to glucocorticoid resistance (Silverman and Sternberg, 2012).

1.2.8.3 Actions on the metabolism

States of either endogenous or exogenous glucocorticoid deficiency or excess are characterized by severe perturbations in systemic energy metabolism. Conditions associated to increased glucocorticoid levels, such as patients suffering from Cushing's syndrome, ACTH-producing tumors or under chronic systemic glucocorticoid treatment display central obesity, increased breakdown of skeletal muscle mass, hyperglycemia, fatty liver development, hypertension, elevated cholesterol, immunodeficiency, and insulin resistance. Interestingly, several of the aforementioned complications of glucocorticoid excess also represent features of the metabolic syndrome. On the other hand, suboptimal glucocorticoid levels, as in Addison's disease or as a result of autoimmunity against the adrenal cortex, inherited glucocorticoid synthesis dysfunction or pituitary disease, are associated with impaired stress resistance, weight loss and hypoglycemia. Besides the glucocorticoid status itself, the sensitivity of GR and MR receptors (also influenced by genetic mutations, as discussed on page 38) seems to play an important role in determining these effects (Rose and Herzig, 2013, Ferrau and Korbonits, 2015). An overview of the main metabolic actions of glucocorticoids is presented below.

Glucocorticoids are counter-regulatory opponents of insulin's anabolic functions. Under normal conditions, feeding promotes insulin secretion by the pancreas, triggering the fast uptake and oxidative catabolism of glucose in liver, muscle, and adipose tissue. At the same time, it inhibits glycogenolysis and gluconeogenesis in the liver. Insulin's actions are mediated by its membrane-bound receptor, which upon insulin binding exerts its tyrosine kinase activity, driving the transduction of the insulin signal to downstream cytoplasmic and nuclear effectors. This signal leads to the stimulation or inhibition of metabolic target gene transcription. Low plasma glucose levels during fasting and exercise trigger a series of hormonal cues that promote a switch in whole body energy usage. Along with a drop in insulin levels, counter-regulatory hormones – such as glucagon and glucocorticoids – gain metabolic control to antagonize anabolic insulin actions (Vegiopoulos and Herzig, 2007).

The capability of the liver to induce gluconeogenesis – the *de novo* glucose production – during times of nutrient deficiency is a prerequisite for the maintenance of stable blood glucose levels and thus proper energy availability for the brain, the renal medulla, and red blood cells. Genome-wide analysis of glucocorticoid-regulated target gene networks has identified more than 50 genes that are directly controlled by GR in the context of hepatic energy metabolism. Glucocorticoids have also been shown to act on the pancreas, more specifically on β -cells – the only insulin-producing cells in the mammalian organism. High levels of glucocorticoids have been shown to mediate the starvation-triggered impairment of β -cell numbers by restraining β -cell development *in utero*. Indeed, deletion of GR in pancreatic precursor cells at the fetal stage counteracts these effects. In adult mice, time-restricted over-expression of GR in mature β -cells did not affect β -cell numbers, but led to impaired glucose tolerance in mice, correlated with impaired insulin secretion. Consistent with these *in vivo* findings, it was demonstrated in experiments with cultured β -cells that glucocorticoids are able to suppress insulin secretion *in vitro* (Lambillotte et al., 1997, Phuc Le et al., 2005, Rose and Herzig, 2013).

Studies suggest that glucocorticoids, mainly through its interaction with GR, play a central role in skeletal muscle insulin sensitivity and, thus, glucose handling. Impaired insulin action in skeletal muscle is believed to account for the majority of the defect in insulin-stimulated glucose disposal in obese, insulin resistant as well as diabetic individuals. Glucocorticoids, through their GR-mediated actions, have been shown to critically trigger muscle insulin resistance and – as a consequence – to suppress glucose uptake by inhibiting the translocation of the glucose transporter type 4 (GLUT4) to the cell surface. In addition, glucocorticoid treatment has been described to reduce glycogen synthesis through a mechanism involving the suppression of glycogen synthase activity. Interestingly, GR mRNA levels in skeletal muscle of diabetic patients correlates with the degree of insulin resistance, with diabetes treatment leading to a normalization of GR levels associated to an improvement of insulin sensitivity. Therefore, it seems that aberrant activation of glucocorticoid signalling in the skeletal muscle can at least partially explain the dysfunctional glucose metabolism found in the insulin resistant and diabetic states (Rose and Herzig, 2013).

Finally, the effects of glucocorticoids on adipose tissue metabolism are conflicting, because despite glucocorticoids having long been accepted as being lipolytic in nature, patients with elevated levels of glucocorticoids usually display central adiposity. There is considerable evidence to support a pro-lipolytic effect of glucocorticoids. Much of the literature agrees that GCs increase lipolysis in mature adipocytes as a result of increased transcription and expression of the lipase proteins adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) (Slavin et al., 1994, Xu et al., 2009, Campbell et al., 2011, Peckett et al., 2011). The pro-adipogenic function of glucocorticoids, on the other hand, was clearly observed in transgenic mice over-expressing HSD11B1 in adipocytes, increasing active local glucocorticoid levels. In these mice, a significant increase in abdominal fat was observed whereas peripheral fats were less affected (Masuzaki et al., 2001). Glucocorticoids can affect the body as a whole to increase circulating fatty acids through an increase in dietary fat intake, increased lipogenesis and lipoprotein secretion from the liver, as well as increases in lipoprotein lipase (LPL) activity. With a greater amount of lipids in circulation, there is more to be taken up by the adipose tissue for storage. Glucocorticoids have also been shown to promote differentiation *in vitro* (Gregoire et al., 1998). Furthermore, by increasing adipogenesis, there are a greater number of adipocytes in which these lipids can be stored. Recent reports where the GR function was rendered deficient pharmacologically or genetically, adipogenesis was shown to be inhibited *in vitro* (Shteyer et al., 2004), suggesting an important role of GR on the adipogenic functions of glucocorticoids.

More recent work suggests that, when present in high amounts, glucocorticoids may inhibit lipolysis and increase lipid accumulation. Locally elevated levels and/or activation of glucocorticoids in the abdominal adipose tissue – through elevated HSD11B1 activity or GR expression – may favour the anti-lipolytic effects of glucocorticoids and support lipid accumulation. Overall, it seems likely that both pro-lipolytic and anti-lipolytic mechanisms exist, but some may play larger roles than others depending on the adipose depot in question and the dose and duration of exposure. A possible explanation for these divergent effects is the occurrence of both genomic and non-genomic glucocorticoid effects, which would culminate differential acute and long-term results (Campbell et al., 2011, Peckett et al., 2011, Rose and Herzig, 2013). Moreover, increasing numbers of studies address the interactions of glucocorticoids and insulin, and the impact of synergisms between these two major hormones in the

regulation of fat distribution and function *in vivo* and *in vitro*. Glucocorticoids differentially affect lipid storage depending on nutritional or hormonal conditions. While glucocorticoids decrease lipogenesis and free fatty acids uptake in basal or fasting conditions (Volpe and Marasa, 1975, Gathercole et al., 2011), they can also act additively or synergistically with insulin to upregulate lipogenesis after feeding (Minshull and Strong, 1985, Wang et al., 2004, Gathercole et al., 2011).

1.2.8.3.1 The impact of chronic stress on body weight

Although glucocorticoids – through their metabolic actions and multiple effects on the brain's response networks – appear to have evolved to make animals and humans fitter to handle stressors, these mechanisms do not seem to be adapted to an optimal functioning in our current society. The incidence of chronic social stress is increased, high calorie palatable foods are readily available and the physical effort used to acquire them is decreased. This seems to be turning a long-refined set of mechanisms that have enabled many species to survive, and even thrive under the influence of chronic stress, into a potential source of disease. Glucocorticoid hormones are not only highly implicated on the control of metabolism, but also have a central role on the control of feeding behaviour. Therefore, a misbalance of HPA axis function is likely to have an impact on weight. In fact, regardless of the influence of confounding factors – such as species, sex and social status – many studies performed in both humans and animal models have found an association between obesity and HPA axis hyperactivity (Dallman et al., 2006, Bose et al., 2009, Kyrou and Tsigos, 2009).

As some examples, Lenglos and colleagues have found that male rats have their body weight significantly decreased by a weekly 1-hour session of restraint stress for 7 weeks, but not females. Nevertheless, both males and females from the stressed groups presented higher plasma corticosterone levels when compared to control groups. Increased adiposity, but no changes in body weight, has been reported in mice submitted to nine weeks of social overcrowding as a chronic stressor. Consistent with the increased fat content, serum leptin was markedly elevated in stressed mice. Interestingly, higher corticosterone levels have been reported for weeks one and three of social overcrowding, but not for week nine, with the authors suggesting a habituation to the chronic stressor. Karagiannides and colleagues also found that stressed rats showed dramatically increased adiposity – despite no weight gain – compared to control animals. Chronic stress also increased plasma corticosterone levels and the expression of genes involved in the glucocorticoid gene network. Regarding studies in humans, a literature review performed by Bergmann and colleagues has examined 19 studies performed in adults from February 1990 to October 2013 investigating the relationship between chronic stress and adiposity levels. The results varied according to the type of stress analyzed: studies regarding stress from relationships and overall perceived stress generally supported an association between stress and weight gain, especially among women; distress is supported as a risk factor for weight gain among both genders; working stress presents conflicting results, with approximately half of the studies finding no association, and the other half finding some association among either men or women. Using waist circumference or body mass index as the outcome variable seems to influence the results. For example, one study found waist circumference to have a bidirectional profile, associated to both weight gain and weight loss depending on baseline body mass index (Lenglos et al., 2013, Bergmann et al., 2014, Karagiannides et al., 2014, Lin et al., 2015).

Chronic stress and HPA axis hyperactivity seem to influence obesity in many different ways. Studies have shown that the high levels of corticosterone induced by stress act in synergy with insulin to promote a higher intake of palatable food, such as those rich in fat and sugar. This combination of high corticosterone and high insulin levels also favours the accumulation of white adipose tissue. These fat depots send signals to the brain that reduce the stress response, notably by a decrease in CRH secretion, having an anxiolytic effect. This mechanism was termed by MF Dallman's team "the comfort food theory". Moreover, the adipose tissue is no longer regarded as a passive energy storage depot, but rather as a highly active endocrine gland that produces and secretes a wide variety of hormones and factors that signal the metabolic status of the adipocytes. This signaling would allow the formation of a complex reciprocal network between the adipose tissue, the other organs and tissues of the periphery and the central nervous system to regulate the body energy intake and expenditure. It has been suggested that the derangement of this network could be implicated in the onset of obesity. Furthermore, the accumulation of adipocytes seems to promote a persistent, generalized, low-grade inflammatory state, due to the capacity of these cells to express and secrete pro-inflammatory factors and cytokines that are responsible for the activation of the acute-phase inflammatory response. The secretion of pro-inflammatory factors by the adipocytes is proportional to the accumulated fat mass and correlates with the body mass index. This way, chronic stress would contribute to the development of obesity and, conversely, the accumulation of visceral adipose tissue would constitute a chronic stressful state due to the persistent low-grade inflammatory state (Kyrou et al., 2006, Warne et al., 2009).

Nevertheless, the effects of chronic stress on body weight are complex, and may present in bidirectional ways. By using male rats submitted to a 1-hour session of restraint stress for 90 days and/or a high-fat (HF) diet, Oliveira and colleagues found that stress significantly decreases body weight, but the association of this chronic stressor with a HF diet prevents this decrease. Retana-Márquez and colleagues have evaluated the effects of stressors of different kinds and intensities throughout 20 days. They found that all the stressors evaluated significantly decreased the weight gain of stressed rats when compared to controls. Interestingly, the effects of these stressors on plasma corticosterone levels were varied, but a general tendency towards higher levels was observed in all stressed groups, which usually presented significantly higher values than control groups when stressed and sacrificed in the light (inactive) phase. Thompson and colleagues found that moderate chronic stress decreased body weight gain of male rats in the first three weeks only, with stressed rats recovering their weight and maintaining it at the same level as control rats after the third week. No alterations in food intake or body adiposity were observed. A recent review of the literature by Razzoli and Bartolomucci has proposed the differential activation of the brown adipose tissue (BAT) metabolism by stress as a possible factor explaining the variability of the effects of chronic stress on weight gain and fat accumulation. In the presence of hypophagia, or when hyperphagia is associated with BAT recruitment and enhanced thermogenesis, stress results in weight loss and/or obesity resistance. On the other hand, hyperphagia associated with no changes in BAT recruitment leads to weight gain and obesity (Retana-Marquez et al., 2003, Oliveira et al., 2015, Thompson et al., 2015, Razzoli and Bartolomucci, 2016).

1.2.8.4 Actions on the central nervous system

1.2.8.4.1 Glucocorticoids and mood regulation

The stress response does not only affect the peripheral organs, but it also has an important impact on brain and can influence several brain functions. In interaction with other stress hormones, NA and corticosteroids differentially alter neuronal activity in areas that play a role in attention, vigilance, in the determination of appropriate behavioral strategies and storage of memories for future use (Joels and Baram, 2009). This can be regarded as the nervous system's contribution to the general stress response, overall resulting in successful behavioral adaptation (Joels, 2011). Repeated exposure to stressful situations, especially when these are unpredictable, uncontrollable and/or take place at vulnerable periods in life, can increase the risks of genetically vulnerable individuals to develop psychopathologies. As an example, the chances of developing an episode of depression in people carrying two alleles of the serotonin transporter s-type gene variant increase with the number of stressful life events they have experienced, while l-type gene variant carriers are relatively protected against depression even under adverse life conditions (Caspi et al., 2003).

Disturbance in the HPA functionality has been found over the past years as a characteristic feature in major depression. A classic example are the patients suffering Cushing's syndrome, which very often also present with from depression, and frequently seek medical care primarily because of their depressive state (Newell-Price et al., 2006). In fact, many individuals diagnosed with depression show elevated circulating corticosteroid levels (especially during the circadian trough) and relative insensitivity to dexamethasone-induced suppression of the HPA-axis. Interestingly, the normalization of these functions generally precedes relief of depressive symptoms and the degree of normalization predicts the likelihood to relapse. Furthermore, glucocorticoid antagonists given to patients with psychotic depression and hypercortisolism accelerate and increase the chances of success in their treatment with antidepressants (DeBattista and Belanoff, 2006, Blasey et al., 2009). Bipolar disorder also has been associated with HPA axis alterations, notably a significant degree of hyperactivity of this axis (Belvederi Murri et al., 2016). This observation is most prominent during the manic phase, but also persists in remission. The hyperactivity of the HPA axis in these cases has been associated with an increased CRH (and possibly AVP) secretion, which is thought to be a result from an impairment in this axis' feedback mechanism. In fact, a multitude of studies have found alterations in GR expression or function (reduced expression and impaired GR-mediated feedback inhibition) in patients suffering from depression and other psychiatric conditions, such as bipolar disorder and schizophrenia. Still it is not clear if these alterations stem from the conditions themselves or are indeed a result of the stress that having a psychiatric disorder may cause. Moreover, HPA axis activity was also described to be related to suicide attempts. This association was positive for subjects that were 40 years old or less, while negative in older subjects. These finding were true for both morning and evening cortisol levels, and no influence of gender was detected (Pariante et al., 2004, Uchoa et al., 2014).

Nevertheless, it is difficult to precisely pinpoint the mechanism by which chronic stress and the increased glucocorticoid activity affect the brain. The effects may result from gradual cumulative changes taking place at the cellular or circuit level in the brain, but may also be caused by peripheral adaptation to the increased HPA axis activation, indirectly impacting on the brain. Some of the disturbances observed include increased

secretion and reactivity of cortisol, elevated basal cerebrospinal fluid CRH levels, and increased size as well as activity of the pituitary and adrenal glands. An interesting candidate to explain the effects of chronic stress on depression-related symptoms is the serotonergic system. Chronic stress is known to affect responsiveness to serotonin. In control animals, brief exposure to corticosterone induces a delayed GR-dependent enhancement of responses via the 5HT1A receptor (Karst et al., 2000). After 3 weeks of exposure to high levels of corticosterone or to unpredictable stress, 5HT1A receptor mediated responses were found to be attenuated, both under basal corticosterone conditions and several hours after a brief exposure to corticosterone. The changes in 5HT1A receptor mediated responses were not coupled to reduced expression of this receptor. Instead, a transient change in MR/GR ratio was observed which may contribute to the gradual attenuation in serotonin responsiveness (Karten et al., 1999, van Riel et al., 2003).

Another noteworthy possibility is the downregulation of brain-derived neurotrophic factor (BDNF) by glucocorticoids. Animal depression models induced by a variety of chronic stress paradigms elicit increased levels of glucocorticoids and alteration in expression and/or function of BDNF, notably in the hippocampus (Xu et al., 2006, Bravo et al., 2009, Yan et al., 2011). Chronic glucocorticoid exposure has usually a negative impact on neuronal survival and function, and since BDNF contributes to cell survival and neuronal function, it is likely that downregulation of BDNF following chronic glucocorticoid exposure is involved in depression (Numakawa et al., 2012, 2013). Given that the signalling cascades activated by antidepressants closely associate with BDNF mediated signalling, and if GR or MR acts as a regulator of neuronal function including BDNF-stimulated intracellular signalling, the regulation of survival and synaptic plasticity through the actions of glucocorticoids on BDNF remains as an interesting target for psychiatric conditions treatments (Coyle and Duman, 2003, Castren and Rantamaki, 2010).

1.2.8.4.2 Glucocorticoids and memory

Memories are highly dynamic entities that are built in stages. Recently perceived stimuli are first encoded, and then the new and fragile memory trace is stabilized during a consolidation process. When a consolidated memory is retrieved, the memory trace can re-enter an unstable state that will require a reconsolidation process to stabilize it anew. Stress may have an effect on all these processes – encoding, consolidation, retrieval, and reconsolidation (or extinction), but how stress will influence these memory formation stages will depend largely on when and for how long an individual is stressed (Joels et al., 2006, Schwabe et al., 2012).

Encoding is affected when the individual is exposed to stress or glucocorticoids before learning. To estimate how these factors affect solely memory encoding is challenging since possible effects on encoding are inevitably confounded with effects on consolidation processes and memory retrieval, depending on the interval in which the memory is tested. This matter brings inconsistent results to the studies that investigate memory encoding, showing that pre-learning stress or glucocorticoids can actually have bi-directional effects on it. Encoding is usually enhanced by stress and glucocorticoids (Domes et al., 2002, Schwabe et al., 2008, Smeets et al., 2008), but the output can be influenced by variables such as the emotional meaning of the learned material, the interval between the stressful episode and the learning experience, or the time when the newly acquired memory is tested. As an example, stress administered prior to encoding

impairs neutral but enhances emotional long-term episodic memories (Payne et al., 2007).

Consolidation processes are typically enhanced by stress and glucocorticoids. Both human and rodent studies point towards a facilitated consolidation when stress or glucocorticoid is administered within a short-time window after learning and this effect can be even more pronounced if the learned information induces emotional arousal (Cahill et al., 2003, Andreano and Cahill, 2006, Beckner et al., 2006, Miranda et al., 2008, Liao et al., 2013). Adrenal hormones, released by emotional arousal, play a crucial role in regulating the strength of these memories, and the enhancing effects of glucocorticoids on memory consolidation appear to require a simultaneous occurrence of noradrenergic activity in the BLA (Roosendaal et al., 2006, Segal et al., 2014).

Memory retrieval, on the other hand, seems to be mostly impaired by stress, although this impairment could be alternatively interpreted as competitive encoding of new information, related to the stress exposure (de Kloet et al., 1999). Memory retrieval performance has been shown to be reduced in both humans and rodents by exposure to stress or administration of glucocorticoids shortly before testing (Roosendaal et al., 2004, Dorey et al., 2011, Atsak et al., 2012). Similarly to the effects of stress on memory consolidation, these effects are most pronounced for memory related to emotionally arousing material (Kuhlmann et al., 2005, Buchanan et al., 2006, de Quervain et al., 2007). Likewise, there is ample evidence that the effects of stress on memory retrieval also need co-occurring glucocorticoid and noradrenergic activity in the basolateral amygdala (Roosendaal et al., 2004).

Finally, recent evidence indicates that stress can also influence reconsolidation and/or extinction processes. Stress and glucocorticoid administration after memory retrieval were shown to impair later recall in both rodents and humans (Cai et al., 2006, Maroun and Akirav, 2008, Zhao et al., 2009, Schwabe and Wolf, 2010).

1.2.9 Variability in the HPA axis responses

1.2.9.1 Sex and gender

Males and females of many species secrete different levels of stress hormones following the same stimulus. The biological factors underlying the sex differences in stress responses are complex and involve several regions of the central and peripheral nervous systems. Significant and important differences between males and females are present at all points along the HPA axis, including inputs to the PVN of the hypothalamus, neuromodulatory regulation of CRH neurons, and the relative size and steroidogenic activity of the adrenal gland cortex. Gonadal steroids, which can impact gene expression, protein synthesis, and cellular excitability through their actions on androgen and estrogen receptors, are largely responsible for the development and expression of sex differences in HPA axis activity. Estradiol seems to exert the most important modulating effects on HPA axis functioning, including HPA axis responsiveness and sensitivity to glucocorticoid negative feedback. Animal studies show that estradiol has a strong stimulatory influence on HPA axis functioning, with modulatory effects on GR and MR, besides also directly enhancing CRH gene transcription in the hypothalamus. Sexual dimorphisms in brain function can also be underlying these differences. There is evidence indicating that brain limbic regions, including the PFC, the hippocampus, and amygdala – involved in the processing of psychological stress – are sexually dimorphic. For instance, female rats in proestrus

have a greater density of spines in area CA1 of the hippocampus than males, and this difference is affected in opposite directions by a stressful experience – spine density was enhanced in the male hippocampus but reduced in the female hippocampus (Shors et al., 2001). Moreover, brain imaging studies in humans have observed sex differences in the lateralisation of the activation in the amygdala. In response to either emotionally arousing film clips or to happy face perception, male subjects displayed enhanced activity of the right, but not the left, amygdala, while the opposite was found for females (Cahill et al., 1999, Killgore and Yurgelun-Todd, 2001, Kudielka and Kirschbaum, 2005, Goel et al., 2014, Bale and Epperson, 2015).

A persistent trend from animal studies is the constitutively higher responses of corticosterone secretion after stress in adult females compared to males. The HPA axis is also activated for a longer period after acute stress in females, and females have been shown to have higher corticosterone output from adrenals, even at baseline conditions. In fact, the sexually dimorphic development of brain circuits that are involved in eliciting sex differences in adult stress responses begins early in life. Animal studies have shown that these differences in HPA axis output are present from the immediate new-born period (Kudielka and Kirschbaum, 2005, Goel et al., 2014, Panagiotakopoulos and Neigh, 2014).

The literature regarding sex differences in the stress response in humans is less consistent than that from animal studies. This is probably due to the diversity in methodology and demographic composition of subjects, including type of stressor, time and source of hormone sampling (plasma vs. saliva), and age and health status of subjects. Still, sex differences in stress responses can be found at all stages of life, and these differences are related to both the organizational and activational effects of gonadal hormones and to genes found on the sex chromosomes. During gestation, sex differences in placental and embryonic responses to maternal stress and environmental perturbations are well documented in humans, where males are at a greater risk of suffering short-term and long-term negative outcomes. In contrast, adversity during childhood appears to preferentially increase the risk for affective disorders in women, manifested especially during their reproductive years. In adult humans, while some studies quote no sex differences in the stress response, others find higher glucocorticoid or ACTH levels for either sex. In fact, the type of stress stimulus adopted by each study, and the ability of this stimulus to trigger a proper stress response in both sexes, seems to play an important role in these inconsistencies. Stroud and colleagues showed that, compared to women, men had significantly greater cortisol responses to mathematical and verbal challenges, while women showed greater cortisol elevations to social interaction challenges (Stroud et al., 2002, Kudielka and Kirschbaum, 2005, Lupien et al., 2009, Panagiotakopoulos and Neigh, 2014, Bale and Epperson, 2015).

Furthermore, stress seems to have a greater negative impact on women's psychological health, with stress-related mood disorders – such as major depression and general anxiety – being approximately two times more prevalent in women than in men. Interestingly, the sex difference in the rate of depression first emerges during puberty, suggesting a role for gonadal hormones in the sex differences in stress and mental health. Stress exacerbates the risk of depression in girls with early onset puberty, but not in boys, and stress is correlated to symptoms of anxiety and depression to a greater degree in women than men. Women are also more likely to develop psychopathology when faced with the same stressful events as men. Thus, women and men seem to have substantially different strategies in coping with the challenges of stress (Stroud et al., 2002).

1.2.9.2 Genetic factors

It is widely accepted that physical and psychological stressors do precipitate the so-called stress-related disorders. Why, however, certain subjects thrive and others succumb following stress episodes is still unclear. As previously described in this introduction, there is a great variability in the HPA axis response between species as well as between individuals from the same species. Some of this variability has been shown to have a genetic origin. To date, most studies on the genetic variability of the stress response have focused on the GR and MR receptors' polymorphisms. Studies in human populations have pointed out that carriers of GR and MR genetic variants show remarkable differences in HPA axis and autonomic output. GR variants were shown to influence not only negative feedback of the HPA axis and stress glucocorticoid responses, but also basal glucocorticoid levels. On the other hand, the MR variants seem to affect both autonomic and HPA axis responses, besides basal glucocorticoid levels. Furthermore, MR and GR variants have been associated with different pathologies, including psychiatric, metabolic, cardiovascular and inflammatory disorders, indicating the importance of the central stress-response for a wide variety of physiological responses. These findings place GR and MR receptors as interesting pharmacological targets to potentially treat a wide range of disorders (see (DeRijk et al., 2002, Wust et al., 2004, Derijk and de Kloet, 2008) for reviews).

An approach that has been used to detect the source of individual variations in the HPA axis response is the QTL mapping. This method is widely used to detect genome regions influencing phenotypes that vary continuously inside a given population, and that are typically the result of the interaction between multiple genes and the environment. Such phenotypes are frequently referred to as “quantitative traits” and the genomic regions that contribute to their variation are known as *quantitative trait loci* (QTL). It consists in associating scores for a given phenotype to variations in genotypic data (usually molecular markers), which can either be spread genome-wide or limited to a region of interest. For being a non-hypothesis-driven method, it has the advantage of looking simultaneously at a great number of *loci* and eventually allows the identification of influences from genes that *a priori* were not thought to influence the phenotype studied. QTL analyses have been used to identify genomic regions containing genes likely to be influencing the stress response.

In the mid-80s, P Mormède and colleagues performed a QTL study using pigs that were generated from the intercross between the strains European Large White and Chinese Meishan. These two strains differed regarding their behavioral and endocrine responses to stress, with Meishan pigs presenting higher basal and after stress cortisol levels, higher adiposity and a less active behavior when compared to Large White pigs. This study has led to the identification of a *locus* on chromosome 7 that explained 20% of the variability found for after stress cortisol levels. MP Moisan's team has pursued this study, proposing the gene coding for CBG – *Serpina6* – as a candidate gene influencing these behavioral and endocrine alterations. The location of *Serpina6* was demonstrated to be inside the previously found QTL, and a highly significant genetic linkage between CBG plasma binding capacity values and the chromosome 7 markers flanking the cortisol-associated QTL was detected. CBG capacity has also been shown to be positively correlated to fat and negatively correlated to muscle content in that hybrid population. Finally, CBG capacity and gene expression were found higher in Meishan when compared with Large White pigs, pointing out *Serpina6* as a key regulator of blood

cortisol levels (Mormede et al., 1984, Desautes et al., 1999, Desautes et al., 2002, Ousova et al., 2004, Moisan, 2010)

Since that seminal study, other independent groups have confirmed the influence of CBG on the variability of glucocorticoids levels. A QTL analysis performed in rats by Solberg and colleagues has identified several *loci* for HPA axis-related traits, including after stress cortisol levels. *Serpina6* was pointed out as a candidate gene to explain the effect of one of these *loci*, and further analyses have identified a sequence alteration resulting in an amino acid substitution in the final protein, which was thought to lead to decreased binding affinity. More recently, Bolton and colleagues carried out a genome wide association meta-analysis for plasma cortisol values in over 15 thousand participants. They found that genetic variation in a single region of chromosome 14 significantly accounted for less than 1% of variance in plasma cortisol. Among the genes comprised in this *locus* is the human variant of the previously mentioned SERPINA6 gene, as well as SERPINA1, encoding α 1-antitrypsin (a type of serine protease inhibitor which inhibits cleavage of the reactive centre loop that releases cortisol from CBG). Three common single nucleotide polymorphisms (SNPs) were identified in this genomic region, which were associated with variation in total cortisol binding activity in plasma (Solberg et al., 2006, Bolton et al., 2014). Altogether, these studies reinforce the key role of CBG in determining plasma cortisol levels and highlight the study of CBG as an interesting approach to better understand the effects of stress on health and disease.

1.2.10 Animal models of HPA axis alterations

As previously discussed, altered HPA axis activity is found in several pathological states, among which psychiatric and metabolic disorders. Despite such abnormalities not being universally observed in human conditions, nor being adequately specific to these states to provide diagnostic criteria, they still inspire the development of several animal models of disrupted glucocorticoid homeostasis. Some examples of genetic models of HPA axis alteration and their associated phenotypes - with emphasis to neuropsychiatric and metabolic traits - are described below and summarized on Table 1.

1.2.10.1 CRH and CHR receptor

CRH underexpression

Given the clinical evidence that CRH is elevated in hypothalamic PVN neurons and in the cerebrospinal fluid of depressed humans (Raadsheer et al., 1994), the question of whether these changes have consequences on behavior has been addressed in using mice with specific genetic manipulations in the CRH/CRH receptor signaling pathways. Muglia and colleagues were the first to generate a mouse model of CRH deficiency and showed that life without CRH is possible. In these mice, basal pituitary ACTH secretion is sustained by an increased expression of the AVP. This way, CRH deficiency impairs but does not block HPA responses to stressors. No gross behavioral abnormalities have been reported in the CRH knockout mice, which underscores how flexible the stress response system is in allowing for such compensation (Muglia et al., 1995, Jacobson et al., 2000, Venihaki and Majzoub, 2002).

CRH overexpression

Transgenic mice overexpressing rat CRH were also developed. These animals display increased anxiety-related behavior in various behavioral tests, such as the light-dark box and the elevated plus maze. However, the interpretation of behavioral results in these CRH overexpressing mice is limited in that the animals exhibit prominent endocrine and physical changes similar to the changes seen in patients with Cushing's syndrome, such as hypercortisolemia, muscle atrophy, excess fat accumulation, thin skin, and hair loss. In an attempt to meet these demands, a transgenic mouse line in which CRH is overexpressed in neurons of the postnatal and adult brain was developed. CRH overexpression was verified in the PVN, the central nucleus of the amygdala and the bed nucleus of the stria terminalis. These mice also display a Cushing-like phenotype, but only after the age of 6 months. Peripheral concentrations of ACTH are only marginally elevated, but still lead to increased plasma corticosterone levels. No behavioral alteration was observed in the light-dark box or the elevated plus maze, but significantly reduced freezing was reported in the fear conditioning paradigm. Although these mice were developed to regionally increase CRH expression, the profound neuroendocrine alterations in these animals still leads to the development of Cushing's syndrome after several months, and limits the value of these mice as a model for the behavioral and neuroendocrine symptoms of depression (Stenzel-Poore et al., 1992, Stenzel-Poore et al., 1994, Dirks et al., 2002a, Dirks et al., 2002b, Groenink et al., 2002, van Gaalen et al., 2002, Groenink et al., 2003).

CRHR1 underexpression

Two conventional knockout mouse lines for CRHR1 have been independently generated. Both deficient lines display a severe impairment of stress-induced HPA system activation and marked glucocorticoid deficiency. Basal plasma ACTH concentrations in homozygous CRHR1 mutants are similar to those found in wild-type controls, suggesting that basal ACTH secretion can be maintained via signaling pathways other than CRH/CRHR1. Similarly to the CRH knockout model, the hypothalamic AVP system was shown to be significantly activated to maintain pituitary ACTH secretion. At the behavioral level, CRHR1 deficient mice exhibit increased exploratory activity and significantly reduced anxiety-related behavior. Behavioral analyses of CRHR1 null mutants are limited by the fact that these mice display a severe glucocorticoid deficiency. As glucocorticoids play important roles in modulating anxiety-related behavior, the anxiolytic effect observed in conventional CRHR1 knockout mice may result from either CRHR1 deficiency itself or be influenced by the reduction in circulating glucocorticoid levels in these animals (Smith et al., 1998, Timpl et al., 1998, Muller et al., 2000).

Conditional CRHR1 knockout mice were later generated. In these mice, the inactivation of CRHR1 takes place postnatally and is restricted to the forebrain and limbic system. Disruption of CRH/CRHR1 signaling pathways in the aforementioned neuronal circuitries significantly reduced anxiety-related behavior. In contrast to conventional CRHR1 knockout mice, basal plasma ACTH and corticosterone levels of the conditional mutants were similar to those from wild-type mice. Their behavioral phenotype, therefore, is not likely to be influenced by central nervous system effects of circulating stress hormones. Moreover, conditional mutants showed a normal stress response to different durations of immobilization stress. However, hormone levels

remained significantly elevated in the conditional mutants following a short period of restraint stress, providing evidence that limbic CRHR1 is required for central control of HPA system feedback and hormonal adaptation to stress (Muller et al., 2003, Muller and Holsboer, 2006).

1.2.10.2 AVP and AVP receptor

AVP underexpression

Following its identification in 1954, AVP was considered the principal factor in the regulation of ACTH release. However, for several reasons, including the identification of CRH and the domination of Dale's Law (one neuron/ one transmitter), the interest in AVP at the vanguard of stress research waned. The Brattleboro rat is a rodent model for AVP deficiency. Its consists in a strain of laboratory Long Evans rats descended from a litter born in West Brattleboro in 1961 carrying a naturally occurring genetic mutation impairing their ability to produce AVP. Experiments carried out in this model suggest that AVP deficiency leads attenuated depression-like behavior in the forced swim test (FST) and sucrose preference tests. AVP deficiency had no influence on basal levels of ACTH and corticosterone, and had only mild impact on hormonal activation in response to stress, which also depends of the type of stressor. Furthermore, AVP deficient rats seem to be protected from the endocrine – but not from the behavioral – consequences of chronic stress. Pharmacological studies have shown that AVP and its analogues can produce either improvements or impairments in memory functions, effects that depend upon the agent administered, the memory process measured and the task employed. AVP deficient rats show weaker performance in the course of social and object discrimination tests and a higher escape failure during the shuttle box experiment. Studies in partial AVP deficient (heterozygous for the Brattleboro mutation) rats showed that it can trigger deficits of spatial working memory performance in both males and females. However, the absence of AVP in early development as well as the diabetes insipidus provoked by AVP deficiency limits the conclusion of these studies (Aarde and Jentsch, 2006, Mlynarik et al., 2007, Varga et al., 2011, Makara et al., 2012, Varga et al., 2014).

AVP receptors underexpression

In the brain, the AVP signal is conveyed via two different receptors: AVP receptor 1A (AVPR1A) and 1B (AVPR1B). Some attempts were made to determine which of these receptors accounts for the behavioral changes induced by AVP. AVPR1A receptor knockout and knockdown mice have deficits in social recognition and show marked decreases in anxiety-like behavior, suggesting that AVPR1A receptors might be interesting targets for drugs designed to treat mood disorders. AVPR1B knockout mice were shown to take longer to attack an intruder in the resident/intruder paradigm, what was interpreted as a reduced level of aggression. Stress has been shown to increase AVPR1B receptor mRNA levels in the rat brain, and synthetic AVPR1B receptor antagonists have been developed and produced behavioral changes comparable to those observed after administration of antidepressants, further underlining that AVP receptors are potential targets for drugs to treat stress-related disorders. Interestingly, AVP receptors are also implicated in metabolic processes. AVPR1B receptors have been demonstrated to mediate the AVP-stimulated insulin release from islet cells, with

mice lacking the AVPR1B losing these effects, which are preserved in mice lacking the AVPR1A receptor. Furthermore, AVPR1B receptor deficient mice present with enhanced insulin sensitivity and increased insulin signaling in adipocytes. AVPR1A receptor deficient mice, on the other hand, were shown to have increased lipid metabolism, since it is normally modulated by the antilipolytic action of AVP and the synthesis of bile acid via AVPR1A (Landgraf et al., 1995, Griebel et al., 2002, Wersinger et al., 2002, Bielsky et al., 2004, Oshikawa et al., 2004, Griebel et al., 2005, Fujiwara et al., 2007, Hiroyama et al., 2007, Tanoue, 2009).

1.2.10.3 ACTH receptor

MC2R underexpression

ACTH stimulates steroidogenesis in the adrenal gland via its specific cell-surface receptor, MC2R. MC2R knockout mice have been developed under B6/N5 background, but most of the homozygous mice for the null mutation die shortly after birth due to hypoglycemia (around 94-100% between generations N4 and N8). To allow a further characterization of MC2R deficient mice, the mutation was transferred onto a B6/Balbc background, what decreased the lethality of the homozygous null mutation to 50%. These animals are fertile and deliver their offspring with similar litter size compared to heterozygous mice. Interestingly, even in the B6/Balbc background, homozygous pups that are born from homozygous dams die shortly after birth due to lung hypoplasia, similarly to what happens to CRH null mice. One functioning allele seems to be enough to guarantee lung maturation, since both heterozygous pups from homozygous null dams and homozygous null pups from heterozygous dams are viable. Serum corticosterone after 30 min of restraint stress was undetectable in MC2R knockout mice (Chida et al., 2007, Chida et al., 2009).

1.2.10.4 Mineralocorticoid and glucocorticoid receptors

MR underexpression

Targeted disruption of the MR gene (nr3c2) results in pseudohypoaldosteronism type I, with failure to thrive, severe dehydration, hyperkalemia, hyponatremia, and high plasma levels of renin, angiotensin II and aldosterone. Although both aldosterone and glucocorticoids bind MR with high affinity, this receptor is protected from glucocorticoids in aldosterone target cells – such as those in the kidneys and colon – by the increased activity of the enzyme HSD11B2, which inactivates cortisol and corticosterone. In MR knockout mice in which the salt loss syndrome has been corrected, a marked decrease in neuron density, mainly in the upper blade of the hippocampal granule cell layer, is observed. Moreover, a significant reduction of granule cell neurogenesis was reported (Berger et al., 1998, Gass et al., 2000, Muller and Keck, 2002).

A recent study has examined social approach and social discrimination in male and female mice lacking MR from hippocampal-amygdala-prefrontal circuitry and controls. In the social approach task, which allows the assessment of time spent with an unfamiliar mouse and the ability to discriminate between familiar and unfamiliar conspecifics, they observed that deletion of MR from the limbic circuitry of male and female mice intensified the interest for their conspecifics in spite of an empty cage. Furthermore, male knockout mice did not show the expected stronger interest in the

unfamiliar mouse compared to the familiar mouse as male control mice did. Both findings can be viewed as a demonstration of impaired inhibitory control in mice lacking MR in limbic areas. Previous studies with these same mice have shown that they display increased perseveration (visiting the same holes repeatedly) on the spatial task of the circular hole board and make more re-entry errors on the radial arm maze, suggesting that the absence of functional MR in limbic regions leads to a lack of behavioral flexibility. Finally, these mice were also shown to present spatial memory impairments on the Morris water maze, with slower swim speeds and deficits in working memory on the radial arm maze (Berger et al., 2006, ter Horst et al., 2012, Ter Horst et al., 2013, Ter Horst et al., 2014).

MR overexpression

Transgenic mice with overexpression of forebrain MR have also been generated. These mice exhibit overexpression at levels 20–25% above endogenous levels in the cortex and hippocampus. MR levels in the amygdala and PVN are normal. They present with decreased CA1 GR and increased CA1 5HT1A expression, with no changes in PVN CRH. In these mice, basal HPA axis function appears unaffected, as the corticosterone release after mild stress. The corticosterone response to restraint stress is moderately suppressed in female, but not in male mice. Increased MR activity in the forebrain led to decreased anxiety and, in some circumstances, to improved spatial memory capability (Lai et al., 2007, Rozeboom et al., 2007, Kolber et al., 2008).

GR underexpression

The first mouse model with impaired GR function had a DNA stretch of the GR inserted into the mouse genome but in reverse direction and under control of a neuron-specific promoter. By this mechanism, antisense directed against the GR gene (nr3c1) mRNA is produced, resulting in a markedly impaired GR function in the brain. These mice showed several behavioral and neuroendocrine depression-related signs, such as reduced negative feedback sensitivity to dexamethasone and enhanced stress hormone response following pharmacological or emotional stimuli. Perhaps the most interesting feature of these mice is their response to antidepressants, which include increases in GR mRNA, reduced HPA-activity, improved cognitive function and enhanced measures in hippocampal long-term potentiation (LTP), an *in vitro* model for synaptic plasticity and learning. The GR-antisense transgenic mouse has also limitations which include the molecular interaction of GR antisense and the GR transcription machinery and related adaptations (Pepin et al., 1992a, b, Stec et al., 1994, Montkowski et al., 1995, Barden et al., 1997, Steckler and Holsboer, 2001, Muller and Keck, 2002).

Although the conventional GR null mutant was not viable due to severely impaired lung development and subsequent respiratory failure, a small fraction of these mice survived, showing elevated plasma ACTH and corticosterone levels and spatial learning disability that were comparable to that observed in GR antisense transgenic mice. GR-heterozygous mutant mice, where only one GR allele was ablated proved to be particularly useful for studies of the role of incapacitated GR. These mice show increased helplessness after stress exposure, a behavioral correlate of depression in rodents, and an abnormally elevated stress hormone response in the combined dexamethasone/CRH-test. Moreover, these mice have decreased hippocampal BDNF

content (Cole et al., 1995, Oitzl et al., 1997, Steckler and Holsboer, 1999, Tronche et al., 1999, Ridder et al., 2005).

Mutants with central nervous system specific deletions of GR were also generated, allowing a refined analysis of GR function in the brain. Because the GR deficit occurred also in the hypothalamus, these mice displayed excessively plasma-corticosterone secretion with the development of Cushing's syndrome. Another study directed the GR deficit in a time-dependent way into the mouse forebrain of mice from three weeks old. Forebrain-specific GR deletion produced non-suppression of corticosterone secretion following dexamethasone administration, altered circadian HPA axis activity, and increased hypothalamic AVP expression, which are all hallmarks of depression. Also, behavioral changes in behavioral-despair tests – increased immobility in the FST and tails suspension test (TST) – were observed. Both HPA axis abnormality and the depression-like symptoms disappeared once these mice were treated with imipramine. Altered behavior was also observed in an anxiety-related test (elevated plus maze), as well as increased CRH levels in the central amygdala. The expression of 5HT1A, considered to be crucial for mediation of stress responses, is elevated in the hippocampus of these mice, which is further indication that this receptor depends on the MR/GR balance (Lopez et al., 1998, Tronche et al., 1999, Wei et al., 2004, Boyle et al., 2005).

In order to investigate the effects of GR mediating glucocorticoids effects on adipose tissue expansion and obesity, an adipocyte specific GR knockout mouse model was created. GR deletion did not affect body weight gain or adipose tissue formation and distribution, but the lack of GR in adipocyte promoted a diet-induced inflammation, determined by higher pro-inflammatory genes expression and macrophage infiltration in the fat pads. The adipose tissue inflammation was not correlated with insulin resistance or dyslipidemia, but with disturbed glucose tolerance (Desarzens and Faresse, 2016).

GR overexpression

A mouse model overexpressing GR employed a transgenic technique using a yeast artificial chromosome. This GR overexpressing mouse was more resistant to developing behavioral helplessness, had lower stress hormone response in the dexamethasone/CRH test, and increased hippocampal BDNF content, underscoring the close link between HPA-axis activity and BDNF gene expression (Reichardt et al., 2000, Ridder et al., 2005).

1.2.10.5 11- β -hydroxysteroid dehydrogenases

HSD11B1 underexpression

To evaluate the importance of the enzyme HSD11B1 in regenerating active glucocorticoids from circulating inert forms, mice were produced with targeted disruption of the gene coding for this enzyme. Despite compensatory adrenal hyperplasia and increased adrenal secretion of corticosterone, these mice have lower intracellular corticosterone levels. They display attenuated activation of the key hepatic gluconeogenic enzymes when under starvation, presumably because of intrahepatic glucocorticoid deficiency, and increased enzymes of lipid oxidation. HSD11B1 knockout mice were found to resist hyperglycemia provoked by obesity or stress. Furthermore, HSD11B1 deficiency produces an improved lipid profile (lower serum triglycerides and

high high-density lipoprotein (HDL) cholesterol) and hepatic insulin sensitization, showing HSD11B1 deficiency protects these mice from the metabolic consequences of obesity. When the null mutation is inserted into an obesity prone genetic background, HSD11B1 deficient mice are further protected from weight gain on a HF diet. Data obtained from brain-specific HSD11B1 knockout mice suggest that the protection from the metabolic consequences of obesity is not mediated by the central nervous system. Recent studies suggest that the degree of metabolic protection presented by these mice are strain-dependent (Kotelevtsev et al., 1997, Morton et al., 2001, Morton et al., 2004, Carter et al., 2009, Harno et al., 2013a, Harno et al., 2013b).

Spatial learning and memory are enhanced in adult and aged HSD11B1 deficient mice compared with age-matched controls. No alterations in other cognitive (working memory in a spontaneous alternation task) or affective (anxiety-related behaviors) domains were observed in HSD11B1 knockout mice, nor differences in plasma corticosterone or glucose levels, or increased age-related pathologies when compared to control mice. LTP was found significantly enhanced in hippocampal slices from aged HSD11B1 knockout mice, suggesting that increased synaptic potentiation in the aged hippocampus may underlie the better maintenance of learning and memory with aging in HSD11B1 deficient animals. By measuring spatial memory before and during intracerebroventricular infusion of a MR antagonist or a GR antagonist, it was shown that HSD11B1 deficiency in aging mice leads to lower intracellular glucocorticoid concentrations in brain, particularly in the hippocampus, which activate predominantly MR to enhance memory, while in aging controls the increased intracellular glucocorticoids saturate MR and activate predominantly GR, thus impairing memory (Yau et al., 2007, Yau et al., 2011).

HSD11B1 overexpression

Adipose tissue-specific overexpression of HSD11B1 produces a phenotype analogous to the metabolic syndrome, with central obesity, hypertension, insulin resistance, and dyslipidemia. This is associated with high intra-adipose corticosterone, high levels of plasma leptin, TNF, and adipose LPL mRNA. Liver overexpression of HSD11B1 results in fatty liver, dyslipidemia, mild insulin resistance, increased angiotensinogen expression, and hypertension. However, these mice do not show increased adiposity or glucose intolerance (Masuzaki et al., 2001, Masuzaki et al., 2003, Paterson et al., 2004).

HSD11B2 underexpression

Fewer studies have addressed the effects of HSD11B2 deficiency. Homozygous mutant mice lacking HSD11B2 appear normal at birth, but approximately 50% show motor weakness and die within 48 hours. Both male and female survivors are fertile but exhibit hypokalemia, polyuria, and hypertension. Young adult HSD11B2 deficient mice are markedly hypertensive. Subsequent breeding shifted the HSD11B2 knockout model onto a C57BL/6J background, largely obviating the neonatal phenotype. On this background, low birth weight was clear, indicating that the absence of HSD11B2 during the development was retarding fetal growth. Studies on this model have shown that adult HSD11B2 offspring generated from a homozygous mating approach exhibit high anxiety indexes in comparison with wild-type littermates. HSD11B2 deficient offspring generated from a heterozygous mating display increased depressive-like behavior. More

recently, a brain-specific knockout of HSD11B2 was generated to investigate the importance of the high expression of HSD11B2 within the midgestational fetal brain. Brain-specific knockout mice have markedly diminished fetal brain HSD11B2, but intact fetal body and placental HSD11B2 and normal fetal and placental growth. Despite normal fetal plasma corticosterone, they exhibit elevated fetal brain corticosterone levels at midgestation. As adults, brain-specific HSD11B2 knockouts show depressive-like behavior and have cognitive impairments, but no anxiety-like behavioral deficits are observed (Kotelevtsev et al., 1999, Holmes et al., 2006, Wyrwoll et al., 2011, Wyrwoll et al., 2015).

Table 1 – Genetic models of HPA axis alteration and their associated phenotypes.

Transgenic line	Type of modification	Phenotype observed	References
CRH knockout	Full knockout mice	<ul style="list-style-type: none"> - Compensatory increase in AVP secretion - Glucocorticoid deficiency and HPA hyporeactivity to stress - No gross behavioral abnormalities observed 	Muglia et al 1995 Weninger et al 1999 Jacobson et al 2000
CRH overexpression	Nonselective overexpression in mice	<ul style="list-style-type: none"> - Cushing-like phenotype (increased ACTH and corticosterone levels, muscle atrophy, excess fat accumulation) - Increased anxiety-related behavior 	Stenzel-Poore et al 1992 Stenzel-Poore et al 1994 Heinrichs et al 1997a van Gaalen et al 2002
CRH overexpression	Exclusive overexpression in neurons of the postnatal and adult mice brain	<ul style="list-style-type: none"> - Increased corticosterone levels and development of Cushing’s syndrome after 6 months of age - No alteration of anxiety-related behavior - Reduced freezing in the fear conditioning paradigm 	Dirks et al 2002a Dirks et al 2002b Groenink et al 2002 Groenink et al 2003
CRHR1 knockout	Full knockout mice	<ul style="list-style-type: none"> - Compensatory increase in AVP secretion - Basal glucocorticoid deficiency and HPA system hyporeactivity to stress - Increased exploratory activity - Reduced anxiety-related behavior 	Timpl et al 1998 Smith et al 1998 Müller et al 2000
CRHR1 knockout	Postnatal CRHR1 deficiency restricted to the forebrain and limbic systems of mice	<ul style="list-style-type: none"> - Normal HPA system regulation under basal and stress conditions - Sustained HPA axis activation after stress - Reduced anxiety-related behavior 	Müller et al 2003
AVP-deficient	Congenital deficit of vasopressin due to a spontaneous mutation in a rat strain (Brattleboro rats)	<ul style="list-style-type: none"> - Attenuated depression-like behavior - No influence on basal levels of ACTH or corticosterone - Mild impact on hormonal activation in response to stress - Prevents the endocrine – but not the behavioral – consequences of chronic stress - Weaker performance in the course of social and object discrimination tests and higher escape failure during the shuttle box experiment 	Aarde and Jentsch, 2006 Mlynarik et al., 2007 Varga et al., 2011 Makara et al., 2012 Varga et al., 2014
AVP V1a receptor knockout	Full knockout mice	<ul style="list-style-type: none"> -Deficits in social recognition - Decreased anxiety-like behavior - Increased lipid metabolism 	Bielsky et al., 2004 Hiroyama et al., 2007 Tanoue, 2009

Transgenic line	Type of modification	Phenotype observed	References
AVP V1b receptor knockout	Full knockout mice	<ul style="list-style-type: none"> - Longer latency to attack an intruder in the resident/intruder paradigm/ reduced level of aggression - Lower insulin levels, enhanced insulin sensitivity and increased insulin signaling in adipocytes 	<p>Oshikawa et al., 2004 Fujiwara et al., 2007 Griebel et al 2005 Griebel et al 2002 Guillon et al 2004 Wersinger et al 2002</p>
MC2R knockout	Full knockout mice	<ul style="list-style-type: none"> - Almost fully lethal under B6/N5 background, 50% lethality under B6/Balbc background - Fertile and deliver their offspring with similar litter size compared to heterozygous mice - One functional MCR2 allele seems to be necessary to guarantee lung maturation in the pups - Serum corticosterone is undetectable in baseline conditions or after restraint stress 	<p>Chida et al., 2007 Chida et al, 2009</p>
GR knockout	Full knockout mice	<ul style="list-style-type: none"> - Most are not viable due to severely impaired lung development and subsequent respiratory failure - Homozygous survivors present elevated plasma ACTH, elevated corticosterone levels and spatial learning disability - Heterozygous display increased helplessness after stress exposure, abnormally elevated stress hormone response in the combined dexamethasone/CRH-test and decreased hippocampal BDNF content 	<p>Cole et al., 1995 Oitzl et al., 1997 Steckler and Holsboer, 1999 Tronche et al., 1999 Ridder et al., 2005</p>
GR knockout	Specific deletion in the forebrain of mice from 3 weeks old	<ul style="list-style-type: none"> - Non-suppression of corticosterone secretion after dexamethasone administration - Altered circadian HPA axis activity - Increased hypothalamic vasopressin expression - Increased CRH levels in the central amygdala - Increased 5-HT1A expression in the hippocampus - Increased behavioral-despair - Increased anxiety-related behaviors 	<p>Boyle et al 2005</p>
GR knockout	Adipocyte-specific GR knockout mice	<ul style="list-style-type: none"> - Increased diet-induced inflammation in fat pads - Disturbed glucose tolerance - No effect on body weight gain or adipose tissue formation 	<p>Desarzens and Faresse, 2016</p>

Transgenic line	Type of modification	Phenotype observed	References
GR knockdown	Antisense directed against the GR mRNA specifically in the mice brain	<ul style="list-style-type: none"> - Reduced negative feedback sensitivity to dexamethasone - Enhanced stress hormone response to stimuli - Response to antidepressants: increase in GR mRNA, reduced HPA-activity, improved cognitive function and increase in LTP 	Stec et al., 1994 Barden et al., 1997 Müller et al., 2002 Pepin et al., 1992a,b Montkowski et al., 1995 Steckler et al., 2001
GR overexpression	Yeast transgene, mice expressing 2-fold overexpression of GR	<ul style="list-style-type: none"> - Resistance to developing behavioral helplessness - Lower stress hormone response in the dexamethasone/CRH test - Increased hippocampal BDNF content 	Reichardt et al., 2000 Ridder et al., 2005
MR knockout	Full knockout mice	<ul style="list-style-type: none"> - Normal prenatal development but death around postnatal day 10 due to severe dehydration and pseudohypoaldosteronism - Reduced density of neurons and neurogenesis in the hippocampus - High aldosterone and corticosterone plasma levels 	Berger et al., 1998 Hubert et al., 1999 Bleich et al., 1999 Gass et al., 2000
MR knockout	Forebrain-specific MR knockout mice	<ul style="list-style-type: none"> - Impaired social discrimination and decision-making in male, but not in female mice - Decreased behavioral flexibility - Impaired spatial and working memory 	Ter Horst et al., 2014 Berger et al., 2006
MR overexpression	Forebrain-specific MR overexpressing mice	<ul style="list-style-type: none"> - Normal MR levels in the amygdala and PVN - Decreased GR and increased CA1 5HT1a expression in CA1 - Unaffected basal HPA axis function and corticosterone release after mild stress - Decrease corticosterone response to restraint stress in females - Decreased anxiety-related behaviors - Improved spatial memory capability (not consistently found)) 	Lai et al., 2007 Rozeboom et al., 2007 Kolber et al., 2008
HSD11B1 knockout	Full knockout mice	<ul style="list-style-type: none"> - Compensatory adrenal hyperplasia and increased adrenal secretion of corticosterone - Decreased intracellular corticosterone levels - Protection from the metabolic consequences of obesity - Enhanced memory and spatial learning, associated with increased LTP in the hippocampus <p>*Phenotype shown to vary according to the background strain</p>	Kotelevtsev, 1997 Morton et al, 2001 Morton et al, 2004 Harno et al, 2013 Carter et al, 2009 Yau et al, 2007 Yau et al, 2011
HSD11B1 overexpression	Adipose tissue-specific HSD11B1 overexpressing mice	<ul style="list-style-type: none"> - Phenotype analogous to the metabolic syndrome: central obesity, hypertension, insulin resistance and dyslipidemia - Increased intra-adipose corticosterone, high levels of plasma leptin, TNF, and adipose lipoprotein lipase mRNA 	Masuzaki et al, 2001 Masuzaki et al, 2003

Transgenic line	Type of modification	Phenotype observed	References
HSD11B1 overexpression	Liver-specific HSD11B1 overexpressing mice	<ul style="list-style-type: none"> - Fatty liver - Dyslipidemia - Mild insulin resistance - Hypertension - No sign of increased adiposity or glucose intolerance 	Paterson et al, 2004
HSD11B2 knockout	Full knockout mice on a C57BL/6J background	<ul style="list-style-type: none"> - Low birth weight, indicating retard in fetal growth - Offspring generated from a homozygous mating approach exhibit high anxiety indexes - Offspring generated from a heterozygous mating display increased depressive-like behavior 	Kotelevtsev et al, 1999 Holmes et al, 2006 Wyrwoll & Holmes 2011
HSD11B2 knockout	Brain-specific HSD11B2 knockout mice	<ul style="list-style-type: none"> - Diminished fetal brain HSD11B2 - Intact fetal body and placental HSD11B2, and normal fetal and placental growth - Increased fetal brain corticosterone levels at midgestation - Increased depressive-like behavior and cognitive impairments as adults 	Wyrwoll et al, 2015

1.3 Corticosteroid binding globulin (CBG) or transcortin

As already mentioned, CBG is a plasma glycoprotein synthesized by the liver which has an important role in regulating glucocorticoid availability. Both in humans and in rodents, 80-90% of the glucocorticoids in the bloodstream are found strongly bound to CBG, while about 10% are weakly and nonspecifically bound to albumin and only 5-10% are found in their free and active form. Since – according to the free hormone hypothesis – only the free fraction of the hormone presents biological activity, CBG stands as an important regulator of glucocorticoid activity (Hammond et al., 1980, Siiteri et al., 1982, Mendel, 1989).

1.3.1 Structure

The presence of a human plasma protein binding corticosteroids with high affinity ($5-8 \times 10^7 \text{ M}^{-1}$) and low capacity ($10^{-9} - 10^{-8} \text{ M}$) was discovered in 1956 independently by IE Bush and by WH Daughday. A couple of years later, a similar protein which was named transcortin was first described, and later turned out to be identical to the previously described CBG. CBG was purified in the end of the 1960s by several independent groups from human and rat blood samples. More recently, the gene coding for CBG, named SERPINA6 due to the similarity of its sequence with that from other serine protease inhibitors (serpins), has been cloned and characterized in more than 20 species according to ENSEMBL database (www.ensembl.org). Its existence in a wide variety of species together with a highly conserved structure (the degree of sequence similarity among species is 60-70%) supports the idea that CBG plays a crucial role in corticosteroid physiology (Breuner and Orchinik, 2002, Moisan, 2010, Murphy, 2010).

The primary structure and the molecular composition of CBG were first described by Hammond and colleagues in 1987 through the isolation of CBG cDNA from human liver and lungs. In the years that followed, the structure of the human CBG gene has been determined, as well as its chromosomic location. The CBG gene is composed of 5 exons, and in humans it locates on the long arm of chromosome 14, in the region q32.1, inside a 370-kb region containing 11 genes belonging to the clade A serpins superfamily. The serpins are a group of highly conserved proteins that are capable of inhibiting proteases, notably serine proteases as its acronym indicates. It has been postulated that members of the serpin superfamily have arisen by duplication of a common ancestral gene, but serpin genes are located on several different chromosomes, and their structural organization varies considerably. Still, it is noteworthy that the two serpins that exhibit the closest similarity with CBG in terms of gene organization and primary structure – α 1-antitrypsin (A1AT) and α 1-antichymotrypsin (α 1AC) – are located in the same cluster on chromosome 14. In fact, SERPINA6 probably derives from the same common ancestor as the other 10 genes from this cluster on human chromosome 14, which are partially regulated by a common *locus* control region that recruits liver-specific transcription factors (Hunt and Dayhoff, 1980, Hammond et al., 1987, Underhill and Hammond, 1989, Seralini et al., 1990a, Zhao et al., 2007, Moisan, 2010).

The molecular weight of CBG is 50-60 kDa (42.646 kDa for human CBG), and there is one cortisol binding site per molecule. It is a monomeric protein (with the possible exception of New World primates in which CBG may circulate as dimmers) consisting of approximately 42 000 amino acids, and the differences in molecular size among species are probably due to differences in glycosylation. The human CBG

sequence is thought to contain six consensus sites for N-glycosylation, two of which have been retained throughout evolution and seem thus to be functionally important (Lin et al., 2010, Murphy, 2010).

CBG, just like the other members of the serpin superfamily, presents a loop structure with 30 to 40 amino acids in the C-terminal extremity of the protein. This region is called reactive central loop (RCL) and is susceptible of cleavage by a specific protease. Studies have shown that once the glucocorticoid molecule binds to CBG's binding site, CBG adopts a stressed (S) conformation, fully exposing the RCL. The crystal structure of cortisol-bound rat CBG displays a typical serpin S conformation. It contains a 5-stranded β -sheet A (termed s1A, s2A, s3A, s5A and s6A), a 6-stranded β -sheet B (s1B-s6B), a 4-stranded β -sheet C (s1C-s4C), and 10 intervening α -helices (hA-hJ). The RCL in the CBG steroid complex is fully exposed from the β -sheet A, and this particular feature is shared by other serpins which also have known ligand-binding (Figure 11). Neutrophil elastase specifically cleaves CBG at its RCL, which is completely inserted into the central β -sheet A as a novel β -strand (s4A), resulting in a significant conformational change from the native S form ($K_d = 1.6 \cdot 10^8 \text{ M}^{-1}$) to a relaxed (R) form ($K_d = 1.4 \cdot 10^9 \text{ M}^{-1}$) of the protein. Cleaved CBG (374 to 408 amino acids according to the species) presents a ten-fold lower affinity for glucocorticoids, and cleavage triggers the release of about 80% of the glucocorticoids that were previously bound to CBG. This observation was mainly made in inflammation sites, where a proteolytic attack of the circulating CBG-glucocorticoid complexes takes place. This complex is locally cleaved, leading to a local increase of glucocorticoid concentration in the inflammatory site. This observation has led to the view of CBG as an important transporter of anti-inflammatory steroids to inflammation sites. Recently a new cleavage site in human CBG's RCL has been identified, offering the perspective that yet unknown proteases might exist, which are able to trigger the S-to-R transition in human CBG. Even more recently, chymotrypsin has been shown to be able to cleave of the RCL of CBG, resulting in undetectable levels of the intact protein, whereas total CBG levels remained unchanged. Another serpin, α 1-antichymotrypsin, is the natural inhibitor of chymotrypsin, and this could also limit the exposure of CBG to chymotrypsin cleavage since plasma concentration of α 1-antichymotrypsin rise in the acute inflammatory response. Yet, comprehending the physiological significance of these findings demands further studies (Pemberton et al., 1988, Hammond et al., 1990, Hammond et al., 1991, Potempa et al., 1994, Irving et al., 2000, Klieber et al., 2007, Lin et al., 2010, Gardill et al., 2012, Lewis and Elder, 2014).

1.3.2 Biosynthesis

The main site of CBG synthesis is the hepatocytes. CBG is secreted from hepatocytes as a 383-amino acid peptide after cleavage of a 22-amino acid signal peptide at the endoplasmic reticulum and circulates in the bloodstream at concentrations ranging from 30 to 52 pg/ml. *In vitro*, CBG can also be produced and secreted by hepatocytes in culture. Despite the liver being the main production site, CBG has also been detected in other tissues such as the spleen, kidney, lungs, ovaries and testicles of many species. Still, the amount of extra-hepatic CBG is very low and its significance is uncertain (Hammond et al., 1987, Torpy and Ho, 2007, Murphy, 2010).

The kidneys represent the main extra-hepatic site of CBG synthesis. In mice, kidney tubules are the main site of CBG synthesis until the 10th postnatal day. Nevertheless, this expression is transitory, reaching its peak at the third postnatal day

and progressively disappearing and becoming undetectable after the 6th postnatal week. Kidney CBG expression has also been detected in the fetus of other species, such as the sheep and the rabbit. Moreover, CBG expression has also been detected in the pancreas of mice fetus (Seralini et al., 1990b, Scrocchi et al., 1993a, Scrocchi et al., 1993b, Berdusco et al., 1995).

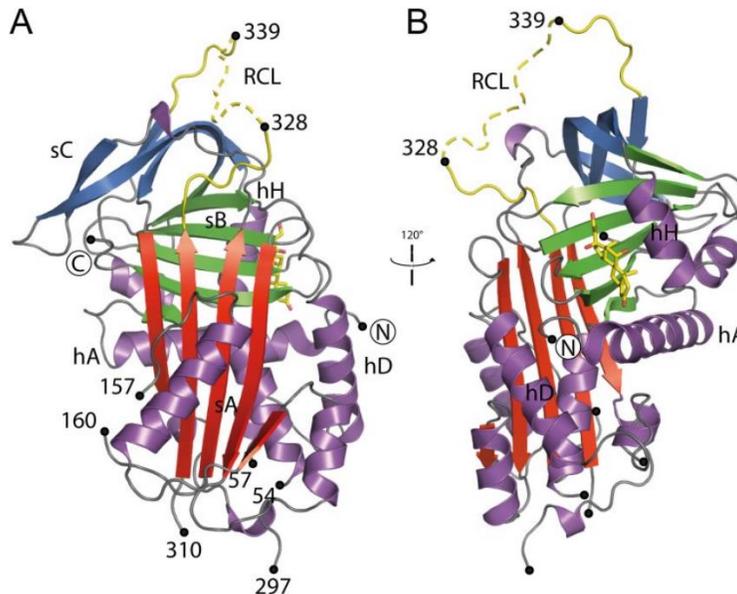


Figure 11 – Crystal structure of rat CBG. Ribbon representation of rat CBG viewed from two different angles (A, B). Secondary structure elements are named according to the common serpin nomenclature. The β -sheets A, B, and C are colored in red, green, and blue, respectively, while the α -helices are represented in purple. The trace of the residues of the RCL is modeled by a dashed line. The steroid is bound on top of sheet B and shown in yellow.

As for the brain, studies have detected the presence of CBG in magnocellular neurons in the rat hypothalamus, co-expressed with AVP and oxytocin. Furthermore, it has been observed through the axon projections of the lateral hypothalamus, median eminence and posterior pituitary and in a more widespread manner throughout the rat brain, including the lateral hypothalamus, preoptic region, bed nucleus of the stria terminalis, periaqueductal zone of the mesencephalon, cerebellum and the dorsal horn of the spinal cord. It has been suggested that the different locations of CBG in the rat central nervous system indicate it has various different functions, which should exceed that of a mere transporter for serum glucocorticoids. CBG is also expressed in many different regions and in different cell types in the mouse brain. Many neuronal systems, such as the hypothalamus, the amygdala, the PFC and the hippocampus are positive for CBG-immunostaining. Interestingly, these areas are implicated in an interconnected way in the central stress response, suggesting that CBG might have a local role in regulating glucocorticoid signaling in these areas during the stress response. Double immunostaining for CBG and for glial markers shows that a considerable portion of non-neuronal cells in the brain contains CBG, such as ependymal cells, tanycytes, epithelial cells and astrocytes, all known to be derived from astroglia. A quantitative analysis of brain and liver Cbg mRNA revealed that liver Cbg mRNA is about 200 times more abundant than pituitary or hippocampal Cbg mRNA. In hypothalamus, cortex and amygdala, the presence of Cbg mRNA appeared to be very low (Figure 12). These low

levels question the putative role of CBG as an efficient glucocorticoid buffer in brain. Nevertheless, brain CBG levels might be upregulated in some conditions such as after an intense stress. Also, the quantification of Cbg mRNA in large brain regions by quantitative PCR might underestimate CBG levels within particular cell nuclei. The exact role and relevance of brain CBG in regulation brain glucocorticoid levels is still to be clarified (Mopert et al., 2006, Jirikowski et al., 2007, Sivukhina et al., 2013).

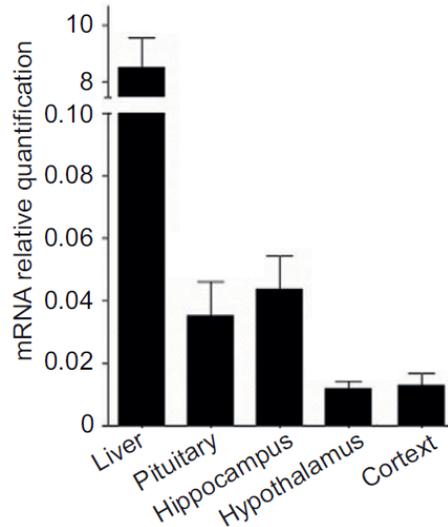


Figure 12 - Quantitative analysis of Cbg mRNA by real-time PCR. mRNA expression is shown as relative expression. N=4 in pituitary, hypothalamus and cortex, N=5 in liver and hippocampus.

Despite the inherent technical difficulties, the presence of CBG in the human brain has also been investigated. Similarly to rodents, these studies also revealed the presence of CBG in the hypothalamus, co-localized with AVP and oxytocin. Since CBG has been shown not to cross the blood-brain barrier, its presence in the hypothalamus – a brain region rich in MR and GR receptors – suggests that it might have an important role in the fine modulation of glucocorticoid accessibility in this region, being particularly important for the glucocorticoid negative feedback. Finally, human astrocytoma cell line has also been shown to synthesize and secrete CBG in the presence of glucocorticoids. Since CBG has been found in other astrocyte-derived cell types, it has been proposed that glia cells could actually be the source of intracerebral CBG (Pardridge et al., 1986, Mopert et al., 2006, Sivukhina et al., 2006, Pusch et al., 2009, Henley and Lightman, 2011).

1.3.3 Glycosylation

Glycosylation is a classic protein modification in eukaryotes. It adds a new level of structural complexity that can influence the biosynthesis, the secretion and the biological activity of the protein. A typical glycoprotein contains one or several chains of glycans bound to asparagine through N-linked glycosylation bounds. The glycosylation of CBG, as for every other protein, takes place in the endoplasmic reticulum through enzymes located in the luminal side of the membrane, originating a protein that is rich in mannose. Once bound to the protein, the oligosaccharides are promptly modified. Trimming is initiated in the endoplasmic reticulum (one mannose and three glucoses are

removed from most glycoproteins) and subsequent processing and modification of the oligosaccharide chain is carried out in the golgi apparatus (Avvakumov, 1995).

CBG in vertebrate species invariably contains 20-30% carbohydrate, and there are about 5 moles of oligosaccharides per mole of CBG isolated from human serum. The carbohydrates associated with CBG may have diverse biological functions, such as improving CBG survival in the circulation, influencing its interaction with plasma membranes and promoting the proper folding of newly synthesized CBG. Furthermore, some eukaryotes use different glycosylation patterns to direct some proteins towards target organs. The carbohydrate composition of the glycosylations varies between species and can change according to different physiological situations – such as pregnancy, fetal development or disease – to modify the protein's properties (Avvakumov et al., 1993, Berdusco et al., 1993, Avvakumov, 1995, Mitchell et al., 2004).

There are six consensus sites for N-glycosylation in the human CBG amino acid sequence. The role of these N-glycosylations is not clear, but it appears that a successful glycosylation is essential for an adequate folding of the protein, allowing the changes in conformation of CBG according to the glucocorticoid binding. It has been observed that if the first glycosylation step at the endoplasmic reticulum is blocked, the resulting CBG has its binding capacity disrupted. Moreover, the effect of mutations on carbohydrate chains is site dependent. In humans, while the absence in some glycosylation sites has little or no effect on the final protein activity (as in sites I, III, IV and VI), in others it can lead to a complete loss of the steroid-binding activity (as in site IV, one of the two glycosylation sites which is phylogenetically conserved), suggesting some sites are more important than others for the formation of the CBG steroid-binding site (Hammond et al., 1987, Ghose-Dastidar et al., 1991, Avvakumov et al., 1993, Avvakumov, 1995).

1.3.4 Clearance

The metabolic clearance of CBG can be defined as the amount of CBG molecules eliminated by the organism in a given unit of time. Circulating CBG is progressively degraded and the speed of its degradation depends on its glycosylation level and on the activity of neuraminidases. The speed of CBG clearance from plasma is particular of each species. For example, if both human CBG and rat CBG are injected in rats, the half-lives of each protein vary: human CBG will be eliminated five times slower than rat CBG. This difference in half-life is likely due to differences in the metabolism of these two molecules by the endogenous neuraminidases of the two species. In humans, the half-life of CBG is five days, which is higher than what has been reported for other species: 13 hours in the lamb, 14.5 hours in the rat, and around 13 hours in the rabbit (Brien, 1981, Hossner and Billiar, 1981, Seralini et al., 1989, Smith and Hammond, 1991, Berdusco et al., 1993).

Some variations in the plasmatic concentrations of CBG can be explained by changes in its clearance rate. An interesting example is the experiment carried out by Smith and Hammond throughout the development of rats. In newborn rats, plasma CBG levels are relatively low. These levels only attain normal adult values after the 6th postnatal week, despite the levels of liver CBG mRNA being already equivalent to adults' from the 3rd postnatal week. The authors have demonstrated that the origin of this gap is a higher clearance rate in the newborn rats. In fact, the half-life of CBG is of approximately 6.9 hours in the newborn rat, versus 14.5 hours in the adult rat. This difference in clearance rate is not due to differences in the protein's composition, since it

was found to be identical regardless of the age of the animal. Instead, a higher activity of serin proteases or an alternative glycosylation pattern for CBG in newborn rats have been proposed as a possible sources for this difference (Smith and Hammond, 1991).

1.3.5 Binding specificity

CBG binds glucocorticoid hormones (cortisol, corticosterone) with a very high affinity. The affinity constant for the binding of cortisol to human CBG is approximately $1 \times 10^9 \text{ M}^{-1}$ at 4°C and $5 \times 10^7 \text{ M}^{-1}$ at 37°C . The CBG of most other species are in the range of 10^7 - 10^9 M^{-1} at 4°C and are also lower at 37°C . Although the specificity and affinity of the steroid-binding site vary among species, maximal binding usually coincides with the biologically more important glucocorticoid – either cortisol or corticosterone – in a given species. The ratio of cortisol to corticosterone varies widely among species, and the relative binding affinities for the two steroids also differ widely. Thus, in both humans and pigs, CBG will preferentially bind cortisol, while in rodents and birds it will preferentially bind corticosterone. For humans, the binding distribution of endogenous steroids in the plasma of normal men and in pregnant and non-pregnant women are represented on Table 2. The affinity of CBG for synthetic steroids largely depends on each molecule. In humans, prednisolone binds to CBG with an affinity, comparable to that from cortisol, while dexamethasone does not bind to CBG. CBG has a really low affinity for estrogens. The displacement of cortisol from CBG was evaluated after incubation with several different steroids, and the results – which reflect a relative affinity between these steroids and CBG – are illustrated in Table 3. Pregnancy can alter the binding capacity of CBG, and it is possible that the high levels of progesterone and testosterone in the reproductive organs compete with glucocorticoids for CBG binding (Brien, 1981, Dunn et al., 1981, Pugeat et al., 1981, Siiteri et al., 1982, Murphy, 2010).

Table 2 – CBG-bound endogenous steroids in human plasma (adapted from (Dunn et al., 1981)).

Steroid	Men		Women					
	nM ^b	% ^c	Late pregnancy		Follicular		Luteal	
	nM ^b	% ^c						
Cortisol	400	90	740	95	400	90	400	90
Cortisone	72	38	110	5	54	39	54	38
Corticosterone	12	78	48	90	7.0	78	13	78
4-Androstene-3,17-dione	4.1	1.4	13	2.3	5.4	1.4	5.4	1.3
5-Pregnen-3 β -ol-20-one	2.2	0.4	15	0.4	2.2	0.2	4.4	0.2
17-Hydroxyprogesterone	1.8	41	14	66	1.8	42	5.9	41
Androsterone	1.5	0.5	1.5	1.4	1.5	0.5	1.5	0.5
Testosterone	1.3	3.4	4.7	4.7	1.3	2.3	1.3	2.2
Etiocholanolone	1.2	0.4	1.2	1.3	1.2	0.5	1.2	0.4
Aldosterone	0.4	21	5.8	41	0.2	22	0.5	21

^bTotal concentration.

^cPercentage bound to CBG.

Table 3 – Influence of various steroids *in vitro* on human CBG binding to cortisol (adapted from (Brien, 1981)).

Steroid	Cortisol displaced (%)^a
Aldosterone	6
Betamethasone	5
Dexamethasone	10
Oestradiol	11
Prednisone	11
Prednisolone	41
Progesterone	23
17-OH-Progesterone	46
Testosterone	25
Tetrahydrocortisol	4

^a Corticoid-poor plasma was overloaded with cortisol at 0.5 µg/ml together with an equal amount of other steroids. CBG bound cortisol was determined after 15 min of incubation. Fifty percent displacement indicates an equal ability to bind CBG as that from cortisol.

1.3.6 Binding dynamics

Although CBG is present in much lower concentrations in plasma than albumin, its high affinity and specificity for steroids enables them to play much more dynamic roles in determining the plasma concentrations of glucocorticoids. The dynamics of cortisol binding is crucial and can determine the level of free glucocorticoids at the tissue level. Yet, the levels of glucocorticoids can quickly vary since these hormones are secreted in a pulsatile manner, according to circadian and ultradian rhythms. CBG's RCL binding activity is thermosensitive, as it is also observed for other serpins. It has been suggested that this property would allow the modulation of the binding affinity to glucocorticoids in different physiological states (Mickelson et al., 1981, Zhou et al., 2008, Hammond, 2016).

Binding and release of glucocorticoid hormones normally occurs in a reversible equilibrium that ensures their appropriate tissue concentration. This equilibrium can be modulated by temperature, which affects CBG binding capacity. A study performed by SL Lightman's team has investigated the effects of the temperature (32°C, 37°C, 42°C) on the binding between CBG and cortisol. For that, fractions of free cortisol – reflecting the levels of CBG – were measured in human plasma treated with different doses of cortisol. Their findings show that at 37°C (physiological temperature) the fraction of free cortisol remains the same for cortisol doses between basal levels and 400 nM. From 600 nM on, the fraction of free cortisol increases according to the dose. This indicates that at physiological temperatures CBG becomes saturated between 400 nM and 600 nM. High temperatures (42°C) increase free cortisol fraction, suggesting that the CBG binding capacity decreased by the temperature increase. On the other hand, the binding between cortisol and CBG has been shown to increase as temperature decreases, as observed at 32°C. Albumin is practically unaffected by these temperatures or to the different cortisol concentrations providing its low affinity and low specificity. These observations suggest that CBG acts as a thermosensitive protein, which can deliver cortisol promptly and locally to specific tissues, as for example, during fever states or local inflammation. Interestingly, the binding affinity of glycosylated CBG to cortisol is

more sensitive to changes in temperature than that of unglycosylated CBG. In particular, the KD of glycosylated S-state CBG increases by over 4 times when the temperature increased from 37°C to 42°C, while the corresponding change for the unglycosylated protein is a mere two-fold (Burton and Westphal, 1972, Lewis et al., 2005, Zhou et al., 2008, Cameron et al., 2010, Henley and Lightman, 2011, Chan et al., 2013).

This balanced equilibrium between CBG and glucocorticoids can exceptionally tip to favor the release of glucocorticoids when in a proteolytic milieu, such as in sites of inflammation. Although CBG has lost its function as a protease inhibitor, the exposed RCL is susceptible to proteolytic cleavage by elastases and other proteases released by inflammatory cells. As previously mentioned, when CBG binds to glucocorticoids it adopts an S conformation, exposing the RCL. The cleavage of the RCL results in a profound S-to-R conformational change, typical from serpins. The cleaved loop will be fully incorporated in the main β -sheet of the molecule with an accompanying transition in the overall conformation of the molecule from an initial stressed S state to a more ordered relaxed R state. This S-to-R change in CBG results in a ten-fold decrease in its binding affinity for corticosteroids, with their consequent release. Recent crystal structures of hormone-complexed native CBG show that there is unlikely to be any direct contact between the cleaved reactive loop and the hormone binding site (Pemberton et al., 1988, Zhou et al., 2008).

1.3.7 Interaction with cellular membranes

As previously mentioned, CBG expression has already been detected extra-hepatically, notably in target tissues for glucocorticoid action, such as liver, kidney, pituitary, uterus, prostate, lung, brain and fat. It has been questioned whether the detected CBG would result from a *de novo* synthesis of CBG on these sites or if it was actually the result of the internalization of the plasmatic protein. Recently, CBG mRNA has been found to be produced locally within several tissues, despite in considerably smaller quantities than in the liver, implying local synthesis and not merely transport from blood (Sivukhina and Jirikowski, 2014, Meyer et al., 2016).

In humans, membrane-bound CBG has been observed in hepatic, prostate, endometrium and syncytiotrophoblast cells. In other primates, CBG has been detected in the membranes of kidney, liver, spleen, and uterus cells. The same observation has been made for membranes of kidney, spleen, liver and endometrium of rats, but this binding was only observed when CBG was in its free form, not bound to glucocorticoids. Interestingly, it had been previously found that the binding between CBG and cell membranes was not affected by the binding status of the molecule. Nevertheless, it is important to consider that the former study was performed at 4°C, while the most recent one was carried out at 37°C. This detail can be crucial in the interpretation of these results, since CBG is a thermosensitive protein. Although many of these results await confirmation, it appears that CBG does bind to some cell membranes with a KD of approximately 0.9 μ M at physiological temperatures. This binding requires calcium and/or magnesium ions and is negligible at 4 or 23°C (Strel'chyonok and Avvakumov, 1983, Hryb et al., 1986, Krupenko et al., 1991, Maitra et al., 1993, Murphy, 2010).

Despite the rising number of studies in this topic, the CBG receptor has not been characterized or cloned in humans, with only indirect *in vitro* evidence of its existence, and has only been partially characterized in rats. Moreover, the physiological significance of this binding is still unknown. Some alternatives have been proposed regarding the role of a CBG membrane receptor. The first one attributes an intra-cellular

function to CBG, and a membrane receptor would allow the entrance of CBG inside the cells. A second one sees this mechanism as a mean to transport the CBG-glucocorticoid complex inside the cells, allowing a better control of the distribution of glucocorticoids in several tissues, especially in those poorly-vascularized. Once inside the cell, CBG could release the active glucocorticoid. This would consist in an active transport in alternative to the classic diffusion through the membrane. Finally, CBG could also serve as a pro-hormone capable of activating a second messenger system. Free CBG (but not complexed CBG) would bind the cell membrane receptors and, once fixated, glucocorticoids would bind to this CBG-receptor complex. This would quickly increase adenylyl cyclase and cAMP levels and trigger a non-genomic action of glucocorticoids (Hryb et al., 1986, Rosner, 1990, Maitra et al., 1993, Hammond, 1995, Orchinik et al., 1997, Gagliardi et al., 2010, Meyer et al., 2016).

1.3.8 Variability of CBG levels

CBG binds mainly to glucocorticoids, and thus regulates the bioavailability and metabolic clearance of these hormones to their target tissues. The levels of plasma CBG are the result of their production and elimination rates, and every factor influencing either of these processes can potentially influence the plasma levels of this glycoprotein. Other factors may also influence CBG's activity, by modulating its binding affinity. All these processes may vary normally under physiological conditions, but some variations are associated with pathological states. Age, development, hormonal influences, stress and sex are some of the factors that can influence CBG levels and activity.

1.3.8.1 Variability among species

Plasma levels of CBG vary from 10 to 500 nM in most vertebrate species. They are exceptionally high in the green iguana (3000 nM) and squirrel (2500 nM), and they are low in New World monkeys - only 1-10% of those in other primates. The affinity is also lower in New World monkeys, resulting in a much higher fraction of unbound plasma cortisol. In the squirrel monkey, the only mammal known that appears to lack CBG, half the cortisol is albumin-bound and half is free (Dunn et al., 1981). A study that compared the CBG binding of cortisol measured by equilibrium dialysis in seven species revealed that CBG maximal capacity (Bmax) was approximately three-times more than the plasma cortisol levels for most species, with cow, dog and ewe exhibiting the lowest and cynomolgus monkey exhibiting the highest values. The free (6 to 14%), CBG-bound (67 to 87%), and albumin-bound (7 to 19%) cortisol fractions are also similar within species. Also in most species, as much as 68% of plasma CBG was found to remain free of cortisol under physiologic conditions (Gayrard et al., 1996).

1.3.8.2 Physiological variability

The concentration of CBG in the lymph is slightly lower than that in the blood, whereas concentrations in the synovial fluid and pleural effusion are higher and those in the spinal fluid and amniotic fluid are very low. CBG passes into milk - in human milk the concentration has been reported to be 1-15% that of plasma and in rat milk approximately 25% that of plasma. Because there is little or no CBG or albumin in saliva, salivary cortisol is often used as an index of free cortisol. However, the salivary gland contains HSD11B2, which converts approximately half the cortisol to cortisone, so

salivary values are approximately one-half those of free cortisol in plasma (Murphy, 2010).

CBG production is apparently independently regulated in mother and fetus. Whereas fetal corticosteroid levels rise in many species during pregnancy, CBG levels vary considerably with species; in many, the levels fall rapidly postpartum, and in most species the early postpartum fetal CBG levels are lower than adult values. Compared to the adult (700 nM), CBG levels are lower in the human fetus and neonate: approximately 250 nM in late gestation and immediately postpartum. These levels rise to approximately 500 nM by 30 days postpartum, and reach adult levels by several months of age.

The level of CBG in the fetal rat rises from approximately 1.3 nM at 16 days gestation to a maximum of 2 nM at 18 days. Between gestation day 18 and postnatal day 3 these levels fall and reach almost undetectable values, and start rising again at postnatal day 10 until reaching adult values by 6 weeks. Despite the low neonatal values of CBG in rats, hippocampal glucocorticoid receptor occupancy was noted to be similar at all ages, both under basal conditions and following stress. The decline in fetal CBG before birth has been suggested to promote an increase in free corticosterone levels necessary for lung maturation (Brien, 1981, Viau et al., 1996, Murphy, 2010).

CBG levels are higher in younger adults than in older adults. Furthermore, CBG levels do not differ greatly among young men and women, but levels are higher in older women compared to older men (Kudielka et al., 2004). Slight changes in CBG concentrations have been reported to occur with diet, with a high carbohydrate diet resulting in a fall in CBG concentrations, and low fat feeding resulting in higher plasma CBG concentrations and lower free cortisol values (Garrel, 1996, Murphy, 2010).

1.3.8.3 Sexual dimorphism

CBG binding capacity of men and women is similar (600-700 nM). It is questionable if this lack of difference is real or an artifact of the many uncontrollable variables from human studies which can potentially modulate CBG levels. Still, the few studies in humans which have reported sex differences in CBG levels always point out women as having higher CBG levels than men. Similarly, the presence of variations in CBG levels during the estrous cycle in women is not clear. Some studies have found higher levels during the luteal phase when compared to the pre-follicular phase, while others only observe some small non-significant variations. In rats, mice and guinea pigs, female CBG levels are approximately twice those of males, a difference that is abolished by gonadectomy (Angeli et al., 1978, Moore et al., 1978, Brien, 1981, Faict et al., 1986, Jansson et al., 1989, Jones et al., 1998, Lewis et al., 2004, Fernandez-Real et al., 2005, Kudielka and Kirschbaum, 2005, Murphy, 2010).

Gonadal steroids seem to be highly enrolled in these differences. Estrogens positively regulate CBG plasma levels, through a mechanism requiring the presence of estrogen receptor alpha (ER α) (Wiegratz et al., 2003, Nader et al., 2006, White et al., 2006, Qureshi et al., 2007). Females taking oral contraceptives were shown to have their CBG levels increased accordingly to their contraceptives' doses (Doe et al., 1967). These results suggest that estrogens could be interesting markers of the circulating CBG levels. Nevertheless, although this hypothesis of estrogen-induced synthesis of CBG is attractive, it is important to notice that other studies found no evidence of cyclical changes in cortisol-binding capacity in either normal women or women taking oral contraceptives (Durber et al., 1976, Moore et al., 1978, Brien, 1981).

Pregnancy

Elevated levels of CBG, up to 30-fold, have been observed to occur during pregnancy in mammals with a hemochorial type of placentation (rodents, cats, dogs, moles, shrews, manatees, and most primates) but not in those with other types of placentation, and the biological significance of this difference is not known. The increase in CBG is minimal in pregnant rats, while up to three- to four-fold in the guinea pig (Murphy, 2010).

In human pregnancy, CBG rises to levels more than twice of those from non-pregnant women, particularly in the last trimester, but no changes in affinity have been described (De Moor et al., 1966). Doe and colleagues measured CBG in women from early pregnancy to term and found that the mean level increased from a non-pregnant value of 777 nM to 1720 nM at 6 months, after which the level remained relatively constant until term (Doe et al., 1964). These changes in CBG were accompanied by the rise in the levels of cortisol, ACTH and CRH also observed during this period (Batra and Grundsell, 1978, McLean et al., 1995, de Weerth and Buitelaar, 2005, Ho et al., 2007). After delivery CBG values tend to fall to normal non-pregnant levels, what seems to favor the increase in free cortisol levels. Another study found no significant changes in CBG levels between 3 weeks ante-partum and 5 days post-partum (Batra and Grundsell, 1978). Since exogenous estrogens are a very potent stimulus to CBG synthesis it is generally held that the increase in CBG seen in pregnancy is a result of endogenous estrogen production. Interestingly, the intervillous blood levels of CBG from placentas of women undergoing cesarean sections were approximately 50% lower than those undergoing spontaneous delivery. It is unlikely that CBG has a role in regulating free progesterone levels within the placenta or the adjacent maternal uterine cells since concentrations of progesterone in the intervillous blood are always approximately 20 times greater than those of CBG. Moreover, a variant of CBG containing slightly altered glycosylation pattern (possessing only triantennary oligosaccharides) occurs in pregnant women, accounting for 4-14% of total plasma CBG at term, but its function is not yet clear (Mitchell et al., 2004). The role of this increase in CBG levels throughout pregnancy would be, thus, to protect the fetus against an overexposure to free glucocorticoids, besides constitute a greater glucocorticoid reservoir than in “normal” conditions, promoting the anti-inflammatory, immunitary or developmental function of these hormones (Brien, 1981, Lei et al., 2015)

1.3.8.4 Circadian variability

Since the diurnal variation in total plasma cortisol concentration has been long known it was of obvious interest to discover whether any similar variation occurred in CBG levels. Circadian variations in CBG levels have been described in several species, such as birds (Lynn et al., 2003), rats (Ottenweller et al., 1979, Meaney et al., 1992), mice (Ottenweller et al., 1979) and humans (Doe et al., 1964).

In humans, while some authors find no changes in CBG levels throughout the day, others find drops in CBG levels during the nocturnal period. Doe et al. noted that a slight drop of about 10% occurred in males at 03.00 h as compared with 09.00 h, whereas no change was evident at 15.00 h or 21.00 h (Doe et al., 1964). Subsequent work carried out by Angeli et al. has reported the existence of a unimodal daily rhythm in CBG for both sexes. In this study, CBG levels peaked in the early afternoon, with mean binding capacity peaks of 590 nM at 16.00 h, while values of 348 nM ml and 290 nM

were recorded at midnight and at 04.00 h respectively. The authors have also found a phase shift of approximately 4 hours between the rhythmicity of the cortisol binding capacity and that of the total cortisol (Angeli et al., 1978).

Accordingly to the results obtained in human studies, the levels of CBG in rodents also peak during their active phase and decrease during their resting phase, reflecting the circadian variations of glucocorticoids. Hsu and Kuhn found a diurnal variation in CBG levels, with the highest levels of binding protein at the beginning of the dark period. CBG levels then decreased until a nadir was reached shortly after the beginning of the light period (Hsu and Kuhn, 1988). These findings have been reproduced by several groups in rats and mice from both sexes (D'Agostino et al., 1982, Meaney et al., 1992). It is important to notice that these circadian variations in CBG levels are much more subtle than that from glucocorticoids (a 1.4- to 1.5-fold increase in CBG levels during the active phase, while glucocorticoids may present an increase of 13-fold), which might explain why the circadian variation of CBG is less consistently found than the circadian pattern of glucocorticoid hormones. The fluctuation in CBG levels seems to be dependent of the circadian rhythms of glucocorticoid secretion, since studies have shown that adrenalectomy is able to abolish these oscillations. Furthermore, replacing corticosterone in adrenalectomized rats in a continuous manner increases CBG levels but does not recover its circadian pulsatility (Hsu and Kuhn, 1988).

1.3.8.5 Changes in response to stress

Acute stress responses classically trigger an increase in glucocorticoids due to HPA axis activation. Most studies carried out in both humans and animal models evaluate the effects of stress by measuring total corticosterone concentrations, despite measuring the free fraction stands as a more accurate alternative reflecting the biologically active fraction of these hormones. Some studies that have measured CBG levels indicate that it can vary after stress, and thus highlight the importance of this observation.

Acute severe stress in rats (such as restraint for 1 hour or tail shocks over 2 hours) causes a fall in CBG levels, which is apparent by 6 hours, maximal at 24 hours, and persists for 24 to 48 hours. Interestingly, despite the low values of CBG in the neonatal rat, when submitted to stress (restraint maternal separation stress) their levels of ACTH and of free corticosterone increase, and this increase persists for 48 hours. Such increases are associated with increased glucocorticoid receptor occupancy, similar to that observed in stressed adults. In rats in which an inflammatory process (endogenous stressor) was induced by a subcutaneous injection of turpentine, CBG levels were shown to decrease by two-thirds, whereas the corticosterone levels attain very high levels (Savu et al., 1985). Although the decrease CBG levels could have been caused by the high corticosteroid levels, this decrease was still present after adrenalectomy, suggesting that changes in glucocorticoid concentration are not necessary for the changes in CBG levels (Faict et al., 1983).

A study using pigs has found a negative relationship between the levels of glucocorticoids and those of CBG after an acute stress. CBG, free and total corticosterone were measured 1 hour after systemic ACTH administration, physiologically mimicking the effects of a stressor. The ACTH administration was found to increase free and total corticosterone levels, but to decrease CBG levels (Adcock et al., 2007). Horses submitted to social stress (horses new to a herd were confined in a

small yard with dominant resident horses) present a decrease in CBG binding capacity. This decrease in CBG, seen after 3 to 4 days and further decreasing over the 2-week period, was accompanied by an increase in free cortisol, so that the total cortisol concentrations remained unaltered (Alexander and Irvine, 1998).

Among the few studies that have been carried out in humans to measure CBG levels in stress conditions, either this same pattern of negative association between CBG and glucocorticoid/ACTH levels or no alterations on CBG are found. A study has shown that increases in cortisol and ACTH following a psychosocial stress can be modulated by CBG levels. In women, CBG levels were negatively associated with ACTH and salivary (free) cortisol following stress. In men, positive associations were observed between CBG and ACTH and total cortisol levels following stress, while no association between CBG and salivary cortisol levels were observed. Furthermore, high baseline CBG levels were associated with higher peak levels of total cortisol in male individuals with identical pre-stress levels (Kumsta et al., 2007). These findings corroborate the role of CBG as an important regulatory element of HPA axis response patterns, which should be taken into account as a potential modifier of ACTH and cortisol responses to stress.

1.3.8.6 CBG alterations during disease

Obesity

A genetic mapping analysis study was carried out by Osova and colleagues to reveal genes influencing cortisol genetic variability and its relationships with obesity. In a subset of the Meishan x Large White pig intercross progeny, the authors have identified a strong QTL on chromosome 7 associated with basal and post-stress cortisol levels, explaining 20% of the variance in the F2 population. They have then demonstrated that the gene coding for CBG maps on pig chromosome 7, very close to the peak of the cortisol-associated QTL previously detected, and observed a highly significant genetic linkage between CBG plasma binding capacity values and the molecular markers flanking this QTL. Back to the two parental strains, CBG capacity was three times higher in Meishan than in Large White pigs, and a 7-fold difference was found in Cbg mRNA expression between the two breeds. All this has led the authors to suggest the Cbg gene as a key regulator of cortisol levels and obesity susceptibility (Desautels et al., 2002, Osova et al., 2004).

Grasa and colleagues have evaluated corticosterone binding capacity in visceral (VAT) and subcutaneous white adipose (ScAT) tissue of normal and obese Zucker rats. They found that, unlike organs like liver and kidney, perfusion of rats with saline did not affect corticosterone binding in white adipose tissue. Furthermore, they did not detect corticosterone binding activity in cytosol extracts from isolated cells; however, CBG was seen through immunohistochemistry in a thin layer surrounding the cells, near to the plasma membrane. It was suggested by the authors that the CBG layer surrounding the cells could act as a protective barrier which would limit the access of glucocorticoids to the adipocytes, protecting fat cells from the excessive exposure to glucocorticoids. The authors also found that CBG levels are approximately 2-fold reduced in the obese rats, both in plasma and in white adipose tissue, what could make these animals more vulnerable to the effects of glucocorticoids. A preadipocyte culture from an individual with complete CBG deficiency showed increased proliferation and enhanced differentiation when compared with normal preadipocytes. These characteristics may promote adipose tissue deposition by increasing the number of preadipocytes and their

conversion to mature adipocytes. The mechanism of hyperproliferation and accentuated differentiation in preadipocytes from genetically CBG-deficient individuals is unclear, as CBG gene expression was not detected in cultured human preadipocytes and measures of GR binding and HSD11 activity were normal (del Mar Grasa et al., 2001, Joyner et al., 2003).

CBG levels have been suggested to be an interesting indicator for both insulin resistance and low grade inflammation, since in humans they're negatively associated with indexes of insulin resistance such as BMI, waist to hip ratio, and fasting insulin resistance index, and with inflammatory parameters such as TNF receptors and interleukin 6 (IL-6) concentrations. Furthermore, in the population studied, the authors found that women had higher CBG levels than men, who also had a weaker correlation of CBG with inflammatory indexes than women. Whether the lower CBG level is genetic by nature or directly associated to increased insulin and/or inflammation is not yet clear. Nevertheless, because CBG has been shown to be expressed by the adipose tissue, decreased CBG could create locally increased cortisol disposal, with no change in circulating cortisol, and facilitate fat accumulation, insulin resistance, and type 2 diabetes. Interestingly, a decrease in CBG levels associated with an increase of free cortisol has been observed after massive weight loss in morbidly obese subjects. The cause of this decrease is not clear, but it might be an adaptive phenomenon relating to environmental changes. Changes in CBG and free cortisol levels might also be related to the improvement of the low-grade inflammatory status in these patients, as suggested by the decrease of c-reactive protein (CRP) (Fernandez-Real et al., 2002, Manco et al., 2007).

Liver disease

The effect of hepatocellular hypoproteinemia on CBG has been the subject of a number of studies. Murphy and Pattee found a considerable decrease in CBG in cirrhotic patients compared with healthy subjects even though the total plasma cortisol was only slightly decreased (Murphy and Pattee, 1963). McCann and Fulton have found similar results (McCann and Fulton, 1975). Quite contradictory results (increased total cortisol and normal CBG) were found by Keane and colleagues, who suggested that the discrepancy between their findings and those of previous authors could be accounted for by the fact that the patients which they studied were all at an advanced stage of hepatic disease and might therefore be in a more stressed condition than the subjects in other studies (Keane et al., 1969). Similarly, De Moor and colleagues studied ten patients with hepatic disease, five of which presented subnormal levels of CBG, two were within the normal range and three had elevated levels (De Moor et al., 1966).

Protein losing conditions

Sandberg & Slaunwhite found decreased CBG levels in three children with the nephrotic syndrome and hypoalbuminaemia (Sandberg and Slaunwhite, 1959). In a series of patients with hypoalbuminaemia of different aetiologies, Doe and colleagues also found that CBG levels were generally low. Still, these authors point out that hypoalbuminemia is not consistently associated with a low CBG concentration (Doe et al., 1964). Indeed, a study from De Moor and colleagues has found that patients with uraemia almost all had CBG levels within the normal range (De Moor et al., 1962). Other hypoproteinaemic conditions in which decreased CBG levels have been found include

multiple myeloma, amyloid and bowel disease and exudative enteropathy (De Moor et al., 1966).

Thyroid disease

It is well established that thyroid disease can influence corticosteroid metabolism, hyperthyroidism being associated with increased catabolism of corticosteroids while the reverse occurs in hypothyroidism (Pittman et al., 1971, Linquette et al., 1976). It is not clear whether CBG has any role in this phenomenon. Farese and Plager studied the partition of cortisol between plasma proteins and red blood cells and concluded that increased binding to plasma proteins occurred in hypothyroidism and decreased binding in hyperthyroidism (Farese and Plager, 1962). Beisel and colleagues, on the other hand, were unable to show any alteration in protein binding of cortisol in thyroid disease although they did confirm an increased metabolic clearance rate for cortisol in hyperthyroidism and a decreased rate in hypothyroidism (Beisel et al., 1964). Similarly, Doe and colleagues directly measured CBG levels in hypothyroidism and hyperthyroidism and found no change in either condition (Doe et al., 1964). Brien found no alteration from normal in hypothyroidism and hyperthyroidism, while Racadot and colleagues found normal values of CBG in hypothyroidism but decreased values in hyperthyroidism (Brien, 1976, Racadot et al., 1976).

Adrenal disease

Corticosteroid binding globulin levels have been measured in a number of studies in patients with both hyper- and hypo-adrenalism. Doe and colleagues found that patients with Cushing's syndrome due to bilateral adrenal hyperplasia had normal levels of CBG (Doe et al., 1964). Osorio and Schats found that the mean levels of CBG measured as binding capacity was slightly elevated in nine patients with Cushing's syndrome, but De Moor and colleagues found substantially decreased values in four out of eight patients with Cushing's syndrome even after surgical correction of their hypercortisolism (Osorio and Schats, 1964, De Moor et al., 1966). This finding of subnormal values in Cushing's syndrome was confirmed in a study by Racadot and colleagues, but not in another by Murray (Murray, 1967, Racadot et al., 1972). Thus there is evidence that at least in some cases of Cushing's syndrome lower levels of CBG may be encountered but the mechanism responsible for this change remains obscure. The other question to be considered is whether elevated levels of cortisol can, in the long term, inhibit CBG synthesis. In contrast, all reported studies of patients with Addison's disease indicate that normal CBG levels are found in this condition.

Surgical procedures

Murray has investigated patients in stressful situations (post-operative states, infections and burns) but found no evidence of altered CBG values despite raised cortisol levels. Curiously, he found some evidence to suggest that just after death there was a decrease in the cortisol binding capacity of plasma (Murray, 1967). In a series of investigations by Hamanaka and colleagues, Uozumi and colleagues and Kehlet and colleagues on patients undergoing surgery, these authors found decreased levels of cortisol binding activity in plasma. While decreased plasma protein concentration was probably an important factor explaining these findings, particularly in the patients with

extra-corporeal circulation, it could not account for all of the loss of cortisol binding activity. Possible explanations for this phenomenon include partial saturation of CBG with steroids other than cortisol, alteration in the rate of CBG synthesis or degradation, dilution of plasma with electrolyte solution during the procedure, displacement of cortisol from CBG by anesthetics, or redistribution of CBG between intravascular and extravascular compartments during surgery (Hamanaka et al., 1970, Uozumi et al., 1972, Kehlet et al., 1974). Furthermore, patients with septic shock or subjected to multiple traumatic injuries have markedly low CBG levels in the early phase, with levels gradually increasing to normal within a week. In contrast, high levels of free cortisol are observed in the early phase, decreasing toward normal during the second phase. This decline in CBG during extreme physical stress may serve to exaggerate the free cortisol response (Gagliardi et al., 2010).

1.3.8.7 Genetic variants and polymorphisms

1.3.8.7.1 In animal models

Smith and Hammond have identified a lower affinity CBG variant in the BioBreeding rat. In fact, the affinity of CBG for corticosterone in this strain is 50% lower than that from Wistar rats. This variation is caused by a punctual mutation on residue 276, which leads to a methionine → isoleucine substitution. Another study has found a mutation leading to a similar phenotype in mice, more precisely in the strain RIIS/J. A lysine → glutamate substitution in the residue 201 leads to a higher dissociation constant in this strain. In both cases, the variation in affinity only does not fully explain the phenotype observed in these models. The BioBreeding rat is a model of auto-immune diabetes, and no study has ever explained how the decrease in affinity generated by this mutation could lead to this pathology (Smith and Hammond, 1991). Similarly, RIIS/J mice display high mortality rates at birth, and despite the importance of glucocorticoids in the development and maturation of several organs is well known, no direct link between this mutation and death at birth has been demonstrated (Orava et al., 1994).

1.3.8.7.2 In humans

The production of CBG by hepatocytes involves the removal of a secretion signal peptide and the release of a mature protein of 383 residues, which includes a single high-affinity steroid binding site, N-glycosylation sites, and a RCL that acts as a protease bait domain. CBG mutations are extremely rare, but still several different CBG mutants have been identified to influence these properties, which can result in altered production and/or function of the protein. Some of these mutations are associated with a variety of clinical conditions, including chronic pain, fatigue, depression, hypotension, and excess body weight. Nevertheless, the true clinical significance can be confounded since measurement of plasma CBG is not conducted routinely in patients, being usually part of a diagnosis investigation (Torpy et al., 2004, Gagliardi et al., 2010).

For instance, reduced cortisol-binding affinity was observed in CBG Leuven (L93H) and CBG Lyon (D367N) mutations, whereas a complete loss of steroid binding was reported for CBG G237V and CBG Athens (W371S). A decreased capacity for cortisol was reported for CBG E102G. In addition, single nucleotide deletions in codons for residues within the signal polypeptide can cause premature translation termination

and a complete loss of CBG production. Although these mutations occur rarely in general populations, CBG A51V has been found in approximately 1:35 Han Chinese, and it has a secretion defect that causes reduced plasma CBG levels. By contrast, the common variant CBG A224S, as well as the rare CBG R64Q and CBG R64W mutations, do not appear to alter CBG production or function (Simard et al., 2015).

CBG Leuven

This CBG variant was first identified in three unrelated individuals from a population survey of 500 Caucasians. Their CBG levels were normal, but the protein displayed reduced affinity for cortisol. Later, the mutation for Leuven was identified in a septic patient, who had normal CBG levels but reduced cortisol binding affinity, and was subsequently identified in a Polish kindred from that population survey, comprising five heterozygotes and two homozygotes. Familial studies showed that this variant was inherited as an autosomal codominant trait. Sequence analysis of the CBG gene identified a point mutation in exon 2 (433T→A), resulting in a leucine to histidine substitution at amino acid 93. Binding studies from homozygotes showed that the Leuven mutation was sufficient to produce a 3-fold reduction in CBG:cortisol binding affinity. The phenotype associated with this variant was not closely examined. Hypertension has been noted in a few individuals but is not a consistent finding. Thus, the clinical implications of the CBG Leuven mutation remain uncertain (Van Baelen et al., 1982, Smith et al., 1992, Van Baelen et al., 1993, Gagliardi et al., 2010).

CBG Lyon

CBG Lyon mutation has been detected in three kindreds and one isolated patient (Gagliardi et al., 2010). It was first described in a 43-year-old female of northwest African origin who presented with chronic asthenia, depression and hypotension, suggesting adrenal insufficiency. She had very low total plasma cortisol levels and CBG levels, but normal plasma ACTH and urine free cortisol levels. The percentage of free cortisol was increased three-fold, but the free cortisol concentration was at the lower limit of the normal range, suggesting abnormal CBG-binding activity, which was subsequently confirmed. She was found to be homozygous for the CBG Lyon mutation, and had four healthy Lyon heterozygote children (apart from obesity in the eldest). Her kids had a 20% reduction in CBG levels – which could be due to reduced synthesis, increased degradation or impaired antibody binding to CBG – and a 40% reduction in total cortisol levels due to low cortisol binding affinity. The Lyon mutation consists in a point mutation in exon 5, 1254G→A, resulting in an aspartic acid to asparagine substitution at residue 367 (Asp367Asn). *In vitro* studies of the cortisol binding affinity of CBG showed that this mutation leads to a four-fold reduction compared with normal CBG (Emptoz-Bonneton et al., 2000). More recently, a *de novo* Lyon mutation has been reported in a young male with severe muscle fatigue occurring predominantly after stressful events. CBG deficiency was suspected after exaggerated free cortisol increases, but normal serum cortisol concentrations, were detected during the Trier Social Stress Test. The fatigue symptom in this patient presenting a *de novo* Lyon mutation corroborates the reports of fatigue in others with CBG Lyon mutation. The particular increase in fatigue after stressful events reported for this patient suggests endocrine, environmental and perhaps other factors may interact with the genotype,

producing phenotypic heterogeneity amongst individuals with the same genetic variant (Buss et al., 2007).

CBG null

Screening of plasma from more than 10,000 individuals submitted routinely to a biochemistry laboratory did not identify a single individual with absent CBG, suggesting that complete CBG deficiency was either very rare or lethal *in utero*. In 2001, CBG null, a novel, complete loss-of-function mutation, was described in a 39-member Italian-Australian kindred, originating from Calabria, Italy. The proband, a 48-year-old woman, presented with hypotension and fatigue, suggesting possible hypoadrenalism. An elevated plasma free cortisol fraction suggested CBG deficiency, and plasma CBG was undetectable. She was homozygous for a novel point mutation, a 121 G→A base substitution in exon 2, resulting in a premature stop codon at residue-12 of the pro-CBG molecule. CBG gene sequencing of the kindred identified two other null homozygotes, 19 null heterozygotes, and surprisingly, three Lyon heterozygotes and two compound (Lyon/null) heterozygotes. Plasma CBG levels were measured in 32 family members: CBG was undetectable in the null homozygotes, total plasma cortisol levels were very low and free cortisol was increased. There was a 50% reduction in CBG levels in all null heterozygotes, except for one woman taking the oral contraceptive, where CBG levels were normal. Null heterozygotes also had low total, but normal free, plasma cortisol (Rosner et al., 1973, Torpy et al., 2001, Gagliardi et al., 2010).

Hypotension had previously been identified in 8 of 19 affected adult kindred members. Systolic and diastolic blood pressures were significantly lower in CBG null mutation subjects than expected based on population norms. Similarly to other CBG mutations, persistent unexplained fatigue was frequent amongst carriers of CBG null, and a few null mutation carriers also reported chronic pain. Nevertheless, there was no correlation between plasma CBG level and fatigue score, or between blood pressure and fatigue score. These clinical observations of fatigue, hypotension and pain are intriguing because patients with idiopathic fatigue syndromes, as a group, have lower plasma cortisol levels than healthy controls and abnormal blood pressure regulation and hypotension have been observed in chronic fatigue syndrome (Demitrack et al., 1991, Clauw and Chrousos, 1997, Calkins and Rowe, 1998). Furthermore, pain may be associated with chronic fatigue syndrome and reduced neuroendocrine function has been reported in idiopathic pain syndromes. Other less consistent clinical features found among this kindred include obesity, structural cardiac disease and either focal or diffuse thyroid enlargement. Despite the functions of CBG-progesterone during pregnancy, two CBG null females in the kindred – completely devoid of any CBG – have had a total of seven children and had seemingly normal fertility, gestation and pregnancy outcomes. All seven offspring were female, which can indicate an unexplained, selective survival advantage of female embryos (Gagliardi et al., 2010).

A limitation of CBG null genotype-phenotype correlations is that only one kindred was ever described, thus a limited number of clinical observations and of any given genotype that can be made from this family. Nevertheless, this unique report suggests CBG null is an extremely rare variant and contrary to previous data, complete CBG deficiency exists and is not a lethal defect. A study of CBG mutations in the Calabrian community, from which the null kindred originated, has identified a high prevalence of null and Lyon (3.6%) CBG mutations and the presence of either mutation conferred a propensity to chronic pain. Although precise mechanisms are unknown, the association

between CBG loss-of-function mutations and fatigue–pain syndromes suggest that CBG is important in the transport of cortisol to brainstem, activating and anti-nociceptive central nervous system pathways. Hypotension, a key phenotypic feature of the CBG loss-of-function mutations, suggests a role for CBG-cortisol in the central sympathetic or peripheral vascular regulation of blood pressure. Fatigue and hypotension also occur in states of cortisol deficiency, suggesting that CBG loss-of-function mutations may produce a state of “relative cortisol deficiency”, where although free cortisol levels are not different, cortisol function is partly inhibited in the absence of CBG (Rosner et al., 1973, Gagliardi et al., 2010, Cizza et al., 2011).

CBG Santiago

A 9 years old boy of Spanish descent in Santiago, Chile, was referred for long-standing relatively short stature. The patient’s mother also reported that he became excessively tired after playing sport. General examination was normal with no dysmorphic features. Plasma total cortisol levels were reduced, but ACTH levels were normal. DNA sequencing for the SERPINA6 gene revealed a single base deletion, c.13delC, within exon 2. This deletion produces a frame shift at position 5 in the coding sequence and a premature stop codon. As exon 2 encodes the signal peptide of the CBG molecule, no mature CBG is expected. The proband, his sisters, and his father were all heterozygous for this mutation, and their plasma CBG levels were reduced by approximately 50%. The association with fatigue and idiopathic pain is again noted, despite only being related by the proband and not by his father or sisters. This varied symptom expression may have been influenced by a polymorphic CBG allele (c.735G→T) that was also present within the family (Torpy et al., 2012).

Other mutations

A 26-year-old woman who presented symptoms of hypotension and fatigue, associated with extremely low levels of total cortisol was identified as a carrier of a mutation of exon 3 of the SERPINA6 gene. This mutation (CBG G237V) led to an amino acid substitution on the residue 237. This mutation causes a slight change in the beta-sheets disposition and leads to no measurable cortisol-binding activity. The mother of the referred woman is heterozygous for this mutation, while two of her kids are homozygous. CBG levels for homozygous individuals, as well as total cortisol levels in either baseline or stimulated conditions, are below that from normal individuals. On the other hand, free cortisol levels seem to be normal, and pulsatility has been shown to be increased (Perogamvros et al., 2010). A new CBG variant, CBG Athens (CBG W371S), lacks cortisol-binding activity. It has been identified in a woman of Greek origin who was already a carrier of the cortisol-binding deficient CBG Lyon variant. The CBG W371S variant is the second CBG variant identified with no measurable cortisol-binding activity, the first one being CBG G237V (Hill et al., 2012).

In Nutrineuro lab, the association between a Cbg gene polymorphism and obesity and its metabolic markers has been investigated in a human population. The activity and reactivity of the HPA axis, as well as metabolic parameters and the obesity levels were characterized in a population of 44 obese pre-menopausal women. In parallel, a genetic analysis of microsatellite markers in Cbg gene intron 1 was performed. Three different genotypes were detected based on the sizes of the amplified markers: 86/86, 86/90 and 90/90, with the last one being extremely rare (1:44). No significant difference was found

for obesity, metabolic, and HPA axis activity parameters between the genotypes 86/86 and 86/90. Looking for differences in correlations between HPA axis activity parameters and obesity or metabolic parameters between the two genotypes, genotype 86/90 showed a strong correlation between salivary cortisol after dexamethasone suppression test and waist-to-hip ratio, whereas this correlation was weaker for genotype 86/86. These results suggest that polymorphisms in the Cbg gene can modulate the influence of the HPA axis on the distribution of fat mass in obese pre-menopausal women (Barat et al., 2005). In a following study, polymorphisms of this same molecular marker were investigated in a group of 295 men whose body mass index (BMI) was between 19 and 55kg/m². The frequency of CBG allele 90 was markedly increased among men with morbid obesity (BMI > 40) compared to the rest of the population (30 versus 18%, p=0.02), and this allele was associated with decreased CBG levels. CBG genotype was also associated with BMI and waist circumference in this male population (Richard et al., 2009).

A more recent study has characterized naturally occurring CBG mutants encoded by 32 recently reported nonsynonymous human SERPINA6 single-nucleotide polymorphisms. This has led to the identification of eight novel mutations that alter cortisol-binding affinity, cortisol binding capacity, production/secretion, sensitivity to proteolytic cleavage of the RCL, or recognition by monoclonal antibodies used for ELISAs. Most of these variants consist in substitutions in highly conserved residues across mammalian species or in serpins that are structurally related to CBG, such as thyroxin-binding globulin (SERPINA7) or α 1-antitrypsin (SERPINA1). Several are near previously identified naturally occurring CBG mutations that cause defects in CBG production or function, but none are in positions corresponding to mutations previously identified in rat, mouse, or pig CBGs (Simard et al., 2015).

1.3.8.7.3 The Cbg ko mouse: a model of CBG dysfunction

T Willnow's team has generated a mouse model of CBG deficiency and characterized the consequences of the carrier gene defect for the systemic and cellular actions of glucocorticoids. Specific disruption of the CBG gene was obtained by inserting a neomycin resistance cassette in exon 2 of the mouse gene using homologous recombination in embryonic stem cells. The resulting mouse line has a mixed genetic background 129svEmcTer x C57BL/6N. Cbg-null mice are viable and fertile but exhibit distinct defects in glucocorticoid action and adrenocortical stress response consistent with the modulatory role of CBG in hormonal activity. No detectable corticosterone binding was found in male and female CBG deficient animals. The clearance of corticosterone in plasma was found to be markedly increased since levels of radioactive corticosterone injected in the mice were found four times lower after 5min in CBG deficient mice compared to controls. Total corticosterone levels were found to be reduced but free corticosterone levels were 10-times higher in CBG deficient mice compared to controls. Surprisingly, ACTH levels were also found higher (3-fold) in CBG deficient mice. However, these corticosterone and ACTH levels are excessively high for unstressed conditions, especially for morning values (i.e., trough values of the circadian cycle in rodents). The explanation comes probably from the fact that mice were anesthetized before sacrifice. The authors claimed that the same profile was found on non-anesthetized mice for total and free corticosterone, but their values of total corticosterone are still high for wild-type animals (55 ng/ml when trough values on unstressed mice are usually <15 ng/ml) and the free fraction would be 0.7% which is

excessively low (generally 5–10%). According to the authors these results indicate a relative deficiency in sensing higher levels of free corticosterone in the mutant mice. This idea of hyporesponsiveness to free glucocorticoids was again put forward to explain the findings of normal expression of glucocorticoid target genes (gluconeogenic enzymes), aggravated response to septic shock and “fatigue” observed in these mutant animals. The latter was estimated by a behavioral test measuring activity scores which was later questioned by other authors if would in fact constitute an accurate way to assess the fatigue syndrome diagnosed in human patients (Petersen et al., 2006, Moisan, 2010).

This study has also examined metabolic homeostasis in the CBG deficient mice. No differences between genotypes were detected in electrolytes or in metabolites concentrations in the plasma and urine of mice fed *ad libitum* or starved overnight. Additionally, no difference in weight after a 6-week-long HF diet (26% fat vs 4% fat for control diet) period was seen between CBG deficient animals and age-matched controls, but this period under diet did not result in increased body weight for any of the groups. The reported levels of total corticosterone in these HF-fed and then starved animals are astonishingly high but no significant differences were observed for free corticosterone levels. The consequence of CBG deficiency on organ development was also studied, since glucocorticoids play a major role particularly in the maturation of the lung. Furthermore, Cbg mRNA is specifically expressed in liver and kidney during embryogenesis, suggesting a role in the maturation of these organs in rodents. Comparative histological analysis of liver and kidney tissues from CBG deficient mice and their controls in embryos, newborn and adult mice did not detect any differences. Thus, CBG expression does not appear critical for the development of these organs, or alternatively another mechanism occurred in the CBG deficient mice that balanced CBG deficiency to allow normal development (Petersen et al., 2006, Moisan, 2010).

Finally, response to inflammation was assessed by intraperitoneal injection of LPS. Septic shock led to a decreased survival rate in CBG deficient mice, with 50% of death within the first 48 hours whereas all control mice survived that period. Again, free corticosterone levels were found higher in CBG deficient mice. Overall, this study provides interesting data regarding the role of CBG, such as its crucial role in septic shock response which may be related to the serpin structure of CBG allowing local delivery of glucocorticoids at sites of inflammation. It is noteworthy that organ development is normal in CBG deficient animals although it was predictable from the human CBG-deficient patients who do not present with developmental anomalies. Also, the absence of major effect of CBG deficiency in this mouse genetic background after metabolic stress, such as high fat diet or starvation, is important but deserves further exploration with longer diet time and maybe in different genetic backgrounds. Finally, corticosterone levels at the peak of secretion (in the evening for mice) are needed to properly compare the HPA regulation with the human condition (Petersen et al., 2006, Moisan, 2010).

To better understand the role of CBG on glucocorticoid regulation and its influence on the phenotypes known to be modulated by glucocorticoids, MP Moisan's team from Nutrineuro lab has independently developed another CBG deficiency model, the Cbg ko mouse. The Cbg ko line has been obtained by specific deletion of the gene *Serpina6*, encoding CBG, using homologous recombination and the Cre-Lox technology. The resulting mouse line with loxP sites flanking the intron 2 of the *Serpina6* gene was crossed to the CMV-Cre mice allowing the specific deletion of the *Serpina6* gene in every cell of their progeny. While in controls glucocorticoids are found bound to CBG at

80%, and to albumin at 15%, leaving 5% free glucocorticoids, the distribution in the Cbg ko was 46% bound to albumin and 28% free. These data are in good agreement with the values found in the few CBG-deficient patients described in the literature. Although the free fraction is markedly increased in CBG-deficient individuals the free glucocorticoid concentrations acting on target cells are not different from control individuals in basal conditions. The reason for this is that the total glucocorticoid pool is much lower in CBG deficient individuals, thus counterbalancing the higher free fraction. Consequently, both CBG-deficient patients and Cbg ko mice develop normally and do not present any gross abnormality. Thus, CBG is not necessary for survival and the reason of its existence should be searched for in condition of stimulation of the HPA axis (Richard et al., 2010, Moisan et al., 2014).

After a moderate stressor, such as a physical restraint, Cbg ko mice were shown display an increase in total and free glucocorticoids, but in terms of concentration the elevation is lower (around 30%) than in wild-type controls. Thus, in these challenged conditions, the bigger free fraction of glucocorticoids does not compensate for the lower glucocorticoid pool. The authors then looked at how such a reduced glucocorticoid rise would impact on adaptive behavior to stress. They evaluated these mice in the FST, in the learned helplessness and in the TST, which provide indications of despair-like behavior. Altered responses in the Cbg ko mice were detected in all tests, characterized by increased despair-like behavior correlated with a reduced rise of free glucocorticoids. When the authors looked for gene expression in various glucocorticoids target tissues, they found few differences between Cbg ko and controls, but the early expression of gene coding for the early growth response protein 1 (Egr1) was found less induced in the hippocampus of Cbg ko mice. Since Egr1 was shown to mediate stress-related behavioral effects of glucocorticoids in hippocampus, this data further substantiated the hypothesis of a lower glucocorticoid signaling in Cbg ko mice, which could influence their behavior (Richard et al., 2010, Moisan, 2013, Moisan et al., 2014).

OBJECTIVES

CBG has long been considered as a mere transporter of glucocorticoid hormones within the bloodstream. As presented in the introduction of this thesis, studies are increasingly showing that CBG transcends this modest role and its central importance on other processes starts to be unraveled. CBG has been shown to be crucial to the delivery of glucocorticoid hormones to target tissues, notably in cases of inflammation where locally secreted elastases are able to cleave the CBG – glucocorticoid complex and release glucocorticoids in the inflammation sites. It has also been shown to be important in the maintenance of a plasma pool of glucocorticoids, which is needed to attain an optimal stress response. Nevertheless, to date, the role of CBG on the stress response is not completely understood. CBG levels have been classically considered constant, and thus were rarely measured in both animal and human studies, underestimating its possible roles in several different conditions.

The stimulation of the HPA axis in response to environmental stimuli surely has an adaptive value, but on the long term this activation might lead to a wear and tear of the HPA axis. Chronic hyper- or hypo-activity of this axis has been associated to several pathological states, and great variability exists not only in basal but also in stress induced glucocorticoid levels (Gross and Cidlowski, 2008, Kudielka et al., 2009). This intra- and inter-individual variability have been shown to play a prominent role in the vulnerability to various illnesses such as mood disorders (depression and anxiety) (Bremmer et al., 2007, Derijk and de Kloet, 2008), inflammatory and auto-immune diseases (Elenkov et al., 1999), metabolic disorders (Heim et al., 2000), growth (Stratakis, 2006), aging (Lupien et al., 1998) or drug addiction (Piazza and Le Moal, 1998).

One of the sources of variability in glucocorticoid levels is CBG binding capacity, which has been shown to vary according to several conditions, such as physiological and disease states, stress and genetic variants. Genetic mapping studies (a no-hypothesis-driven approach) performed in pigs have detected a *locus* strongly associated with cortisol levels, in particular stress levels, explaining 20% of the variance in the studied population (Desautels et al., 2002). Comparative genomics using human and mouse genomes pointed out *Serpina6* – the gene encoding CBG – as a good positional candidate. Later, this genome region was confirmed to contain the gene coding for CBG in pigs. A second genetic linkage analysis performed with plasma CBG binding as a trait (instead of cortisol) in that same pig population has led to the same QTL, with an even higher significance than that previously found for cortisol levels, reinforcing the hypothesis of a central role of CBG in cortisol variations (Ousova et al., 2004).

The same genetic mapping approaches have been used by other groups to identify genetic factors involved in glucocorticoid levels at rest or after a stress in rodent models (Moisan and Ramos, 2009). An analysis of behavioral and neuroendocrine stress-responsive traits has been performed on a large rat population, and similarly to what was found in pigs, a highly significant QTL associated with stress levels of corticosterone was detected at the CBG *locus*, highlighting again the importance of this gene in glucocorticoid variability. Furthermore, a screening of the rat and mouse genome databases (www.rgd.mcg.edu and www.informatics.jax.org, respectively) reveals the presence of various QTL in the vicinity of the CBG *locus* associated with diseases/traits influenced by glucocorticoids. Nevertheless, in most studies, the

confidence interval of the QTL is too large to propose serious candidate genes and CBG is often not even mentioned (Moisan, 2010).

A recent genome wide association meta-analysis for plasma cortisol in more than 15,000 humans has indicated that less than 1% of the variance in plasma cortisol is accounted for by genetic variation in a single region of chromosome 14, which contained SERPINA6 gene. Three SNPs were identified within this region, which were all associated with variation in total cortisol binding activity in plasma by influencing total CBG concentrations or the immunoreactivity of the RCL. These findings reinforce even more the key role of CBG in determining plasma cortisol levels, and lead to believe that variations affecting CBG can contribute to cortisol-associated disease states (Bolton et al., 2014).

Given the important, yet frequently neglected, role of CBG in modulating glucocorticoid levels and action, our team has developed a mouse model of CBG deficiency to further explore its actions. Previous studies using this model have shown that CBG deficiency blunts the circadian rhythmicity and promote a glucocorticoid hyporesponse to stress (Richard et al., 2010). Furthermore, these mice have been shown to display increased despair-like behaviors and are protected from the deleterious effects of stress on memory retrieval (Richard et al., 2010, Minni et al., 2012). This thesis' objectives were to further explore the physiological and behavioral alterations presented by the CBG-deficient mice in order to better understand the role of CBG in modulating the effects of glucocorticoids. More precisely, in this thesis we aimed at:

- **Investigating if there is a sexual dimorphism in the already described modulation of glucocorticoids by CBG, evidencing an influence of estrogens;**

We hypothesize that, since CBG displays a sexual dimorphism and is known to be regulated by estrogens, the role it plays in emotional reactivity might differ between both sexes, and CBG deficiency might also trigger different consequences in male and female mice.

- **Evaluating if and how CBG modulates the impact of chronic stress on the behavior of mice;**

Chronic stress is known to firstly increase glucocorticoid levels and subsequently dysregulate the HPA axis due to the prolonged activation of the stress response, and this axis' dysregulation is linked to the deleterious effects of chronic stress. We hypothesize that, due to their glucocorticoid hyporesponse to stress, CBG deficient mice would be protected from the deleterious effects of chronic stress.

- **Investigating the importance of CBG in regulating the effects of glucocorticoids on memory formation;**

Brain glucocorticoid concentration is a crucial factor influencing memory processes, and CBG – as an important regulator of glucocorticoid availability – stands as an interesting candidate modulating memory formation. We hypothesize that since Cbg ko mice have suboptimal glucocorticoid response to stress in the brain, and thus have lower glucocorticoid receptors' activation after

performing in emotionally arousing tasks, their memory formation would be compromised and consequently their memory performance impaired.

- **Studying the influence of CBG on weight gain and despair-like behavior induced by the combination of chronic stress and high fat diet (Western lifestyle paradigm).**

A pilot study from our group has shown that CBG-deficient mice display different metabolic and behavioral responses to the combination of hypercaloric diet and chronic stress, and that this difference seems to be linked to an increased lipolysis in Cbg ko mice. In this thesis we want to further characterize these metabolic and behavioral alterations.

STUDY I - ROLE OF CORTICOSTEROID BINDING GLOBULIN IN EMOTIONAL REACTIVITY SEX DIFFERENCES IN MICE

Introduction

As previously mentioned in the general introduction of this thesis, earlier studies from MP Moisan's team have shown an important impact of CBG deficiency on emotional reactivity. Besides having lower total plasma corticosterone levels in baseline conditions and lower total and free corticosterone levels after stress, Cbg ko mice also display more despair-like behavior in several behavioral tests, such as the TST, FST and learned helplessness test (Richard et al., 2010). Nevertheless, all behavioral tests had been performed with male mice, and how CBG deficiency would affect the emotional behavior of female mice remained unexplored.

Differences in the physiology of males and females, and in their response to disease, have been recognized for decades in many species, including humans. Still, despite the obvious relevance of these sex differences to experimental outcomes, male research subjects continue to dominate biomedical studies. Although there is some reasoning behind this choice for males – such as minimizing estrous cycle influences, excluding the protective effect of female hormones on some diseases or even assessing the effects of both chromosomes X and Y – this imbalance surely compromises discoveries on female sex specificities and thereby the safety and effectiveness of drugs in women (Nature, 2010, Wald and Wu, 2010).

When it comes to psychiatric disorders, it is known that diagnoses for anxiety and depression are more than twice as common in women than in men. Nevertheless, less than half of the published studies on these topics mention the use of females (Zucker and Beery, 2010). Since many neuropsychiatric disorders have a sex bias, elucidating what makes the male brain different from the female brain and when in development and maturation it occurs is essential (Bale, 2009).

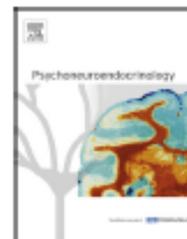
Virtually all sex differences in brain and behavioral phenotypes are attributed to the actions of gonadal steroids. The hypothalamo–pituitary–gonadal (HPG) axis is a neuroendocrine axis that is parallel to the HPA axis and involves the hypothalamus, anterior pituitary gland and gonads. It is comprised of GnRH neurons that release GnRH into the hypothalamo–hypophyseal portal vasculature, where it acts upon the gonadotropes of the anterior pituitary to stimulate the release of the gonadotropins – LH and FSH. In both sexes, circulating LH stimulates the secretion of steroid hormones from the gonads. In the testis, LH will stimulate the testosterone synthesis. In the ovary, FSH and LH will promote the synthesis of estrogens and of their precursors (Handa and Weiser, 2014).

Assessing the effect of stress and stress hormones (e.g. catecholamines and glucocorticoids) on both male and female physiology and behavior is crucial when studying sex differences. Both animal and human studies indicate that gonadal steroid secretion is markedly altered by stress and glucocorticoids in a sex- and hormone-dependent fashion (Becker et al., 2005). For instance, in both rats and mice, stress-induced corticosterone secretion is more pronounced in females than in males. This difference, despite partially buffered by the higher CBG concentration in females, might reflect a greater net impact of stress on glucocorticoid secretion (Viau and Meaney, 1991, Carey et al., 1995, Minni et al., 2014). Furthermore, differences in HPA axis function are observed across the estrous cycle of female rats, which suggests at least a partial role of gonadal steroid hormones on these sex differences.

Estrogens and glucocorticoids have been extensively described to interact in several body tissues, including the brain (Mattsson and Olsson, 2007, Weiser and Handa, 2009, Edgar et al., 2013, Rainville et al., 2015). Nevertheless, the mechanism(s) by which gonadal steroid hormones act to influence HPA function have not been completely resolved, despite evidence pointing at androgens and estrogens acting in the modulation of adrenal, anterior pituitary and hypothalamic functions. Manipulation of estrogen levels have been extensively shown to influence HPA axis reactivity. Suppressing or antagonizing estrogens actions usually leads to a reduced HPA axis reactivity, although some authors have also described the opposite effect or no effect of these manipulations on the HPA axis. Evidence shows that these contrary effects of estradiol on stress reactivity may pass through the estrogen receptor signaling pathway. Limbic structures, as well as the PVN and areas that send direct or indirect projections to this structure contain neurons expressing these receptors. ER α and estrogen receptor beta (ER β) are known to have opposing actions in regulating HPA axis function: ER β activation seems to reduce the gain of the HPA axis response to an acute stressor, while ER α would act enhancing the gain of the HPA axis (Handa and Weiser, 2014).

The study presented in the following article aimed at investigating if the already described modulation of glucocorticoids by CBG could also be influenced by female hormones. For that, female mice were submitted to behavioral tests evaluating despair-like behaviors in order to evaluate if the emotional differences observed between Cbg ko and wild-type males were also present in females. Then glucocorticoid levels throughout the circadian cycle were evaluated, as well as the HPA axis activity, the expression of glucocorticoid-related genes in the hippocampus and glucocorticoid turnover from the plasma after stress. The influence of female hormones, more specifically estrogen, was confirmed by testing ovariectomized mice – replaced or not with estrogen pellets – in the despair-like behavior tests.

All the behavioral data and some of the biochemical analyses were already concluded by the start of this PhD thesis. Nevertheless, I have actively participated on the experiments involving corticosterone turnover from plasma and gene expression in the hippocampus, as well as in the preparation of this manuscript.



Role of corticosteroid binding globulin in emotional reactivity sex differences in mice



A.M. Minni^{a,b}, G.F. de Medeiros^{a,b}, J.C. Helbling^{a,b}, A. Duittoz^c,
N. Marissal-Arvy^{a,b}, A. Foury^{a,b}, V. De Smedt-Peyrusse^{a,b},
V. Pallet^{b,d}, M.P. Moisan^{a,b,*}

^a INRA, Nutrition et neurobiologie intégrée, UMR 1286, 33076 Bordeaux, France

^b Univ. Bordeaux, Nutrition et neurobiologie intégrée, UMR 1286, 33076 Bordeaux, France

^c Université François Rabelais, Physiologie de la Reproduction et des Comportements INRA U85, CNRS UMR7247, IFCE, 37380 Nouzilly, France

^d IPB, Nutrition et neurobiologie intégrée, UMR 1286, 33076 Bordeaux, France

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Summary Sex differences exist for stress reactivity as well as for the prevalence of depression, which is more frequent in women of reproductive age and often precipitated by stressful events. In animals, the differential effect of stress on male's and female's emotional behavior has been well documented. Crosstalk between the gonadal and stress hormones, in particular between estrogens and glucocorticoids, underlie these sex differences on stress vulnerability.

We have previously shown that corticosteroid binding globulin (CBG) deficiency in a mouse model (Cbg k.o.) leads, in males, to an increased despair-like behavior caused by suboptimal corticosterone stress response. Because CBG displays a sexual dimorphism and is regulated by estrogens, we have now investigated whether it plays a role in the sex differences observed for emotional reactivity in mice.

By analyzing Cbg k.o. and wild-type (WT) animals of both sexes, we detected sex differences in despair-like behavior in WT mice but not in Cbg k.o. animals. We showed through ovariectomy and estradiol (E2) replacement that E2 levels explain the sex differences found in WT animals. However, the manipulation of E2 levels did not affect the emotional behavior of Cbg k.o. females. As Cbg k.o. males, Cbg k.o. females have markedly reduced corticosterone levels across the circadian cycle and also after stress. Plasma free corticosterone levels in Cbg k.o. mice measured immediately after stress were blunted in both sexes compared to WT mice. A trend for higher mean levels of ACTH in Cbg k.o. mice was found for both sexes. The turnover of a corticosterone bolus was increased in Cbg k.o. Finally, the glucocorticoid-regulated immediate early gene early growth response 1 (*Egr1*) showed a blunted mRNA expression in the hippocampus of Cbg k.o. mutants while mineralocorticoid and glucocorticoid receptors presented sex differences but equivalent mRNA expression between genotypes.

* Corresponding author at: INRA, Nutrition et neurobiologie intégrée, Université Victor Segalen, 146 rue Léo Saignat, 33076 Bordeaux, France. Tel.: +33 557 579214; fax: +33 557 571227.

E-mail address: mpmoisan@bordeaux.inra.fr (M.P. Moisan).

Thus, in our experimental conditions, sex differences for despair-like behavior in WT mice are explained by estrogens levels. Also, in both sexes, the presence of CBG is required to attain optimal glucocorticoid concentrations and normal emotional reactivity, although in females this is apparent only under low E2 concentrations. These findings suggest a complex interaction of CBG and E2 on emotional reactivity in females.

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1. Introduction

Women and men present differential risks for the occurrence and symptomatology of stress-induced depression, with women of reproductive age being around two times as likely as men to report a lifetime history of a major depressive episode (Kessler et al., 1993; Wittchen et al., 2011). While social factors certainly contribute to these differences, biological sex differences are also known to play an important role (Schotte et al., 2006).

Fluctuations in estrogen levels, rather than their absolute levels, have been shown to be an important risk factor for the onset of depressive symptoms in women. Accordingly, depressive episodes appear more frequently during the premenstrual, postpartum and perimenopausal periods (Solomon and Herman, 2009; Young and Korszun, 2010). Pre-clinical models have confirmed the importance of female sex hormones in affecting emotions and cognition (ter Horst et al., 2012).

Physiologic stress responses, in particular hypothalamic-pituitary-adrenal (HPA) axis reactivity culminating in glucocorticoid release, are also believed to underlie the sex differences in the prevalence of depression. This endocrine system is dysregulated in a significant number of depressive patients and its activity/reactivity displays a sexual dimorphism. Depressed patients generally present a hyperactivity of the HPA axis, i.e. increased circulating cortisol levels, often explained by an impaired feedback regulation and leading to excessive glucocorticoid signaling in the brain (Pariante and Lightman, 2008; Young and Korszun, 2010). Although much less studied, hypoactivity of the HPA axis, leading to low or inefficient cortisol levels, is also observed in some types of depression (Bremner et al., 2007; Gold and Chrousos, 2002; Raison and Miller, 2003).

Sex differences in HPA axis regulation are well documented in animal (Handa et al., 1994; Rhodes and Rubin, 1999) as well as in human studies (Kajantie and Phillips, 2006; Kudielka and Kirschbaum, 2005). Both basal and stimulated HPA axis activities are generally increased in female when compared to male rodents (Rhodes and Rubin, 1999). In human studies, reported sex differences in HPA axis response to stress are inconsistent, as they appear to be influenced by age, type of stressors, phase of the menstrual cycle, or oral contraceptive intake (Kudielka et al., 2009; Young and Korszun, 2010).

Prime candidates for explaining such sex differences in HPA axis activity are circulating gonadal steroids levels, with endogenous estradiol (E2) being known to increase (except during pregnancy) and testosterone to decrease both ACTH and cortisol (corticosterone in rodents) responses to stress (Handa et al., 1994; Seale et al., 2004, 2005; Solomon and Herman, 2009). E2 has been shown to increase

central HPA axis drive in rodents through its stimulating effects on corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) mRNA expression, as well as by increasing adrenal sensitivity to ACTH (Solomon and Herman, 2009). Additionally, estrogens are thought to increase glucocorticoids levels indirectly by positively stimulating corticosteroid binding globulin (CBG) synthesis, as observed during pregnancy (Moore et al., 1978), after the intake of oral contraceptives containing ethinylestradiol (Qureshi et al., 2007; White et al., 2006) or in vitro through a mechanism involving the estrogen receptor alpha (Nader et al., 2006). Hormonal replacement studies have provided conflicting findings about the effects of exogenous E2 on HPA axis response to acute stress (Solomon and Herman, 2009; Young and Korszun, 2010). In most cases, the administration of physiological doses of E2 decreases ACTH and/or corticosterone responses to acute stressors, whereas supraphysiological doses increase these hormones on gonadectomized animals (Solomon and Herman, 2009).

CBG or transcortin is a glycoprotein binding glucocorticoids with high-affinity in vertebrate blood. CBG plays an essential role in glucocorticoid availability and delivery to target tissues (Gagliardi et al., 2010; Moisan, 2010). In our previous studies, we showed that CBG deficiency in male mice led to an insufficient glucocorticoid signaling and delivery to the brain, associated with altered stress-induced emotional and cognitive behaviors. Indeed, we showed that during stress these mutant mice display a lower rise of glucocorticoids than the wild-type (WT) controls, associated with altered emotional reactivity in the forced swim, tail suspension and learned helplessness tests. These data suggested that CBG plays a role in the fast actions of glucocorticoids on behavior. We then demonstrated that stress-induced memory retrieval impairment, an example of the fast action of glucocorticoids on the brain, is abolished in the *Cbg* k.o. mice. This effect of stress on memory retrieval was restored in the *Cbg* k.o. mice by infusing corticosterone directly into the hippocampus. The mechanisms explaining these effects involved an increased clearance but no difference in corticosterone production (Minni et al., 2012; Moisan et al., 2014; Richard et al., 2010).

Considering CBG role in glucocorticoid-dependent emotional behavior in male mice, as well as the importance of estrogens in CBG regulation, the present study aimed at determining the influence of CBG deficiency on emotional behaviors displayed by female mice and its role in the sex differences already described for these behaviors. We first examined emotional behavior in WT and *Cbg* k.o. mice, then we studied the role of estrogens in the behavior of females from both genotypes and finally we

analyzed glucocorticoid activity and reactivity in all animal groups.

2. Methods

2.1. Animals

Cbg k.o. and WT mice (MGI ID: 4833641, *Serpina6*^{tm1.1Mmp}) were produced in our laboratory in Bordeaux (Richard et al., 2010). WT and Cbg k.o. animals were littermates, obtained by breeding males and females heterozygous for the Cbg mutation. The progeny was genotyped from a tail biopsy to select groups of WT and Cbg k.o. mice. Mice used in the present work have a C57BL/6J genetic background above 95%. Animals were maintained in an animal facility with constant temperature (23 °C), under a 12 h light/dark cycle (lights on at 0700 h) and with ad libitum access to food and water. All mice had between 2 and 5 months of age when tested, and were individually housed 1 week before the beginning of the experiments. All the experiments were conducted in strict compliance with current European Conventions (86/609/CEE) and approved by Institutional Regional Committee for animal experimentation (agreement # 5012050-A).

2.2. Experiment 1: emotional behavior analyses

A first batch of male and female WT and Cbg k.o. mice ($n=36$, 8–11 mice per group) were submitted to the tail suspension test (TST) and, 1 week later, to the forced swim test (FST). For the TST, an adhesive tape was fixed to the mouse's tail and hooked to a horizontal ring stand bar placed 30 cm above the floor. The test lasted 6 min, during which immobility time was recorded. Animals that managed to hook onto their tails or to the ring were excluded from the analysis. In the FST, each mouse was placed in a cylinder (height, 32.5 cm; diameter, 15 cm) containing 3 l of water (16 cm) at 25 ± 0.5 °C, changed between subjects. A mouse was judged immobile when it stopped swimming and made only small movements to remain afloat. Immobility behaviors in the FST were expressed in immobility occurrence, assessed every 10 s over a period of 6 min (Bolanos et al., 2008). Recordings were evaluated by two experimenters, blind to the animals' genotypes. Both tests are described in details elsewhere (Richard et al., 2010; Steru et al., 1985).

A second batch of mice ($n=29$, 7–11 males per group and 5–6 females per group) was exposed to the elevated plus-maze and, 1 week later, to the open-field test. The elevated plus-maze apparatus consisted of two opposing open arms (30 cm \times 8 cm) and two opposing closed arms (30 cm \times 8 cm \times 15 cm) extending from a 8 cm \times 8 cm central platform and elevated 1 m from the ground. Each mouse was first placed onto the central platform facing an open arm and then left to freely explore the apparatus for 5 min. The number of entries in the open or closed arms, as well as the time spent on the various sections of the maze, was recorded. The percentage of time spent in the open arms ((time in the open arms)/(time in the open + closed arms) \times 100) was used as an index of anxiety, whereas total number of entries in the closed arms was used as an index of locomotor activity.

The open-field apparatus was a square arena (40 cm \times 40 cm) with 16 cm high walls and its floor divided into 16 squares (10 cm \times 10 cm). Mice were first placed in the central area and allowed to freely explore the arena for 10 min. The total number of squares crossed was considered an index of locomotor activity, while the percentage of time spent in the center (four inner squares) was considered an index of anxiety.

2.3. Experiment 2: effect of ovariectomy and E2 replacement on despair-like behavior

A third batch of female mice ($n=52$, 8–10 per group) underwent either bilateral ovariectomy (OVX) or sham surgery, both performed under continuous anesthesia (2% isoflurane and 2% oxygen). One week later, a silastic implant of E2 was inserted subcutaneously into the dorsum of half of the OVX animals (OVX E2). To activate and stabilize the hormone release, implants were incubated in gelatin-phosphate-buffered saline for 24 h before use. The E2 pellets of 2 cm length were prepared in-house according to the method described by Cohen and Milligan (1993). These pellets produced a slow and continuous release of E2 and have been shown to maintain low plasma levels of estrogens (around 3–4 pg/ml).

Two weeks after the surgeries, mice were exposed to the TST and 1 week later to the FST, as described above. At sacrifice, we confirmed that all OVX and OVX E2 mice had their ovaries removed. Moreover, to estimate the amount of E2 produced, uteri were weighted and then related to mice's body weights. As expected (Cohen and Milligan, 1993), uteri weights were lower for both OVX groups, indicating a diminished or abolished E2 production on OVX mice and lowered levels on OVX + E2 when compared to sham operated mice.

2.4. Experiment 3: HPA axis activity and reactivity analyses

A fourth batch of mice ($n=29$, 5–7 males per group and 8–10 females per group) was used for nycthemeral experiments. Blood and urine samples were collected every 4 h from 0700 h to 2300 h. Blood samples were obtained by tail nick and collected in tubes containing 10% EDTA. This process took less than 2 min in order to obtain basal levels of corticosterone. After 10 min of centrifugation (4000 \times g at 4 °C), plasma was collected and kept at -80 °C. Urine samples were obtained by placing a Petri dish under the mice during blood collection, and were also kept at -80 °C.

Total corticosterone was measured by an in-house radioimmunoassay (RIA) from plasma and urine samples (see Richard et al., 2010 for details). Anti-corticosterone antibody was provided by Dr. H. Vaudry (University of Rouen, France). Prior to RIA, plasma samples were extracted with ethanol while urine samples were extracted with dichloromethane. As the urine volume collected at each time point was variable, creatinine concentration was also measured in with a commercial kit (Kit Biomerieux Creatinine cinétique) in order to normalize corticosterone concentrations between samples.

A fifth batch of animals ($n=76$, 15–22 per group) was used to study corticosterone response to the stress

Table 1 Sequence of primers used in real-time qPCR.

Genes	Primers	Sequence 5'–3'	Amplicon size (bp)
18S RNA	Forward	ACCGCAGCTAGGAATAATGGA	63
	Reverse	GCCTCAGTTCGGAAAACCAA	
Egr1	Forward	AGCCGAGCGAACAAACCC	52
	Reverse	TGTCAGAAAAGGACTCTGTGGTCA	
ER β	Forward	GGACCGAGGAAAGTACGTGTCT	51
	Reverse	CGGGCAGGTGGAATGC	
GR	Forward	GTGGAAGGACAGCACAAATTACCT	61
	Reverse	GCGGCATGCTGGACAGTT	
MR	Forward	GCCGTGGAAGGACAACACA	60
	Reverse	CCTAAGTTCATGCCGGCTTG	

induced by a FST. Females were checked for the phase of their ovarian cycle by performing a vaginal smear analysis immediately after the FST. A cotton swab moistened with 0.9% saline was gently rotated inside the vaginal canal and then smeared on a glass slide. The slides were air dried, stained with hematoxylin–eosin and then fixed with ethanol for cytological examination. The phase of the estrous cycle was determined using the images and tool presented in Byers et al. (2012).

About half of these animals ($n = 39$, balanced for sex and genotype), was euthanized immediately after the FST to measure CBG maximum binding capacity (B_{max}), total and free corticosterone in plasma, as well as mRNA expression of the immediate early gene early growth response 1 (Egr1) and of the estrogen receptor beta (ER β) in the hippocampus. The rest of the animals ($n = 37$) was euthanized 3 h after the FST to evaluate the effect of stress on nuclear mineralocorticoid and glucocorticoid receptors mRNA expression.

Blood samples were collected by cardiac puncture after isoflurane anesthesia. Free corticosterone was measured by isotopic dilution and plasma (100 μ l) ultrafiltration using Centrifree filter device (YM membranes 30K, Millipore, France) as in Richard et al. (2010). The percentage of free corticosterone was calculated as the ratio of cpm filtrate (free corticosterone)/cpm retentate (bound corticosterone). Free corticosterone concentration was calculated as the percentage of free corticosterone multiplied by total corticosterone concentration (measured by RIA as described above).

CBG B_{max} in the plasma of WT animals was estimated from a saturation curve and Scatchard analysis, as described in Richard et al. (2010), using a standard curve of tritiated corticosterone up to 32 nM.

Total RNA were extracted from tissue samples with TRIzol reagent, according to the manufacturer's protocol (Invitrogen, Cergy Pontoise, France). RNA quality and concentration were evaluated with RNA Nano chips onto a Bioanalyser 2100 (Agilent, Boeblingen, Germany). Two μ g of total RNA was reverse-transcribed with Superscript III (Invitrogen, Cergy Pontoise, France) and random hexamers, according to the manufacturer's protocol. PCR reactions were carried out using LightCycler[®] 480II system (Roche), from 5 μ l of cDNA

diluted 1:20, 10 μ l of 2X LC480 SYBER-GREEN I Master solution (ROCHE) and 5 μ l of primer mix at 300 nM in a total volume of 20 μ l. PCR program consisted of 40 cycles of 95 °C for 10 s, 62 °C for 6 s and 72 °C 10 s. All primer pairs were designed using Primer Express software (PE APPLIED Biosystems, Courtaboeuf, France) in a way to avoid genomic DNA amplification. Sequences of the primers used are provided in Table 1. The specificity of the PCR reaction was validated following MIQE (Minimum Information for publication of Quantitative real time PCR Experiments) guidelines (Bustin et al., 2009). mRNA levels of target genes were normalized from 18S RNA expression in each sample (cDNA diluted 1:2000), as 18S proved not to vary in our experimental conditions. The quantification of target mRNA levels was calculated with the LightCycler480 Relative Quantification software (version 1.5). One animal from the WT control group was chosen as a calibrator for the relative quantification ($RQ = 2^{-\Delta\Delta C_p}$).

A sixth batch of animals was used to measure plasma ACTH levels in response to the stress induced by a FST and, a week later, the same animals were used to study corticosterone clearance from plasma ($n = 19$, 4–5 animals per group). Blood samples were collected immediately after the FST from the facial vein. Females were additionally checked for the phase of their ovarian cycle by performing a vaginal smear analysis immediately after the blood collection, as above. Nine females out of 11 were in estrous and 2 were in proestrous phase. ACTH was measured using a RIA kit, following the manufacturer protocol (ELSA ACTH, CIS bio, IBA Molecular, Gif sur Yvette, France). To measure corticosterone turnover, mice were first injected intraperitoneally (i.p.) with dexamethasone (75 μ g/100 g body weight) in order to block corticosterone secretion. Five hours later, these same mice were injected intravenously (i.v.) (in the caudal vein while under isoflurane anesthesia) with a bolus of corticosterone (23 μ g/100 g body weight). Blood samples were collected from the facial vein just before the corticosterone injection to check for corticosterone secretion suppression, and then at various time points after the corticosterone injection, as shown in Fig. 5. Only animals in which the injection in the caudal vein was precisely performed were kept for further analyses ($n = 3$ per group, except Cbg k.o. females where $n = 4$).

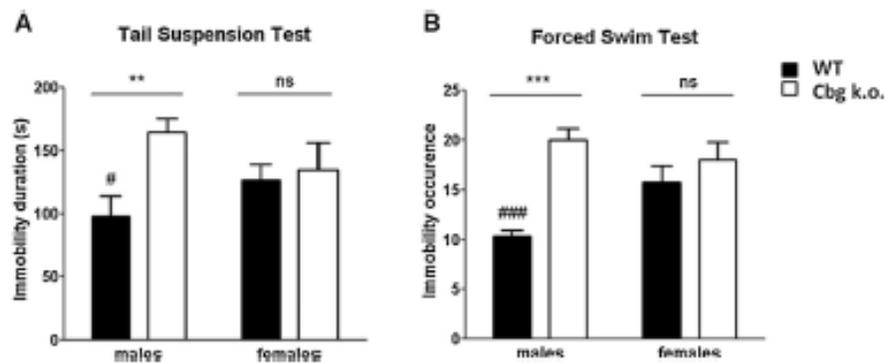


Figure 1 Despair-like behavior in wild-type (WT) and Cbg k.o. mice. Immobility duration in the tail suspension test (A) and immobility occurrence in the forced swim test (B) displayed by males and females from both genotypes ($n = 8-11$ per group). (**) and (***) represent significant genotype effects within male mice (two-way ANOVA followed by Fisher LSD post hoc, $p < 0.01$ and $p < 0.001$, respectively). (#) and (###) represent significant sex effects within WT animals (two-way ANOVA followed by Fisher LSD post hoc, $p < 0.05$ and $p < 0.001$, respectively). ns = not significant.

2.5. Statistics

All results are expressed as mean \pm S.E.M. Statistics were calculated with the software Statistica v.8.

Two-way analyses of variance (ANOVA) followed by Fisher LSD post hoc tests were used to detect sex and genotype effects in experiments 1 and 3, as well as genotype and treatment effects (sham, OVX and OVX E2) in experiment 2. For nycthemeral experiments, two-way ANOVA of repeated measures were performed on genotype and time effects for plasma and urine corticosterone. For other endocrine measures and behavioral tests, genotype effect was detected by unpaired Student's *t*-test. The level of statistical significance was set as *p* values < 0.05 in all tests.

3. Results

3.1. CBG role in emotional behavior sex differences in WT and Cbg k.o. mice

We have analyzed males and females Cbg k.o. and their WT controls in two tests of despair-like behavior: TST and the FST (Fig. 1A and B). A two-way ANOVA for sex and genotype detected a significant interaction between sex and genotype for immobility scores in the TST ($F_{(1,32)} = 5.69$, $p < 0.05$) and FST ($F_{(1,32)} = 9.14$, $p < 0.001$). Post hoc analyses revealed that male Cbg k.o. mice showed higher immobility than WT males in both tests ($p < 0.01$ for TST and $p < 0.001$ for FST). Interestingly, this genotype difference was not observed between WT and Cbg k.o. female mice ($p > 0.05$). Furthermore, WT females showed higher immobility than WT males in both TST ($p < 0.05$) and FST ($p < 0.001$). Regarding Cbg k.o. mice, males and females displayed similar immobility scores ($p > 0.05$ in both tests). A second batch of mice was then evaluated for anxiety related behaviors using the open-field and the elevated plus-maze tests (supplemental Figure S1). The two-way ANOVA detected no genotype differences but revealed that females presented more fear-related behaviors than males, as they spent less time in the center of the open-field and in the open arms of the elevated

plus-maze ($p < 0.001$ for both variables). Females' locomotor activity was found to be lower than males' in the open-field (number of squares crossed, $p < 0.01$) but not in the elevated plus maze test (number of entries in the closed arms, $p > 0.05$).

3.2. Role of estrogens on emotional behavior in WT and Cbg k.o. females

To investigate if the sex difference in despair-like behavior found in WT animals – but not in Cbg k.o. mice – was hormonally modulated, we compared despair-like behaviors in a third batch of animals, consisting of OVX, OVX E2 and control sham-operated female mice from both genotypes.

For the TST (Fig. 2A), the two-way ANOVA revealed a significant interaction between genotype and treatment ($F_{(2,37)} = 7.38$, $p < 0.01$). Among WT females, OVX showed immobility scores 44% lower than sham ($p < 0.01$), whereas immobility scores of OVX E2 and sham did not differ ($p > 0.05$). No difference was observed among Cbg k.o. females regardless of the treatment. A genotype effect was detected only in the OVX group ($p < 0.001$), with WT OVX mice displaying immobility scores 54.4% lower than Cbg k.o. OVX.

In the FST, similar results were observed (Fig. 2B). The two-way ANOVA showed a significant interaction between genotype and treatment ($F_{(2,46)} = 3.27$, $p < 0.05$), and a significant genotype effect ($F_{(1,46)} = 16.02$, $p < 0.001$). Immobility scores of WT OVX mice were significantly reduced by 40.4% ($p < 0.001$) compared to wild-type sham mice, whereas immobility scores of WT OVX E2 mice were not different from WT sham ($p > 0.05$). Differences between genotypes were only found in OVX groups ($p < 0.001$), with immobility scores 50.6% lower in WT OVX compared to Cbg k.o. OVX. Again, no difference was observed among Cbg k.o. females regardless of the treatment. Since our previous studies have shown that low corticosterone levels were associated with the altered emotional behavior in Cbg k.o. males (Moisan et al., 2014; Richard et al., 2010), we have also characterized HPA axis regulation in female Cbg k.o. mice.

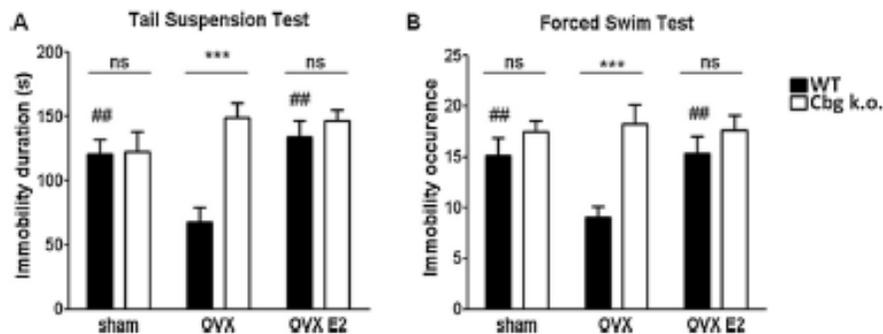


Figure 2 Role of estrogens in despair-like behavior in wild-type (WT) and Cbg k.o. female mice. Immobility duration in tail suspension test (A, $n=6-9$ per group) and immobility occurrence in forced swim test (B, $n=8-10$ per group) displayed by sham-operated (sham), ovariectomized (OVX) and ovariectomized and replaced with E2 pellet (OVX E2) females from both genotypes. (***) represent significant genotype effects within OVX females (two-way ANOVA followed by Fisher LSD post hoc $p < 0.001$). (##) represent significant difference from WT OVX mice (two-way ANOVA followed by Fisher LSD post hoc, $p < 0.01$). ns = not significant.

3.3. CBG and sex differences in HPA regulation

Total plasma corticosterone concentrations were measured during the diurnal cycle in male and female mice from a fourth batch of animals (Fig. 3A and B). A two-way ANOVA on total corticosterone data detected a significant interaction between genotype and time both in males ($F_{(4,24)} = 7.1$, $p < 0.001$) and in females ($F_{(4,28)} = 4.9$, $p < 0.001$). Circadian rhythm was detected in WT animals with a higher peak of corticosterone levels in WT females (216.5 nM vs. 180.5 nM in WT males), whereas Cbg k.o. animals display a

flattened circadian rhythm of corticosterone levels (39 nM in Cbg k.o. females vs. 41 nM in Cbg k.o. males for peak values).

To investigate whether the reduced circulating corticosterone pool in Cbg k.o. mice was due to a difference in production rate, we also measured corticosterone in urine across the circadian cycle of these same animals (Fig. 3C). No significant interaction between sex and genotype was observed. However, a significant genotype effect was detected ($F_{(1,25)} = 6.74$, $p < 0.01$) for the area under the curve (AUC) of urine corticosterone, with female Cbg

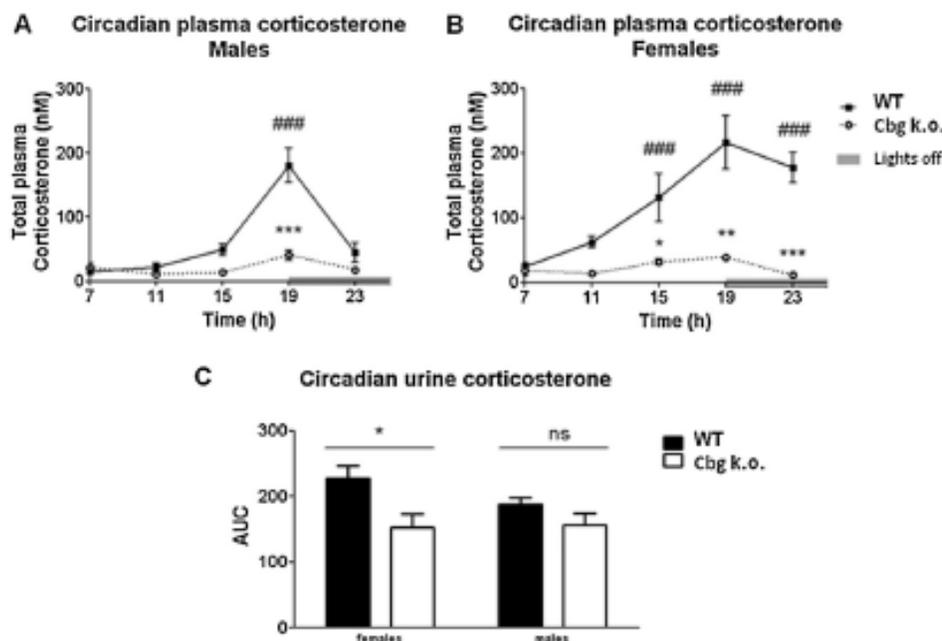


Figure 3 Basal corticosterone levels in wild-type (WT) and Cbg k.o. mice. Total plasma corticosterone (nM) levels across the circadian cycle in male (A, $n=5-6$ per group) and in female (B, $n=10-11$ per group) mice from both genotypes. Area under the curve (AUC) of circadian urine corticosterone levels (C) in male ($n=5-6$ per group) and female ($n=8-10$ per group) mice from both genotypes. (*), (**) and (***) represent significant genotype effects within the time point (two-way ANOVA of repeated measures followed by Fisher LSD post hoc for A & B or t test for C, $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). (###) represent significant difference from WT at time = 7 h (two-way ANOVA followed by Fisher LSD post hoc, $p < 0.001$). ns = not significant.

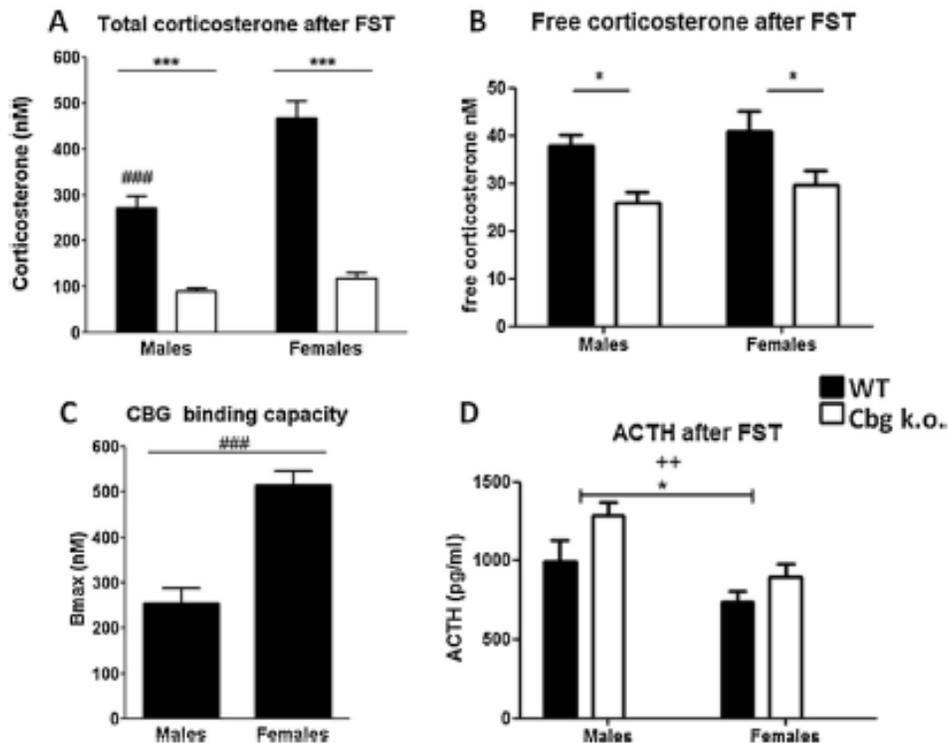


Figure 4 HPA axis activity after stress. Total and free plasma corticosterone concentrations (A, B, $n = 6-10$ per group), CBG B_{max} (C, $n = 7-8$ per group, wild-type (WT) mice only) and ACTH concentrations (D, $n = 4-5$ per group) after FST-induced stress in WT and Cbg k.o. mice from both sexes. (*) and (***) represent significant genotype effects within the same sex (two-way ANOVA followed by Fisher LSD post hoc (A, B), $p < 0.05$ and $p < 0.001$, respectively). (###) represents a significant sex effect within the same genotype (A, C) (two-way ANOVA followed by Fisher LSD post hoc, or Student's t -test (C), $p < 0.001$). (##) represents an overall sex effect ($p < 0.01$) and (*) an overall genotype effect ($p < 0.05$) (D), without interaction between these factors (two-way ANOVA).

k.o. mice presenting a 33% smaller AUC when compared to WT females (152.5 ± 21.0 in Cbg k.o. vs. 227.6 ± 18.9 in WT, $p < 0.05$). The AUC did not differ between males from both genotypes, indicating that the elevated corticosterone secretion of females is CBG-dependent.

Since we had previously shown that the altered behavior in FST observed in Cbg k.o. males was associated with a suboptimal rise in the free corticosterone levels, we have examined this association in females as well. Males were also analyzed in the same conditions for comparison. Because the phase of the estrous cycle may also influence behavior, vaginal swabs were carried out immediately after the FST and analyzed to determine the estrous cycle phase in which each female was. We found that 70% (30/43) of the females in our experimental group were in estrus. Regarding the FST, we observed the same pattern as described in Fig. 1 for immobility behavior in both sexes (regardless of the estrous cycle phase, see supplemental Figure S2). Total and free corticosterone levels, as well as CBG B_{max} , were measured in the plasma of about half of the experimental animals, collected immediately after the FST (Fig. 4). A two-way ANOVA detected a significant interaction between genotype and sex ($F_{(1,31)} = 15.6$, $p < 0.001$) for total corticosterone levels (Fig. 4A). In both sexes, levels were lower in Cbg k.o. mice compared to WT mice ($p < 0.001$). Interestingly, no sex difference in total corticosterone was detected in Cbg k.o. mice (90.4 ± 7.3 nM in males vs. 117.7 ± 13.9 nM

in females), whereas in WT animals, females displayed levels 71.6% higher than those from males (466.9 ± 37.8 nM and 272.0 ± 26.1 nM, respectively; $p < 0.001$). For free corticosterone, no significant effect of sex or interaction between genotype and sex were found, but a genotype effect was detected ($F_{(1,31)} = 12.7$, $p < 0.01$) (Fig. 4B). Free corticosterone levels were found lower in Cbg k.o. (25.9 ± 2.4 nM in males; 39.0 ± 4.8 nM in females) compared to wild type mice (35.1 ± 3.4 nM in males; 33.7 ± 4.0 nM in females) ($p < 0.05$). Finally, CBG binding capacity was found twice as high in WT females (515.9 ± 30.7 nM) than in WT males (254.9 ± 33.7 nM; $t_{(14)} = 5.7$, $p < 0.001$) (Fig. 4C). Plasma ACTH levels were measured in a sixth batch of animals, from blood collected immediately after a FST. No significant sex by genotype interaction was detected, but a sex effect ($p < 0.01$) and a genotype effect were found ($p < 0.05$) (Fig. 4D). Corticosterone turnover was evaluated in this same group of animals by first blocking corticosterone secretion with i.p. administration of dexamethasone, and then injecting an i.v. bolus of corticosterone. Dexamethasone suppression was successful since each animal had, before the corticosterone bolus, a corticosterone concentration of less than 6 ng/ml. Fig. 5 illustrates the levels of corticosterone at different time points after the corticosterone bolus injection. Our results show that, in both sexes, Cbg k.o. mice retained less corticosterone from the bolus than did the WT mice. Two minutes after the bolus, the WT males retained

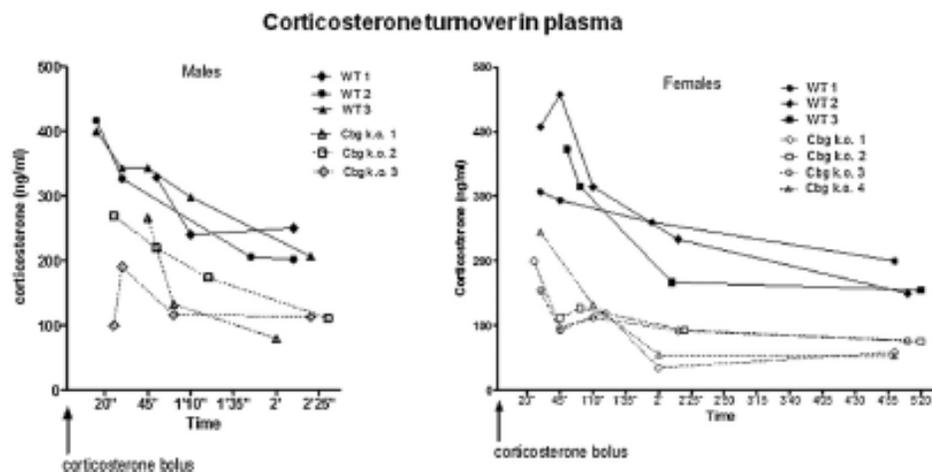


Figure 5 Corticosterone turnover in plasma of wild-type (WT) and Cbg k.o. mice. Mice were first treated with dexamethasone to block corticosterone secretion. A bolus of corticosterone ($23 \mu\text{g}/100 \text{g}$ body weight) was then injected i.v. in the caudal vein and blood samples were collected from the facial vein at various time points after the bolus injection. Each curve represents the plasma corticosterone concentration (nM) for one animal at different time points, being the WT animals represented by black symbols and Cbg k.o. by clear symbols.

twice more corticosterone than Cbg k.o. (219.5 ng/ml vs. 101.3 ng/ml) and WT females retained 3-fold more corticosterone than Cbg k.o. (220.0 ng/ml vs. 68.3 ng/ml).

To further evaluate glucocorticoid activity within the brain, we analyzed the expression of the gene *Egr1* in the hippocampus of animals euthanized immediately after the FST, as this transcription factor was shown to mediate glucocorticoid-dependent behavior in mice (Revest et al., 2005, 2010). In females only, *Egr1* gene expression in the hippocampus was found significantly decreased in Cbg k.o. when compared to WT mice. Regarding hippocampal ER β gene expression in the same animals, no genotype differences were detected (Fig. 6). Hippocampal mineralocorticoid and glucocorticoid receptors gene expression was evaluated 3 h after the FST-induced stress. Here too, no significant genotype effect was observed. For *Egr1*, ER β , and GR a significant sex effect was detected ($p < 0.01$), with males displaying higher expression of these genes (Fig. 6).

4. Discussion

Genetics, environmental factors and their interaction can lead to an inappropriate stress response, embodied by a hyper- or hypoactivity of the HPA axis, that in turn play an important role in the vulnerability to develop depressive disorders (Anacker et al., 2011; Herbert et al., 2006). Sex differences are observed in the prevalence of depressive disorders in humans as well as in glucocorticoid response to stress reactivity in both humans and rodents (ter Horst et al., 2012). However, female rodent models are far less characterized than male rodents in biomedical research, including stress-associated despair-like behaviors (Kim et al., 2010; Wald and Wu, 2010; Zucker and Beery, 2010). CBG is involved in HPA axis sex differences and we have recently shown that its deficiency in males is associated with increased despair-like behavior and glucocorticoid hyposignaling (Minni et al., 2012; Moisan et al., 2014; Richard et al., 2010). In this study,

we examined the role of CBG in emotional behaviors and HPA axis regulation in female mice, and compared them with those in males.

First, we found that WT females have endogenous higher immobility scores compared to WT males in both FST and TST. Sex differences in immobility scores of FST have been found before by some authors in WT mice (Bale and Vale, 2003; Frye, 2011) and rats (Dalla et al., 2010), but not by others (LaPlant et al., 2009). The reasons for these discrepancies between studies are unclear, but may relate either to paradigm differences in the FST (especially between rat and mice), to the age of the animals or to the estrous cycle phase, being increased immobility typically observed in diestrus females (Frye, 2011). In this study we have replicated the sex differences in two independent experiments using young adult females (2- to 5-month old). In the second FST performed, we observed that most WT females were in estrus phase and displayed higher immobility than males. The effect of estrogen hormones was therefore examined as a possible explanation to the sex differences found between WT animals (Becker et al., 2007; McCarthy et al., 2012). Our results showed that ovariectomy decreased immobility behavior in both FST and TST (compared to sham-operated animals), and that replacement with low levels of E2 fully restored the immobility levels in the TST and partially in the FST. Most published studies did not use sham-operated mice as controls for the ovariectomized mice (e.g. Rocha et al., 2005) but those who did (Frye, 2011; Lagunas et al., 2010) reported either no effect of ovariectomy on FST scores or an increased immobility duration on mice tested between 10 and 18 days after surgery (thereafter no more effects of ovariectomy were observed) (Bekku et al., 2006). Of note, we have tested the females 21 days after ovariectomy, a time frame long enough for total clearance of ovarian hormones. This long-term absence of ovarian hormone might mimic an established state of menopause, in which depressive states induced by hormone levels variation do not occur anymore. Along the same lines, E2 administration is

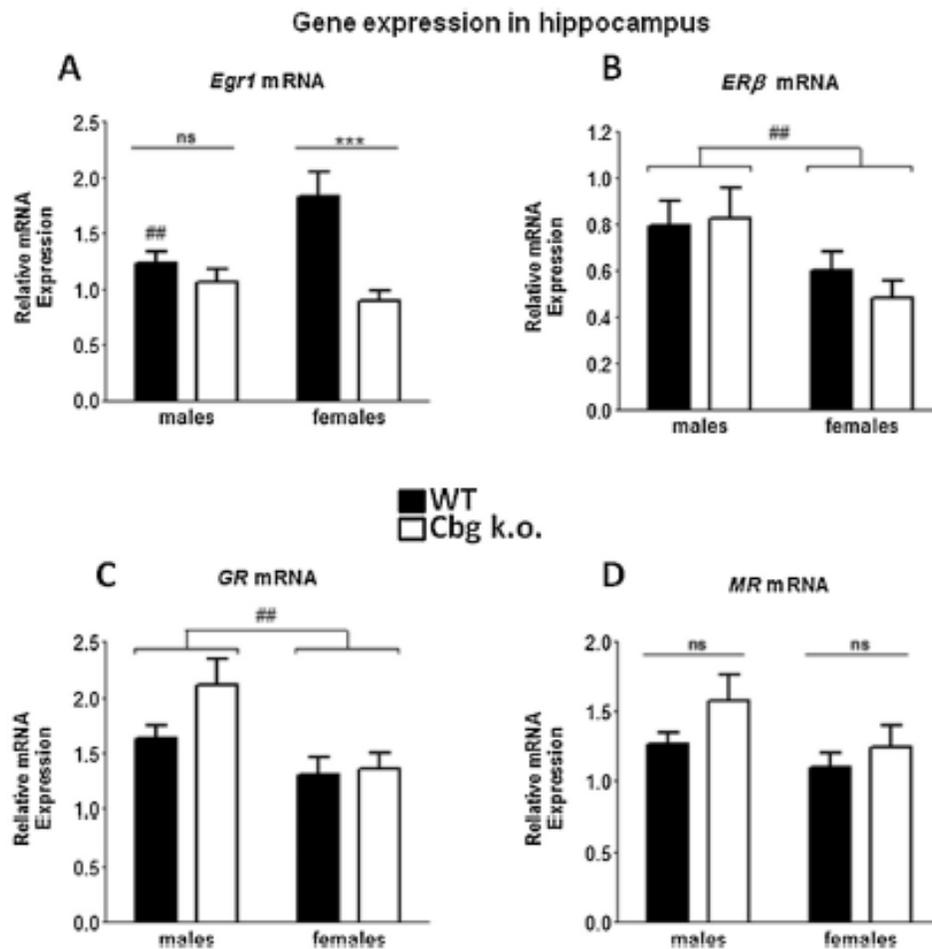


Figure 6 Hippocampal gene expression after FST-induced stress in WT and Cbg k.o. mice. Gene expression was evaluated in the hippocampus of male and female mice from both genotypes by real-time quantitative PCR. mRNA quantification was normalized on 18S gene expression and relative to one animal from the WT male group. EGR1 (A) and ER β gene (B) expression were measured in mice euthanized immediately after FST ($n=7-10$ per group), while GR (C) and MR (D) genes expression were measured in mice euthanized 3 h after FST ($n=6-11$ per group). (***) represents a significant genotype effect within the same sex (two-way ANOVA followed by Fisher LSD post hoc $p < 0.001$). (##) represent either a significant sex effect within WT animals (A) or an overall effect of sex (B, C) (two-way ANOVA followed by Fisher LSD post hoc, $p < 0.01$). ns - not significant.

generally reported as having an anti-depressive effect in mice, while we found that E2 replacement at a low dose restored – at least partially – the despair-like behavior observed in intact or sham-operated mice. The discrepancy with previous studies probably stems again from differences in the paradigm of the FST, or in the time of testing after ovariectomy and in the dose and type of E2 administration. The implants we used were calibrated to release a low dose of E2, while the previously reported anti-depressant effect of E2 in mice was only found efficient in high doses and when injected (Bekku and Yoshimura, 2005; Rocha et al., 2005). Furthermore, the low levels of E2 during the diestrus phase are associated with despair-like behavior, what is consistent with our results.

Interestingly, we found no effect of ovariectomy and E2 replacement on the immobility scores displayed by Cbg k.o. females, which remained elevated both in the FST and in the TST. These results indicate that in females CBG protein interacts with estrogens to regulate emotional reactivity.

The regulation of CBG levels by estrogens has been reported before (Moore et al., 1978; Nader et al., 2006; Qureshi et al., 2007; White et al., 2006) and involves the estrogen receptor alpha (Nader et al., 2006). The question that arose from our results concerned the specific role of CBG in the despair-like behavior displayed by females. Since in Cbg k.o. males suboptimal levels of corticosterone were associated with altered emotional behavior, we investigated whether the same mechanism could be responsible for the alterations observed in Cbg k.o. females.

As already reported by others, we found that basal plasma corticosterone concentrations are higher in WT females than in WT males (Handa et al., 1994; Rhodes and Rubin, 1999). By measuring urine corticosterone levels across the diurnal cycle, we showed that they were also higher in females, indicating, that the well described elevated corticosterone levels in female rodents stems from a different production rate. Interestingly, neither plasma nor urine corticosterone levels, differed between female and

male Cbg k.o mice, indicating that CBG plays an essential role in the sex-related differences in corticosterone secretion observed in WT animals. Regarding the corticosterone response to stress, we found large sex and genotype differences in the total corticosterone surge after the stress induced by the FST. For free corticosterone levels, however, there were no sex differences. In WT mice this is explained by the higher CBG concentrations in females which compensate their higher total corticosterone levels, whereas, the absence of CBG in the Cbg k.o mice results in equivalent free corticosterone levels.

Interestingly, genotype differences in free corticosterone were found both in females and males, suggesting that sub-optimal free corticosterone levels after stress could explain the increased immobility behavior observed in Cbg k.o. in females as previously observed in males. Plasma ACTH levels after FST-induced stress were found slightly higher in Cbg k.o. mice from both sexes, probably reflecting a lower feedback control since free corticosterone levels are lower. However, CBG does not seem to influence the overall HPA axis drive. The origin of lower corticosterone levels in Cbg k.o. was revealed by the turnover analysis. The quantity of corticosterone bound to CBG in the plasma of WT animals constitutes a large pool of this hormone, which is prevented from elimination due to this binding. In Cbg k.o. mice this plasma pool is considerably reduced. Similar data were obtained in humans in which CBG was found to influence positively corticosterone pool size (Bright, 1995). To further characterize the glucocorticoid activity in these mice, we measured *Egr1*, MR and GR mRNA expression in the hippocampus after the stress induced by the FST. *Egr1* was reduced in Cbg k.o. animals when compared to WT, particularly in females, in accordance with the free corticosterone levels measured in plasma. No significant differences between genotypes were detected in MR and GR mRNA expression, similarly to what was previously found in males (Richard et al., 2010). Sex differences in corticosterone receptors in the hippocampus were reported recently (Lin et al., 2011) but lower expression in females was more pronounced for MR rather than GR. The divergent findings might stem from the age of the animals tested, since in their report mice were tested at a young age (7 weeks), whereas we used 2- to 5-month-old mice. The lower expression of ER β mRNA observed in females is also congruent with previous reports, as this receptor was shown to mediate the protective effect of estrogens on despair-like behavior (Rocha et al., 2005).

Finally, no genotype differences were found for other emotional behaviors measured in the elevated plus-maze and in the open-field tests. Furthermore, no difference in the locomotor activity was observed between WT and Cbg k.o. mice. We thus conclude that the effects of CBG deficiency are specific to despair-like behavior. This specificity was also found in our previous study on Cbg k.o. males (Richard et al., 2010).

In conclusion, our results provide new data on the role of CBG in sex differences regarding emotional behavior and HPA axis regulation in mice. In WT mice, estrogens levels (or maybe their fluctuation) are sufficient to explain the sex differences in despair-like behavior. However, in both sexes, CBG deficiency leads to depressive-like behaviors associated with low levels of corticosterone, which overcomes

the negative effect of decreasing estrogens levels that is observed in WT estrous females. Our results also show that CBG is necessary for the sex differences observed in emotional reactivity and that it contributes to the elevated total circulating levels of corticosterone, in females compared to males, by influencing its production rate. As CBG is regulated by estrogens, it seems that this circulating protein is an important intermediary molecule for estrogens and glucocorticoids crosstalk in regulating despair-like behavior. From our data we can infer that CBG presence is necessary to attain optimal levels of glucocorticoids, which in turn are important for normal behavioral response, regardless of the sex. The positive regulation of CBG by estrogens in females necessarily influences glucocorticoids availability in blood. Further analyses are required to understand how *in fine* this estrogens-induced CBG regulation of glucocorticoids availability in females is pejorative for their emotional behavior, but an alteration in glucocorticoid pulsatility may be an interesting lead (Lightman and Conway-Campbell, 2010).

The role of gender has gained recognition as an important factor influencing stress-related mental disorders such as depression. Glucocorticoid and estrogen hormones are both involved and they interact in a complex way to explain sex differences. The results of this study suggest that CBG is a key component in the estrogens-glucocorticoids crosstalk explaining sex differences in emotional reactivity.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.psyneuen.2014.07.029>.

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Complementary studies

The experiments described in this paper have clarified some relevant aspects of CBG influence on emotional reactivity sex differences. One of the main findings was that although wild-type and Cbg ko females differ regarding their plasma corticosterone profiles – both in baseline and after stress – the differences in despair-like behaviors previously described between wild-type and Cbg ko males are not observed among cycling females. Interestingly, this differences appear in females that had their ovaries removed, and therefore do not present an estrous cycle. The influence of reproductive hormones on the modulation of despair-like behaviors by CBG was then confirmed by implanting ovariectomized females with estrogen pellets, which released slow and continuous doses of estrogen and maintained low concentrations of these hormones in the plasma. Females that received these pellets have recovered the high despair-like behavioral profile of cycling females.

Another important finding concern the source of the lower corticosterone levels in the Cbg ko mice. Our results suggest that it stems from a higher plasmatic clearance rate, and not from a lower production or from increased feedback system sensitivity. We have shown that after having their endogenous corticosterone secretion suppressed and receiving a bolus injection of this hormone, wild-type mice are able to retain it two(males)- to three(females)-times more in their plasma than Cbg ko mice. It seems that CBG, through a still unknown mechanism, is able to reduce the elimination of free corticosterone from the plasma.

After the conclusion of this work we have pursued on the study of how CBG influences these sex-differences in emotionality. Since we have observed that Cbg ko male mice, as well as wild-type and Cbg ko female mice, display higher despair-like behaviors than male wild-type mice, we wondered whether their responses to an antidepressant treatment would be different according to the animal's sex or genotype. The drug chosen to perform this experiment was imipramine, a tricyclic antidepressant that is commonly used to treat atypical depression. We chose this drug because the despair-like behavior displayed by Cbg ko mice is associated with low levels of corticosterone, what is also commonly observed among patients with atypical depression.

For this experiment, 40 female mice (20 Cbg ko and 20 wild-type) and 31 male mice (14 Cbg ko and 17 wild-type) between 2 and 6 months old were used. Animals were injected with either imipramine (20 mg/kg, intraperitoneally) or saline solution (sample sizes shown in Table 4) 30 min before being submitted to each test. The first test to be performed was the FST, followed by the TST after a one week interval. As previously described on page 80, these two behavioral tests consist on submitting the animals to an inescapable and stressful situation – as being dropped in a water-filled cylinder in the FST or being suspended by the tail in the TST – for 6 min. After some initial escape-oriented movements, the animals develop an immobile posture known to be reduced by drugs with antidepressant activity. Both tests are also used to investigate depression and antidepressant-related phenotypes (Cryan et al., 2005). The tests' conditions and posterior analyses were performed as described on page 80.

Data obtained in both behavioral tests were analyzed through a two-way ANOVA for the factors treatment and genotype. An extremely significant effect of the treatment with imipramine on the immobility scores (Figure 13A,B,D) was detected, with animals injected with the tricyclic antidepressant persisting longer in the escape-oriented swimming than the controls ($F(1,27) = 15.59$, $p < 0.001$ for the male mice in the TST; $F(1,36) = 25.53$, $p < 0.001$ for the female mice in the FST; $F(1,32) = 15.16$, $p < 0.001$ for the female mice in the TST). No significant effect of treatment was detected for the male mice in the FST (Figure 13C), although the ANOVA revealed a marginal trend towards significance ($F(1,27) = 3.78$, $p =$

0.06). This may be due to the differential sensitivity of both tests (FST and TST) to the immobility-reducing effects of various antidepressants, with the TST being apparently more sensitive to these drugs' effects (Cryan et al., 2005). Also, it's been described that female mice respond to lower doses of imipramine in the FST than male mice (David et al., 2001). The previously found difference between males from both strains in the immobility scores weren't observed in this experiment, not even between the control groups. We hypothesized that this could be due to some methodological differences from the previous experiments, notably the housing conditions, the observer's criteria (in the case of the FST) or the injection that preceded both tests and might have altered the animals' response to the subsequent stress. Some of these factors have already been shown to play an important role in the results' variability (Petit-Demouliere et al., 2005). Further studies should be performed to clarify this issue. Nevertheless, these results demonstrate that Cbg ko mice respond to the treatment with the tricyclic antidepressant imipramine with a decrease in the immobility time, although differences between both strains' responses still need to be investigated.

Table 4 – Sample sizes of groups of animals submitted to the forced swim test (FST) and tail suspension test (TST) after being treated with imipramine (20 mg/kg) or saline solution.

		Saline		Imipramine	
		Wt	Ko	Wt	Ko
FST	♀	10	10	10	10
	♂	8	7	9	7
TST	♀	9	9	8	10
	♂	9	7	8	7

Right after the TST, all animals were anesthetized with isoflurane and blood was collected through a cardiac puncture before sacrifice. Blood samples were placed into 1,5ul microtubes containing EDTA and later centrifuged to obtain plasma samples. Percentage of free corticosterone and total plasma corticosterone were quantified through biochemistry techniques, as previously described on page 160. As expected, the two-way ANOVA for the factors treatment and genotype has revealed a highly significant effect of genotype over the two variables analyzed.

Regardless of the sex, levels of total plasma corticosterone are higher in wild-type mice – which have most of their corticosterone (about 90% under basal conditions) stored in its inactive form, bound to CBG – than in Cbg ko mice (Figure 14A,B; $F(1,16) = 4.39$, $p < 0.001$ for male mice; $F(1,33) = 1.16$, $p < 0.001$ for female mice). On the other hand, the percentage of free corticosterone is higher in Cbg ko animals (Figure 14C,D); $F(1,16) = 1.43$, $p < 0.001$ for male mice; $F(1,32) = 0.10$, $p < 0.001$ for female mice), once they have no corticosterone bound to CBG and only a small fraction of this glucocorticoid (10-15%) is bound to albumin (Breuner and Orchinik, 2002). These findings corroborate those already described on previous work from our group (Richard et al., 2010, Minni et al., 2012). Interestingly, the analyses of the absolute values of free corticosterone revealed a main effect of treatment in males ($F(1,16) = 4.75$, $p < 0.05$), with imipramine increasing plasma free corticosterone concentrations (Figure 14E). No genotype effect or interactions between factors were detected, but the increase in free plasma corticosterone levels seems to be slightly higher in wild-type males treated with imipramine, possibly due to the higher clearance observed in Cbg ko mice. As for the females (Figure 14F), no treatment effect or interaction were detected, but a main overall effect of genotype was observed ($F(1,32) = 6.37$, $p < 0.05$). Despite no interaction being detected, here it is also possible to observe that

the levels of free corticosterone in wild-type females seem to increase with the imipramine treatment, similarly to the profile observed in males, and that trend is not observed for Cbg ko females.

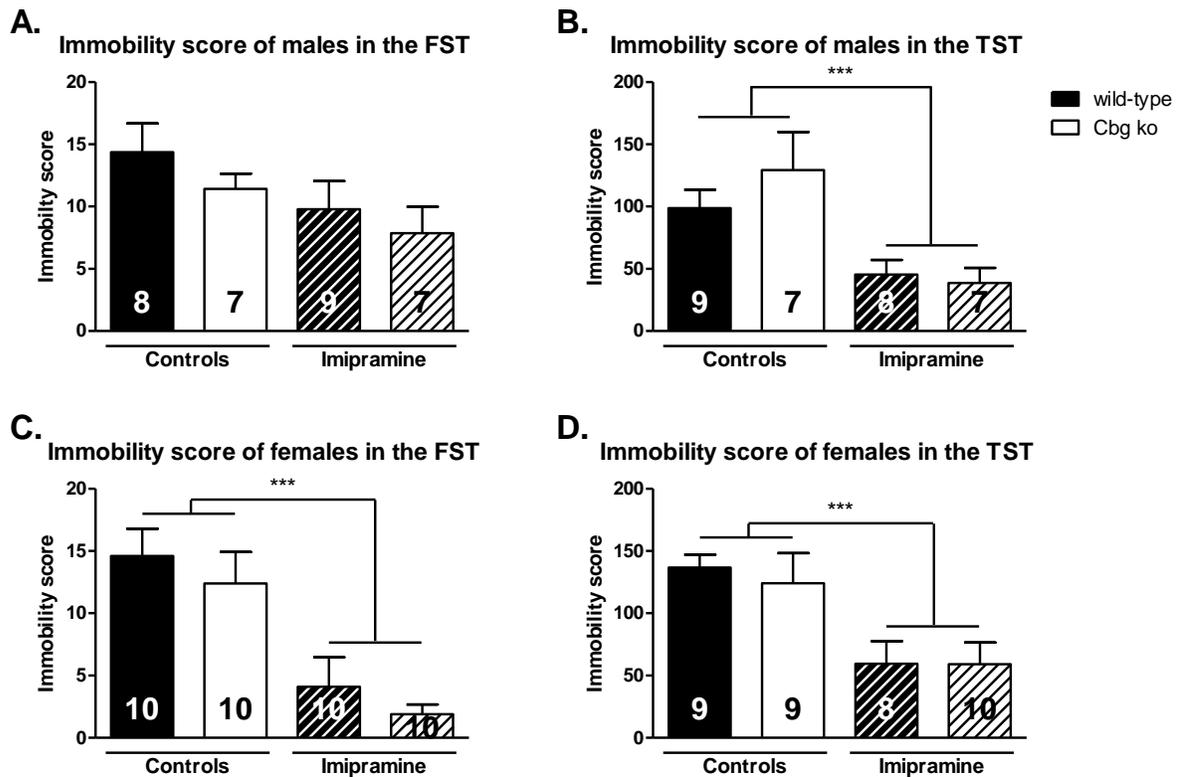


Figure 13 – Immobility scores displayed by male (A,B) and female (C,D) wild-type and Cbg ko mice in the forced swim test (FST, A,C) and in the tail suspension test (TST, B,D). (*) represents significant ($p < 0.001$) overall effect of imipramine treatment. Two-way ANOVA.**

The effects of antidepressants are usually associated with decrease in glucocorticoid levels. The majority of studies using tricyclic antidepressants have shown antidepressant-induced upregulation of brain GR, MR, or both. This, in turn, is associated with enhanced negative feedback by endogenous glucocorticoids, and thus with reduced resting and stimulated HPA axis activity (Pariante et al., 2004). Furthermore, imipramine treatment not only has been shown to attenuate the increase in plasma corticosterone levels after stress, but also prevents behavioral and cellular phenotypes associated to depression (Szymanska et al., 2009, Guan et al., 2014, Solomon et al., 2014, Fenton et al., 2015). No effect of treatment was found for total or percentage of free corticosterone in this study, but the analyses of the free levels have revealed an effect of treatment, with imipramine increasing corticosterone levels in males only. This effect was opposite to what we expected based on the literature, and could be a reflex of the interaction between imipramine and the stress caused by the TST rather than the effect of this antidepressant in the basal activity of the HPA axis. Even though no interaction between factors were detected in males or in females, a discrete trend towards higher levels is seen for both male and female wild-type mice treated with imipramine, while the levels of Cbg ko from both sexes don't seem to be altered. Further studies are necessary to confirm if these trends are in fact reflecting a differential response of the HPA axis of wild-type and Cbg ko mice to imipramine. Furthermore, the previously cited studies which evaluated the impact of imipramine treatment in plasma

corticosterone levels were performed either in semi-chronically (3 injections within 24 hours) or chronically (from 5 to 21 days). Although the effect of imipramine, as that of many other antidepressants, can be easily assessed in despair-like behavior tests after a single acute administration (Cryan et al., 2005, Petit-Demouliere et al., 2005) some physiological effects might demand repeated administration to be observed.

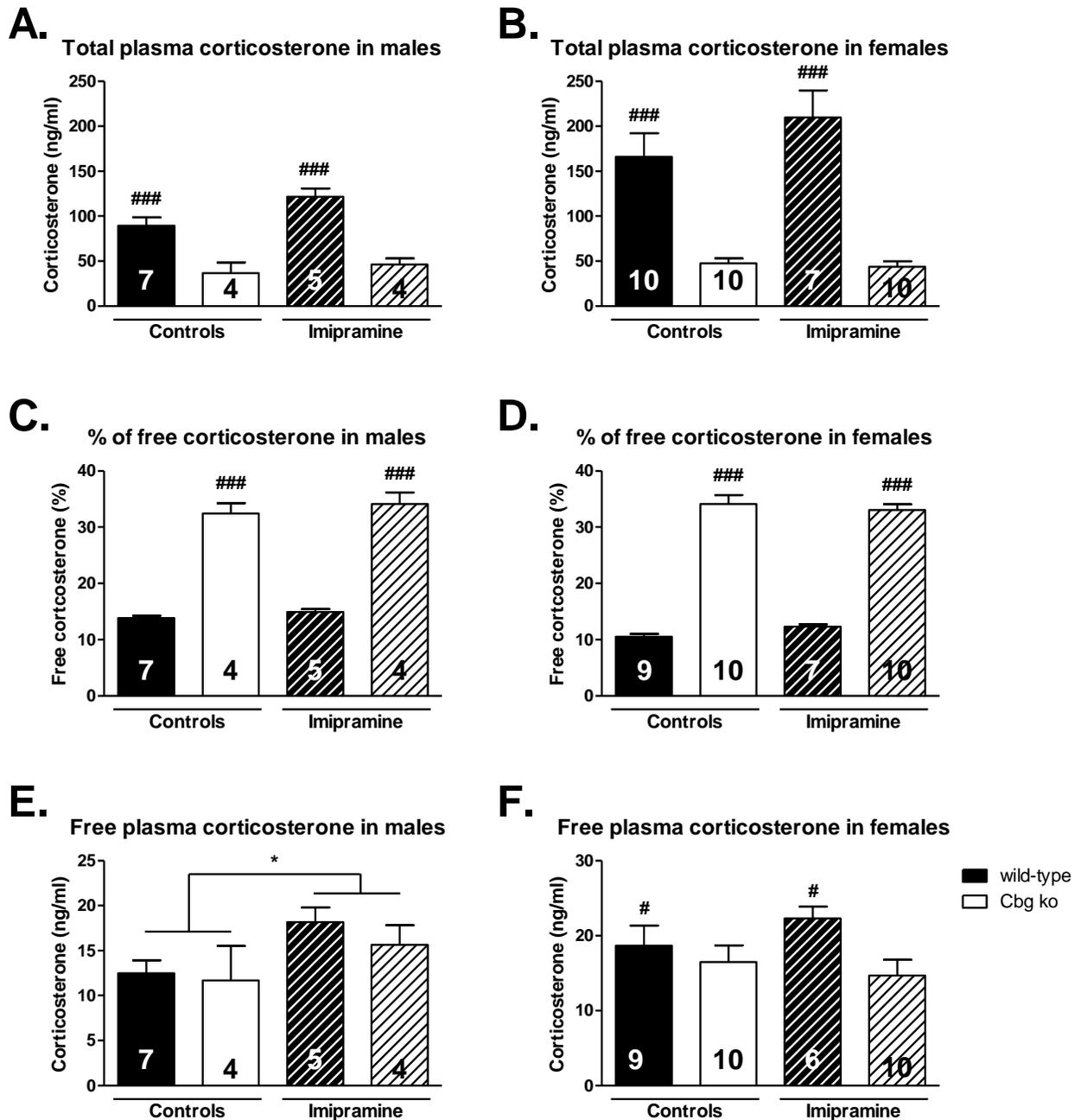


Figure 14 - Total (A,B), percentage of free (C,D) and absolute free (E,F) levels of corticosterone in the plasma of male (A,C,E) and female (B,D,F) wild-type and Cbg ko mice submitted to the tail suspension test. (#) and (###) represent significant ($p < 0.05$ and $p < 0.001$, respectively) overall effect of genotype, while (*) represents an overall effect of treatment. Two-way ANOVA.

Overall we can say that these studies have contributed to a better understanding of the sex differences on the consequences of CBG deficiency, more specifically regarding

emotionality-related phenotypes. We have demonstrated that the increase in despair-like behaviors triggered by CBG deficiency is masked in cycling females by circulating estrogens. We have also shown that Cbg ko mice, regardless of the sex, have a faster corticosterone clearance from plasma than wild-type mice, and that is at least one of the sources of the differences in their plasma corticosterone levels. Finally, we show that the tricyclic antidepressant imipramine is able to reverse the despair-like phenotype of Cbg ko mice from both sexes, and that this effect might be linked to an increase of free corticosterone levels.

STUDY II – CHRONIC STRESS DOES NOT FURTHER EXACERBATE THE ABNORMAL PSYCHONEUROENDOCRINE PHENOTYPE OF CBG-DEFICIENT MALE MICE

Introduction

The effects of stress on physical and psychological integrity are frequently described as variable, being responsible for either enhancing or impairing different body functions. One of the sources of these opposing effects is the duration of the stress response, which can be divided into acute stress – lasting from minutes to hours – and chronic stress – which may last from days to months. The short-term secretion of stress hormones and other mediators, such as neurotransmitters, cytokines, and other hormones, is essential for adaptation to challenges of daily life as well as to major life stressors. When secreted in response to immediate stressors or to lifestyle factors – such as diet, sleep, and exercise – these allostasis mediators promote adaptation and are generally beneficial. However, the failure to shut-down the stress response system, just as an inadequate turn-on during the stress response or even an overuse by different or repeated stressors, can lead to cumulative changes that will deteriorate the stress response system (McEwen, 2004, Moisan and Le Moal, 2012).

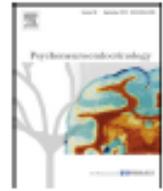
As an example of these opposing effects are the consequences of the secretion of stress hormones – adrenaline and cortisol – in the brain. When in response to an acutely threatening event it promotes and improves memory for the situation, increasing the individual's chances of a successful coping with a similar situation in the future; yet, when the stress is continuous or repeated, it may decrease synaptic plasticity and cause atrophy of dendritic processes (mainly in structures such as the hippocampus and the frontal cortex), and even cause a loss of total volume or gray matter volume, underlying some of the adverse neurocognitive consequences of stress-related diseases. The opposite occurs in the amygdala, particularly the BLA, where stress and glucocorticoids increase synaptic plasticity and promote the expansion of dendritic processes, contributing to the adverse effects of stress on fear acquisition and extinction (McEwen, 2004, Sapolsky, 2015). In fact, prolonged episodes of stress – particularly when experienced as uncontrollable and unpredictable – have been reported to increase the vulnerability to several diseases in genetically predisposed individuals, including psychiatric and neurological illnesses (Joels et al., 2012). Corroborating the importance of time and length of the stressor in determining its effects is a study that shows that hippocampal expression of several genes is altered after either acute stress, chronic stress or stress recovery, but that a large number of these alterations are exclusive to each condition (Gray et al., 2014).

The nervous system regulates the physiological and behavioral coping responses to stressors of different intensities, and the brain is itself a target of the mediators of those responses, such as glucocorticoids, catecholamines and other transmitters. As previously mentioned, the amygdala and hippocampus are two brain structures deeply involved in the interpretation and in the selection of appropriate responses to the received stress stimuli. As examples of the detrimental effects of chronic stress on the amygdala is its hyperactivity in posttraumatic stress disorder and depressive illness, or hypertrophy of its nerve cells. As for the hippocampus, chronic stress has been shown to promote a decrease in dendritic branching and reduction in the number of neurons in the dentate gyrus (McEwen, 2004).

Many animal models have been developed to examine the effects of chronic stress in more details under very controlled experimental conditions. Many studies demonstrated changes in dendritic complexity, spine density and the expression of many molecules involved in neurotransmission after exposure to chronic stress and/or overexposure to

corticosteroid hormones, regardless of the paradigm that was used. All of these changes are expected to affect neural activity. At the same time, the consequences of chronic stress for various behavioral tasks have been addressed in details in numerous animal studies. Chronic stress reduces hippocampal synaptic plasticity in parallel with impaired spatial memory. Working memory and reward anticipatory behavior were also found to be disturbed. Furthermore, the cognitive outcome after chronic stress seems to be task-specific, differing, for instance, accordingly to the level of emotional arousal induced by the task. Finally, the effects of chronic stress on the behavior of male animals may differ from those observed in female animals. Altogether, these studies gather evidence linking several neuronal and behavioral alterations, but the step from altered neuronal activity to disturbed behavior after chronic stress is not fully understood (Luine, 2002, Conrad, 2010, Marin et al., 2011, Joels et al., 2012).

Considering the central role of glucocorticoids on stress response and on the detrimental consequences of chronic stress, and the importance of CBG in regulating the effects of glucocorticoids on the body and on the brain, we wondered whether CBG would also modulate the impact of chronic stress on the behavior of mice. As our group has extensively studied the impact of CBG deficiency in despair-like behavior, which is also widely known to be exacerbated by chronic stress, we have decided to focus on this behavior. The following paper presents a study that was initially performed by A Minni during her PhD under MP Moisan's supervision. All the behavioral tests and blood sampling were performed back then, by A Minni, while the CBG and corticosterone assays, as well as a detailed data analysis and the writing of this article were carried out under the scope of this thesis.



Short communication

Chronic stress does not further exacerbate the abnormal psychoneuroendocrine phenotype of Cbg-deficient male mice

Gabriela F. de Medeiros^{a,b}, Amandine M. Minni^{a,b}, Jean-Christophe Helbling^{a,b}, Marie-Pierre Moisan^{a,b,*}^aINRA, Nutrition et neurobiologie intégrée, UMR 1286, 33076 Bordeaux, France^bUniv. Bordeaux, Nutrition et neurobiologie intégrée, UMR 1286, 33076 Bordeaux, France

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ABSTRACT

Chronic stress leads to a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis which can constitute a base for pathophysiological consequences. Using mice totally deficient in Corticosteroid binding globulin (CBG), we have previously demonstrated the important role of CBG in eliciting an adequate response to an acute stressor. Here, we have studied its role in chronic stress situations. We have submitted Cbg ko and wild-type (WT) male mice to two different chronic stress paradigms – the unpredictable chronic mild stress and the social defeat. Then, their impact on neuroendocrine function – through corticosterone and CBG measurement – and behavioral responses – via anxiety and despair-like behavioral tests – was evaluated. Both chronic stress paradigms increased the display of despair-like behavior in WT mice, while that from Cbg ko mice – which was already high – was not aggravated. We have also found that control and defeated (stressed) Cbg ko mice show no difference in the social interaction test, while defeated WT mice reduce their interaction time when compared to unstressed WT mice. Interestingly, the same pattern was observed for corticosterone levels, where both chronic stress paradigms lowered the corticosterone levels of WT mice, while those from Cbg ko mice remained low and unaltered. Plasma CBG binding capacity remained unaltered in WT mice regardless of the stress paradigm. Through the use of the Cbg ko mice, which only differs genetically from WT mice by the absence of CBG, we demonstrated that CBG is crucial in modulating the effects of stress on plasma corticosterone levels and consequently on behavior. In conclusion, individuals with CBG deficiency, whether genetically or environmentally-induced, are vulnerable to acute stress but do not have their abnormal psychoneuroendocrine phenotype further affected by chronic stress.

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1. Introduction

While an appropriate acute response to stress is crucial for adaptation, intensified and prolonged responses have been suggested to cause or exacerbate several ailments—from common cold to cancer and various psychiatric conditions (de Kloet et al., 2005; McEwen et al., 2015). Given the amount of chronic stressors that individuals living in modern societies are exposed to, the study of its impact on health is of great importance. The hypothalamic-pituitary-adrenal (HPA) axis is the major neuroendocrine system involved in the stress response, and regulates the secretion of glucocorticoids. Prolonged stressor exposure, called chronic stress, leads

to a dysregulation (hyper- or hypo-activity) of the HPA axis which can induce detrimental effects on the whole body and constitute a base for pathophysiological consequences (Chrousos, 2009; de Kloet et al., 2005; McEwen, 2007).

Evidence shows that Corticosteroid binding globulin (CBG) – a plasma binding protein with high affinity for glucocorticoids – is particularly important for an appropriate response to stress. Our team has developed a model of HPA axis hyporesponse due to CBG deficiency, the Cbg ko mice. These animals display altered emotional reactivity and memory function after acute stress, which is associated with a blunted rise of glucocorticoids. We have reported that male Cbg ko mice display more despair-like behaviors than wild-type (WT) mice. This was observed in several depression models that involve acute stressors, such as the forced swim test, the tail suspension test and the learned helplessness paradigm (Richard et al., 2010). These results were confirmed in female Cbg ko mice, although estrogens-glucocorticoids crosstalks take place in WT ani-

* Corresponding author at: Nutrition and Integrative Neurobiology (NutriNeuro)—Université Bordeaux, INRA UMR1286, 146 Rue Léo Saignat, 33076 Bordeaux—France.

E-mail address: mpmoisan@bordeaux.inra.fr (M.-P. Moisan).

mals (Minni et al., 2014). We have also demonstrated that CBG, through its role in regulating GC delivery to the brain, is indirectly involved in memory processes. Indeed, Cbg ko mice are insensitive to the impairments in memory retrieval induced by an acute stress, due to their lower rise of GC levels in the hippocampus (Minni et al., 2012).

In the light of the important role of CBG for an adequate response to an acute stressor, we decided to study its role in chronic stress situations. For that, we have chosen two different paradigms that can be compared to chronic stressors to which humans are regularly submitted to: the unpredictable chronic mild stress (UCMS) – representing the chronic accumulation of daily mild stressors; and the social defeat – as a parallel to more intense and stressful situations promoted by social interactions. Cbg ko mice were submitted to both chronic stress paradigms, and their impact in some neuroendocrine and behavioral responses was then evaluated.

2. Materials and methods

2.1. Animals

Two groups of 6–7 months old mice (MGI ID: 4833641, Serpina6tm1.1Mmp, created in our laboratory in Bordeaux–France) were used in this experiment. Cbg ko and WT mice were littermates, obtained by breeding males and females heterozygous for the CBG mutation, and genotyped from a tail biopsy. As 1 out of 8 pups on average has the male sex and genotype Cbg^{-/-} (ko) or Cbg^{+/+} (WT), and the litter size was of 2–12 pups (mean of 6.5 pups), we used 1 pup per litter per genotype per group. Our mice have a C57Bl/6J genetic background above 95%. Animals were maintained, from birth and throughout the whole experiment, in an animal facility with constant temperature (23 °C), under a 12 h light/dark cycle (lights on at 0700 h) and with *ad libitum* access to food and water. All the experiments were conducted in strict compliance with current European Conventions (86/609/CEE) and approved by Institutional Regional Committee for animal experimentation (agreement# 5012050-A).

2.2. Unpredicted chronic mild stress

A first set of 36 mice, 8–10 mice/group, was evaluated in this experiment. Half of the animals from each genotype were placed into individual cages and submitted to a 4-week protocol of UCMS (adapted from Schweizer et al., 2009) while the other half were kept four mice per cage and served as the control group. The UCMS consisted of random exposure to several mild stressors, twice a day. The stressors included wet cage, tilted cage, no sawdust bedding, lights-on overnight, cage exchange with another mouse and facial air puff. After the four weeks of UCMS, mice were submitted to behavioral tests and sacrificed four weeks after the end of the UCMS protocol.

2.3. Social defeat

A second set of 24 mice, 6 mice/group, was submitted to a social defeat protocol as described in Berton et al., 2006 and modified according to Larrieu et al., 2014. Half of the mice were exposed to a CD1 mouse (aggressor) for 10 days, 5 min a day; while the other half served as the control group. Social defeat was followed by 3 h of protected sensory contact with the aggressor. Different aggressors were used on each day to prevent any habituation, and animals from the same cage were tested simultaneously. During the 3 h of protected sensory contact, mice from the control groups were submitted to a similar display, but in the absence of other mouse.

Animals were sacrificed one week after the end of the social defeat protocol.

2.4. Behavioral tests after chronic stress

Mice from the first (after UCMS) and second (social defeat) experiments had their despair-like behavior (assessed through the forced swim test) and anxiety-related behavior (assessed through the open field test after the UCMS and through the elevated plus maze after the social defeat) analyzed. All three tests were performed as previously described (Minni et al., 2014; Richard et al., 2010). All behavioral sessions were recorded and analyzed by a trained observer blind to the animals' genotypes.

Twenty-four hours after the end of the social defeat protocol, both control and defeated mice were submitted to the social interaction test (Berton et al., 2006; Larrieu et al., 2014). During the first 5 min of test, the mouse was placed in an open field arena containing an empty wire mesh cage in one of the corners. Then, a CD1 mouse was introduced into the wire mesh cage and active investigatory behavior towards the intruder was recorded for another 5 min to assess social interaction. Approach and avoidance zones were defined and the number of visits and time spent in each of these zones were quantified by a trained experimenter blind to the animals' genotypes.

2.5. Sacrifice

Mice from both experiments were anesthetized with isoflurane (Aerrane, Baxter SA, Maurepas, France) and blood samples were obtained by cardiac puncture. Blood samples obtained in the morning were centrifuged at 10,000 rpm for 10 min, and the supernatant (plasma) was stored at –80 °C.

2.6. Total corticosterone and CBG measures

Total corticosterone was measured by an in-house radioimmunoassay (RIA) from plasma samples previously extracted with ethanol. This assay is based on the competition for a specific anti-corticosterone antibody, between endogenous plasma corticosterone and increasing concentrations of a radio-labeled corticosterone (³H) (see Richard et al., 2010 for details). Anti-corticosterone antibody was provided by Dr. H. Vaudry (University of Rouen, France). CBG B_{max} in WT animals was estimated by adding increasing concentrations of radio-labeled corticosterone (³H) (from 0.5 nM up to 32 nM) to plasma that had their endogenous steroids previously removed with charcoal-dextran. Through linear regression we obtained a saturation curve that allowed us to determine CBG's maximum binding capacity (Richard et al., 2010).

2.7. Statistics

All results are expressed as mean ± S.E.M. Statistical values were calculated with the software Statistica v.6.1. Unpaired Student's *t*-test was used for CBG binding capacity assay results. Two-way analysis of variance (ANOVA) was used to detect genotype and chronic stressor effects, followed by Duncan post hoc tests when necessary. A two-way repeated measures ANOVA was used to analyze weight change throughout the chronic stress paradigms, also followed by Duncan post hoc tests when necessary. The level of statistical significance was set as *p* values <0.05 for all tests. Outliers were detected and removed from analyses following Grubbs test.

3. Results

Mice submitted to both types of stress lost weight when compared to unstressed groups [main effect of UCMS, *F*(1,32)=9.72,

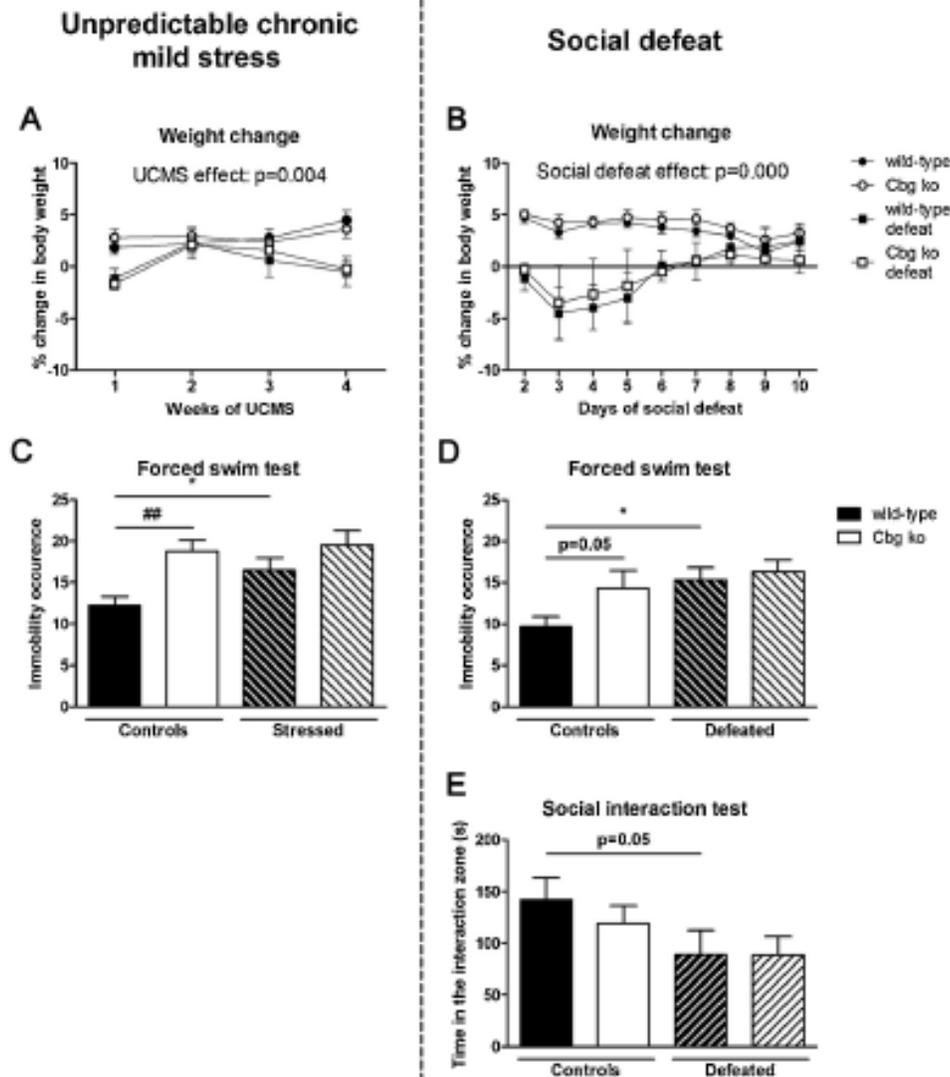


Fig. 1. Weight change percentage (a, b) and performance in the forced swim test (c, d) of wild-type and Cbg ko mice submitted to the unpredictable chronic mild stress (UCMS) and the social defeat chronic stress protocols, and time spent in the interaction zone during the social interaction test after social defeat (e). (##) represents significant difference between control wild-type and Cbg ko mice ($p < 0.01$), while (*) represents significant difference between control and defeated wild-type mice ($p < 0.05$). Repeated measures two-way ANOVA for (a, b). Two-way ANOVA followed by Duncan's post-hoc test for C, D, E ($n = 8-10$ mice/group for UCMS and 6 mice/group for social defeat).

$p < 0.01$; main effect of social defeat, $F(1,20) = 17.50$, $p < 0.0001$]. No effect of genotype [$F(1,32) = 0.02$, $p = 0.89$ for UCMS and ($F(1,20) = 0.23$, $p = 0.64$) for social defeat] or interaction between factors [$F(1,32) = 0.00$, $p = 1.00$ for UCMS and ($F(1,20) = 0.06$, $p = 0.82$) for social defeat] was observed (Fig. 1a, b). No effect of chronic stress or genotype was observed in anxiety-related behaviors, as assessed in the open field test (UCMS) and in the elevated plus maze (social defeat). Also, no effect of chronic stress or genotype on locomotor activity was detected, as assessed in these anxiety tests. (Supplementary Fig. A). Concerning despair-like behaviors, a main effect of genotype ($F(1,30) = 11.4$, $p < 0.01$) but not of UCMS ($F(1,30) = 3.1$, $p = 0.09$) was detected in the forced swim test after UCMS (Fig. 1c). Although no significant interaction between UCMS and genotype was observed ($F(1,30) = 1.58$, $p = 0.22$), a post hoc test revealed that WT mice increase their immobility time after submitted to UCMS ($p < 0.05$), while Cbg ko mice do not. As for social defeat stress, a main effect of social defeat ($F(1,20) = 5.63$, $p < 0.05$) but not the genotype ($F(1,20) = 3.07$, $p = 0.09$) or interaction ($F(1,20) = 1.29$, $p = 0.27$) on despair-like behavior was detected. Similarly to UCMS, a post hoc test revealed

that WT mice increase their immobility time in the forced swim test after submitted to social defeat ($p < 0.05$), while Cbg ko mice do not. Finally, regarding the social interaction test performed after social defeat (Fig. 1e), we found no main effect of genotype ($F(1,20) = 0.00$, $p = 0.99$) or social defeat ($F(1,20) = 3.64$, $p = 0.07$) on the social interaction time. No significant interaction between social defeat and genotype was observed ($F(1,20) = 1.44$, $p = 0.24$), but a post hoc analysis showed that defeated (stressed) WT mice reduced their time interacting with a male CDI when compared to controls ($p = 0.05$).

Considering the putative role of CBG deficiency on these behavioral differences observed between WT and Cbg ko mice, we have looked at corticosterone levels as well as at the CBG binding capacity in animals from both stress experiments (Fig. 2). As previously described (Minni et al., 2014; Richard et al., 2010), levels of total corticosterone under control conditions were found to be lower in Cbg ko than in WT mice (Fig. 2a, b). For the animals submitted to the UCMS, a main effect of genotype ($F(1,31) = 18.62$, $p < 0.001$) but not of UCMS ($F(1,31) = 3.52$, $p = 0.07$) was detected on plasma corticosterone values. Also, no interaction between UCMS and genotype

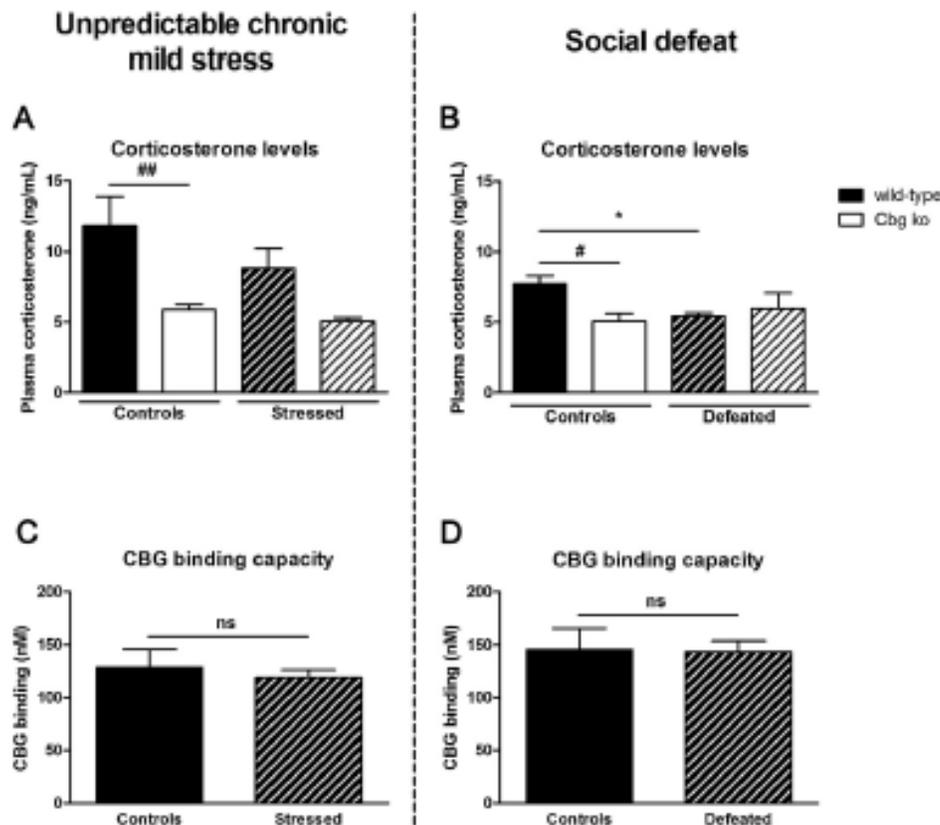


Fig. 2. Total plasma corticosterone levels in the morning (a, b) and CBG binding capacity (c, d) in wild-type and Cbg ko mice submitted to the unpredictable chronic mild stress (a, c) and the social defeat (b, d) stress protocols. (#) represents significant difference between control wild-type and Cbg ko mice (# $p < 0.05$, ## $p < 0.01$, while (*) represents significant difference between control and defeated wild-type mice ($p < 0.05$). Two-way ANOVA followed by Duncan's post-hoc test (a, b) or Student's *t*-test (c, d) ($n = 6$ – 8 mice/group for UCMS and $n = 6$ mice/group for social defeat except WT defeated $n = 4$ in (2)).

was detected ($F(1,31) = 0.00$, $p = 0.95$), but the post hoc analysis showed higher levels of plasma corticosterone in WT mice ($p < 0.01$) under control conditions (Fig. 2a). As for the animals submitted to the social defeat, no main effect of genotype ($F(1,16) = 1.98$, $p = 0.18$) or social defeat stress ($F(1,16) = 0.92$, $p = 0.35$) was found for plasma corticosterone concentration. However, a significant interaction between social defeat and genotype ($F(1,16) = 4.78$) ($p < 0.05$) was detected, with Cbg ko mice displaying lower plasma corticosterone levels than WT mice ($p = 0.05$, post-hoc test) in control conditions (Fig. 2b). We observed that both stress paradigms lowered the corticosterone levels of WT mice although only significantly for social defeat ($p < 0.05$ for social defeat, $p = 0.18$ for UCMS), while those from Cbg ko mice remained unaltered and low. Plasma CBG binding capacity, on the other hand, remains unaltered in WT mice regardless of the stress paradigm (Fig. 2c, d).

4. Discussion

We had previously shown that Cbg ko mice show more despair-like behaviors than WT mice in different depression models (Richard et al., 2010), and these differences have been consistently confirmed in the non-stressed animals from both stress paradigms herein evaluated. We additionally observed an increase in the display of despair-like behavior in WT mice, exposed to chronic stress. However, no noticeable increase of this behavior was observed in CBG KO mice, that show an already high level of despair in control conditions. The same behavioral pattern was found in mice that underwent the social defeat and were later evaluated in the social interaction test: stressed WT mice reduced their interaction time, while Cbg ko mice maintained an

unaltered and low score, comparable to that from stressed WT mice.

Alterations in the HPA axis response due to chronic stress seem to underlie these behavioral changes. The levels of corticosterone followed the same pattern observed for the behavioral data, with variation (decrease) between the stressed and unstressed group being observed in WT animals only. Although the most common assumption is that glucocorticoid levels increase after stressful conditions, a recent review on the effects of chronic stress on corticosterone profiles (Dickens and Romero, 2013) shows that in about half of the studies, performed on lab animals, corticosterone levels are either decreased or unaltered. These same authors propose that a change in glucocorticoid levels might be a better marker for stress conditions than an increase itself. It has been suggested that variations on the kind and length of the stressors, as well as individual features, might explain these apparently controversial findings (Miller et al., 2007). Regarding CBG levels, previous studies in different species have reported either a reduction (Neufeld et al., 1994; Spencer et al., 1996) or no change (Cyr et al., 2007) in its binding capacity after chronic stress. Despite finding no change in CBG levels after our two chronic stress protocols, this protein seems to be crucial in modulating the effects of stress on plasma corticosterone levels and consequently on behavior. This is herein demonstrated through the use of the Cbg ko mice, which only differs genetically from WT mice by the absence of this protein (although subsequent physiological adaptations are likely to occur and cannot be discarded). Since the absence of CBG results in a constantly low reservoir of corticosterone in the blood (Minni et al., 2014), we believe that Cbg ko mice do not present any variation of these hormones' levels during chronic stress, while WT do. This would

explain why no behavioral variations linked to chronic stress are observed in Cbg ko mice.

CBG was detected in several brain regions at low levels, suggesting neuroendocrine and neurohumoral functions (Sivukhina et al., 2013). Thus, CBG expression in the brain could potentially have a role in the observed behavioral differences between Cbg ko and WT mice. However our preliminary results from a brain-specific Cbg ko showed no alteration in stress corticosterone levels and no behavioral differences in the FST test when compared to WT (Helbling et al., 2009). We believe therefore that the effects of CBG deficiency observed on the behaviors tested here are due to plasma rather than brain CBG.

In conclusion, we herein demonstrate that although CBG levels do not change under our chronic stress conditions, it does have a crucial role in modulating the alterations imposed by chronic stress on the HPA axis. Consequently, the behavioral changes caused by these glucocorticoid levels alterations are observed in WT but not in CBG deficient animals. Thus, while individuals with CBG deficiency (induced either genetically or environmentally), show susceptibility to acute stress, there is there is no further impact of chronic stress on their abnormal psychoneuroendocrine phenotype.

Further studies are required to investigate whether pharmacological manipulation of CBG would be efficient in attenuating chronic stress induced despair-like symptoms in WT animals.

Contributors

Moisan M.P., Minni A.M and de Medeiros GF design the experiments and did the analyses

Minni A.M, Helbling J.C., de Medeiros G., performed the experiments.

De Medeiros GF & Moisan M.P wrote the manuscript.

All authors reviewed and approved the manuscript.

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Conflict of interests

All authors of this manuscript reported no biomedical financial interests or potential conflicts of interest.

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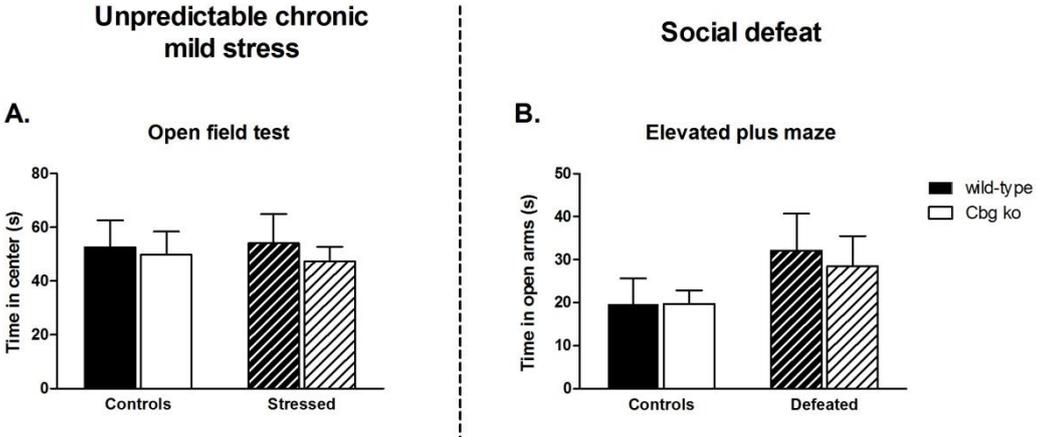
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.psyneuen.2016.04.014>.

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Supplementary material



Suppl. Fig. 1 – Performance of wild-type and Cbg ko mice submitted to the open field test (A, after the unpredictable chronic mild stress) or to the elevated plus maze (B, after social defeat). No statistical difference between groups was detected. Two-way ANOVA followed by Duncan’s post hoc test (n=8-10 mice/group for UCMS and 6 mice/group for social defeat).

Complementary studies

This work has demonstrated the importance of CBG in modulating the alterations imposed by chronic stress on the HPA axis. Although no chronic stress-induced variation in the plasma levels of this protein is observed in wild-type mice, its presence seems to be of great importance to allow variations in the levels of plasma corticosterone. Variations in corticosterone levels, on their turn, seem to underlie the behavioral changes observed in wild-type mice submitted to chronic stress. This would then explain why individuals with CBG deficiency, whether genetically or environmentally-induced, are not further affected by chronic stress.

Besides the data presented in the article, we have additionally investigated some other parameters we thought could be differentially affected by chronic stress on wild-type and Cbg ko mice. One of these parameters was the free – and therefore active – plasma corticosterone fraction (Figure 15). This analysis was carried out in samples from the animals submitted to the social defeat stress - the same samples in which total corticosterone was quantified. The assay protocol was identical to that previously described on page 160. Surprisingly, a two-way ANOVA for the factors genotype and social defeat has only revealed an overall effect of genotype ($F(1,14) = 30.82$, $p < 0.001$), with Cbg ko mice presenting slightly higher (~0.5 nM) levels of free corticosterone than wild-type mice. No effect of social defeat or interaction between both factors was detected.

The absence of an interaction between the factors genotype and social defeat opposes our original hypothesis. If the difference in total corticosterone values between control and stressed conditions – that was detected in wild-type mice only – was indeed responsible for the behavioral effects observed, it should also be reflected in free and active corticosterone values. A possible source for this finding might be the fact that both total and free corticosterone values were measured from plasma obtained in resting conditions, at the nadir of corticosterone secretion. Although chronic stress is known to produce long-lasting effects in the HPA axis (Erburu et al., 2015, Yi et al., 2015), we suspect that if these samples were collected after an emotionally arousing situation, such as the FST, the pattern observed could be different. We believe that the HPA axis activity and reactivity might not follow the same pattern after chronic stress. Furthermore, to support the consistency of this effect, the same assay should have also been performed with plasma samples from the UCMS experiment. Unfortunately, plasma samples from these mice were no longer available for that.

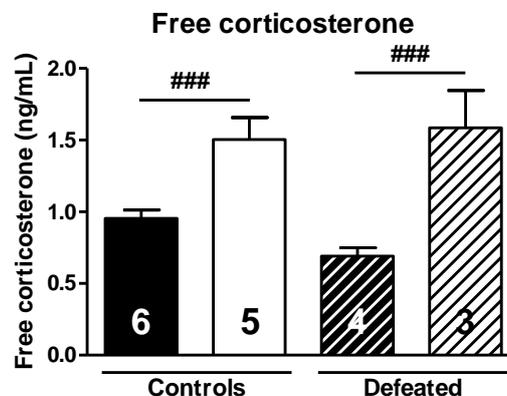


Figure 15 – Free plasma corticosterone levels in the morning in wild-type and Cbg ko mice submitted to the social defeat stress protocol. (###) represents a significant overall effect of genotype ($p < 0.001$). Two-way ANOVA.

The expression of several genes related to the HPA axis activity and/or the behavioral phenotype studied was also evaluated through real-time quantitative polymerase chain reaction (RT-qPCR). This time, only brains from animals that underwent the UCMS protocol were available for the assay. Brains were removed immediately after sacrifice, frozen in dry ice and kept at -80°C . The hippocampus and hypothalamus were dissected at -20°C and stored at -80°C until their use. Total RNA was extracted from both structures with TRIzol reagent (Invitrogen, CergyPontoise, France), according to the manufacturers' protocol. RNA quality was evaluated with RNA Nano chips onto a Bioanalyser 2100 (Agilent, Boeblingen, Germany). 2 μg of total RNA was reverse-transcribed with Superscript III (Invitrogen, Cergy Pontoise, France) and random hexamers, according to the manufacturers' protocol. 5 μL of cDNA diluted 1:20 was PCR amplified onto an ABI7500 thermocycler using 10 μL of Mesagreen qPCR MasterMix (Eurogentec, Seraing, Belgium) and 5 μL of primer mix at 300nM in a total volume of 20 μL . PCR program consisted of 40 cycles of 95°C for 15s and 60°C for 1 min. All primer pairs were designed using Primer Express software (PE APPLIED Biosystems, Courtaboeuf, France). Sequences of primers used are provided in Table 5 and presented a 100% precision. The specificity of the PCR reaction was validated following MIQE (Minimum Information for publication of Quantitative real time PCR Experiments) guidelines (Bustin et al., 2009). mRNA levels of target genes were normalized using 18S RNA expression for each sample (cDNA diluted 1:2000) as 18S proved not to vary in our experimental conditions. The quantification of target mRNA levels was calculated with the SDS2.1 software (Perkin Elmer, Courtaboeuf, France) and expressed as relative mRNA expression.

Table 5 – Sequences of the primers used for gene expression analyses by RT-qPCR

Protein	Gene		Primers' Sequences 5' → 3'
18S	Rn18s	Forward	ACCGCAGCTAGGAATAATGGA
		Reverse	GCCTCAGTTCCGAAAACCAA
Mineralocorticoid receptor	Nr3c2	Forward	GCCGTGGAAGGACAACACA
		Reverse	CCTAAGTTCATGCCGGCTTG
Glucocorticoid receptor	Nr3c1	Forward	GTGGAAGGACAGCACAATTACCT
		Reverse	GCGGCATGCTGGACAGTT
Corticotropin-releasing hormone	Crh	Forward	CAGCCCTTGAATTTCTTGCA
		Reverse	TCACCCATGCGGATCAGA
Corticotropin-releasing hormone receptor 1	Crhr1	Forward	GGAGCATCCGGTGCCTG
		Reverse	AAAGCCGAGATGAGGTTCCA
Pro-opiomelanocortin	Pomc	Forward	ACGGAGAGCAACCTGCTGG
		Reverse	GCGAGAGGTCGAGTTTGCA
11β-hydroxysteroid dehydrogenase type 1	Hsd11b1	Forward	GGAAGGTCTCCAGAAGGTAGTGTC
		Reverse	GAGGCTGCTCCGAGTTCAAG
Interleukin-6	Il6	Forward	ACAAGTCGGAGGCTTAATTACACAT
		Reverse	AATCAGAATTGCCATTGCACAA
Interleukin-1β	Il1b	Forward	AACCAACAAGTGATATTCTCCATGAG
		Reverse	GCCCAAGGCCACAGGTATT
Serotonin receptor 1A	Htr1a	Forward	TCACTTGGCTCATTGGCTTTC
		Reverse	GCGCCAGCCCAGCAT

Brain-derived neurotrophic factor	Bdnf	Forward	CACAATGTTCCACCAGGTGAGA
		Reverse	GCCTTCATGCAACCGAAGT
Cannabinoid receptor 1	Cnr1	Forward	GTGCTGTTGCTGTTTCATTGTG
		Reverse	CTTGCCATCTTCTGAGGTGTG
Neuropeptide Y	Npy	Forward	TCCAGCCCTGAGACACTGATT
		Reverse	CACCACATGGAAGGGTCTTCA

Regarding the expression of genes involved in the HPA axis (Figure 16), the two-way ANOVA did not detect any significant effect of genotype, UCMS or an interaction between these factors for the expression of *Nrc3c2* or *Nr3c1* in hippocampus (Figure 16A,B). Still, a non-significant trend towards a lower expression of the genes coding for both receptors can be observed in wild-type mice submitted to UCMS, but not in *Cbg* ko animals. As for the expression of *Hsd11b1* in hippocampus (Figure 16C), main effects of both genotype ($F(1,25) = 5.65$, $p < 0.05$) and UCMS ($F(1,25) = 7.62$, $p = 0.01$) were observed. Despite no interaction being detected between these two factors, it seems the high mRNA levels of *Cbg* ko controls are greatly responsible for this genotype effect. We observed an overall effect of genotype ($F(1,26) = 5.18$, $p < 0.05$) and a marginally significant interaction between genotype and UCMS ($F(1,26) = 3.01$, $p = 0.09$) for *Crh* expression in hypothalamus (Figure 16D). Duncan posthoc test has revealed a significant increase in *Crh* mRNA expression in the hypothalamus of wild-type mice that went through the UCMS procedure, both when compared to wild-type controls or to *Cbg* ko submitted to the UCMS ($p < 0.05$ for both). A similar profile was found for *Crhr1* expression in hypothalamus (Figure 16E), for which a significant interaction between genotype and UCMS was detected ($F(1,28) = 6.14$, $p < 0.05$). The posthoc analysis detected higher expression in the wild-type UCMS group when compared both to wild-type controls and to *Cbg* ko UCMS ($p < 0.05$ for both). Furthermore, the expression of *Crhr1* in *Cbg* ko controls seems higher than that found in wild-type controls, but no significant effect was detected. No significant effect of genotype, UCMS or interaction between both factors was detected for the expression of *Crhr1* or *Pomc* in pituitary (Figure 16F,G), although a non-significant decrease in *Pomc* levels can be observed in *Cbg* ko control mice when compared to wild-type controls or *Cbg* ko UCMS groups.

As for the expression of genes implicated in mood modulation and that are regulated by glucocorticoids (Figure 17), we found no significant differences in the expression of *Htr1a*, *Bdnf*, *Cnr1* or *Il1b* in hippocampus, or for *Npy* expression in hypothalamus. However, a non-significant trend towards a higher expression of *Htr1a* in the hippocampus can be observed in *Cbg* ko control mice when compared to wild-type controls. Furthermore, a consistent trend towards a lower expression of *Bdnf*, *Cnr1* and *Ilb1* in the hippocampus, as well as of *Npy* in the hypothalamus can be observed for wild-type mice submitted to UCMS when compared both to their controls and to *Cbg* ko UCMS group. Finally, a main genotype effect was detected for the expression of *Il6* in hippocampus ($F(1,27) = 7.72$, $p < 0.01$), with *Cbg* ko mice displaying higher expression regardless of their stress status.

Furthermore, tissue concentrations of monoamines – NA, serotonin, dopamine, DOPAC (3,4-Dihydroxyphenylacetic acid) and 5HIAA (5-Hydroxyindoleacetic acid) – in the frontal cortex were measured by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) as described in (Navailles et al., 2010) to evaluate the impact of HF diet associated or not to the UCMS procedure. Punches of the frontal cortex were homogenized in 100 μ l of 0.1 N

HClO₄, gently sonicated and centrifuged at 13,000 rpm for 30 min at 4°C. Aliquots (20 µL) of the supernatants were injected into the HPLC system. Samples were

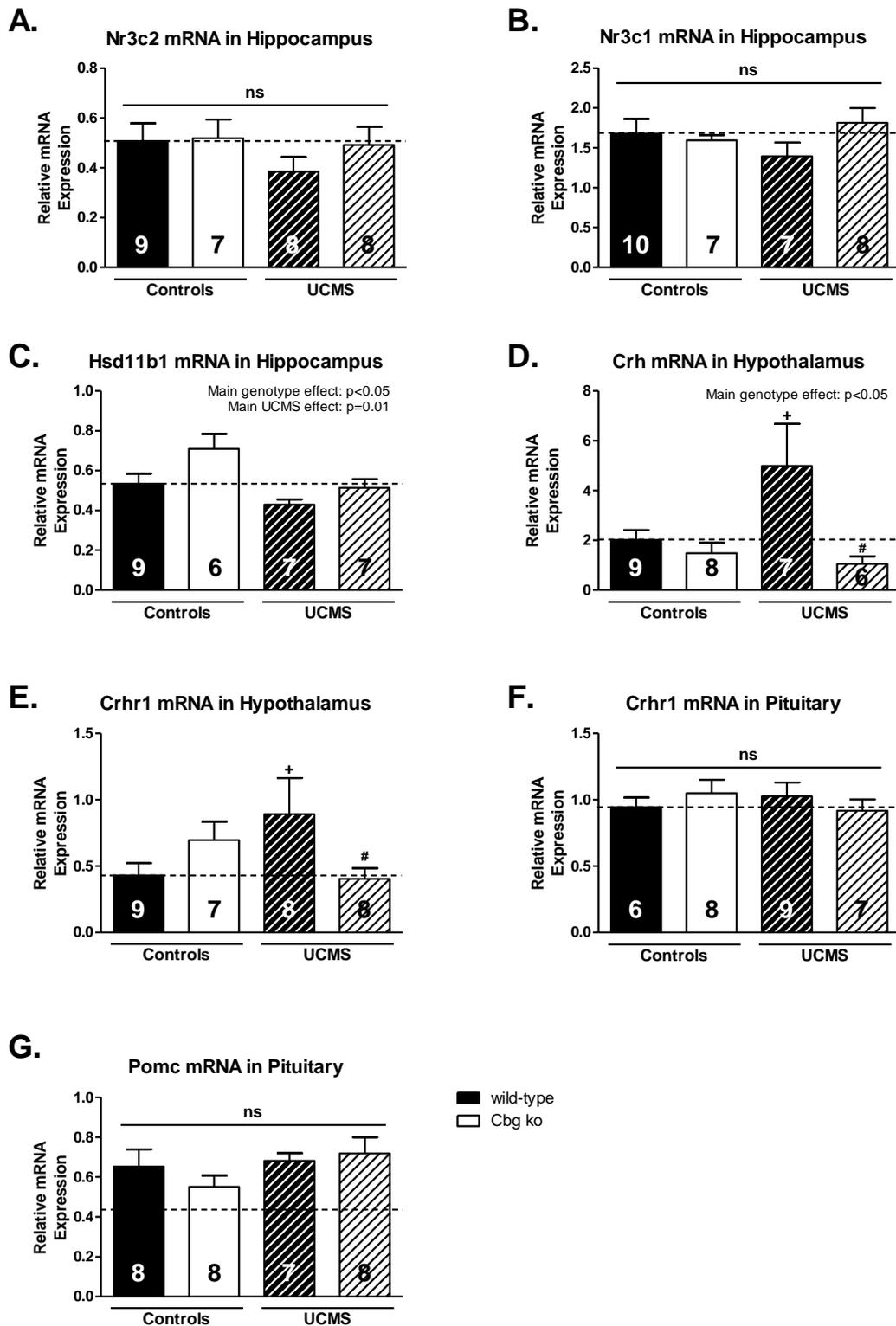


Figure 16 – Expression of genes from the HPA axis. Gene expression evaluated by RT-qPCR for MR (Nr3c2, A), GR (Nr3c1, B) and Hsd11b1 (C) in hippocampus, of Crh (D) and Crhr1 (E) in hypothalamus, and of Crhr1 (F) and Pomc (G) in pituitary. (#) represents significant difference between genotypes within the same conditions and (+) represents significant difference from wild type controls ($p < 0.05$ for both). Two-way ANOVA followed by Duncan posthoc test.

injected via a manual injector (Rheodyne 7725i, C.I.L. Cluzeau, Sainte-Foy-La-Grande, France) into the HPLC column (Kromasyl C8, 150 X 4.6 mm, 5 μ m; C.I.L.-Cluzeau) protected by a Brownlee–Newgard precolumn (RP-8, 15 X 3.2 mm, 7 μ m; C.I.L.-Cluzeau). The mobile phase, was delivered at 1.2 mL/min flow rate using a HPLC pump (Beckman pump 116, Beckman, Paris) as follows (in mM): 60 NaH₂PO₄, 0.1 disodium EDTA, and 2 octane sulfonic acid plus 7% methanol, adjusted to pH 3.9 with orthophosphoric acid and filtered through a 0.22 mm Millipore filter. Detection of monoamines and their metabolites was performed with a coulometric detector (CoulochemII, ESA) coupled to a dual-electrode analytic cell (model 5011). The potential of the electrodes was set at +350 and -270 mV. Signals were recorded on a computer system and analyzed using the Chromnav system (JASCO, France). Results are expressed as ng/mg of tissue.

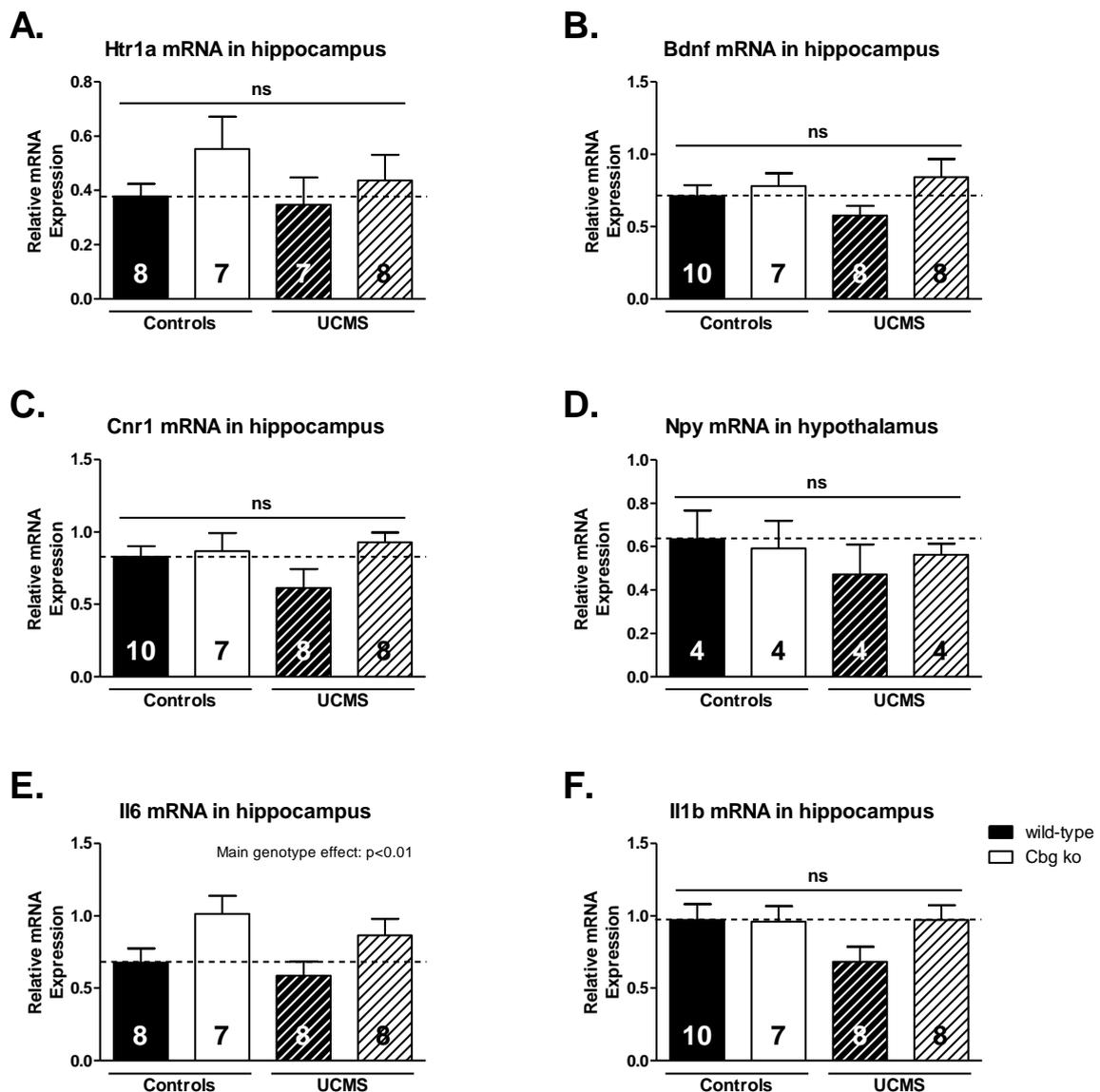


Figure 17 – Expression of genes involved in mood regulation and regulated by glucocorticoids and diet. Gene expression evaluated by RT-qPCR of Htr1a (A), Bdnf (B), Cnr1 (C), Il6 (E) and Il1b (F) in hippocampus and of Npy (D) in hypothalamus. Two-way ANOVA followed by Duncan posthoc test.

For NA levels no main effect of genotype or UCMS were detected, but a marginally significant interaction between both factors was found ($F(1,30) = 3.90$, $p = 0.06$) (Figure 18A). Duncan posthoc test revealed significantly lower levels of norepinephrine in the frontal cortex of wild-type mice submitted to UCMS when compared to either wild-type controls or Cbg ko UCMS ($p < 0.05$ for both). Despite no statistically significant difference were detected for the cortex levels of the other monoamines and their metabolites, several trends were observed. Levels of both serotonin and dopamine metabolites (Figure 18C,E) were slightly increased in Cbg ko controls when compared to wild-type controls. 5HIAA expression in Cbg ko controls seems to normalize with chronic stress, but not that from DOPAC. Furthermore, UCMS seems to decrease the levels of dopamine in the frontal cortex of wild-type mice, and to increase those from Cbg ko mice (Figure 18D).

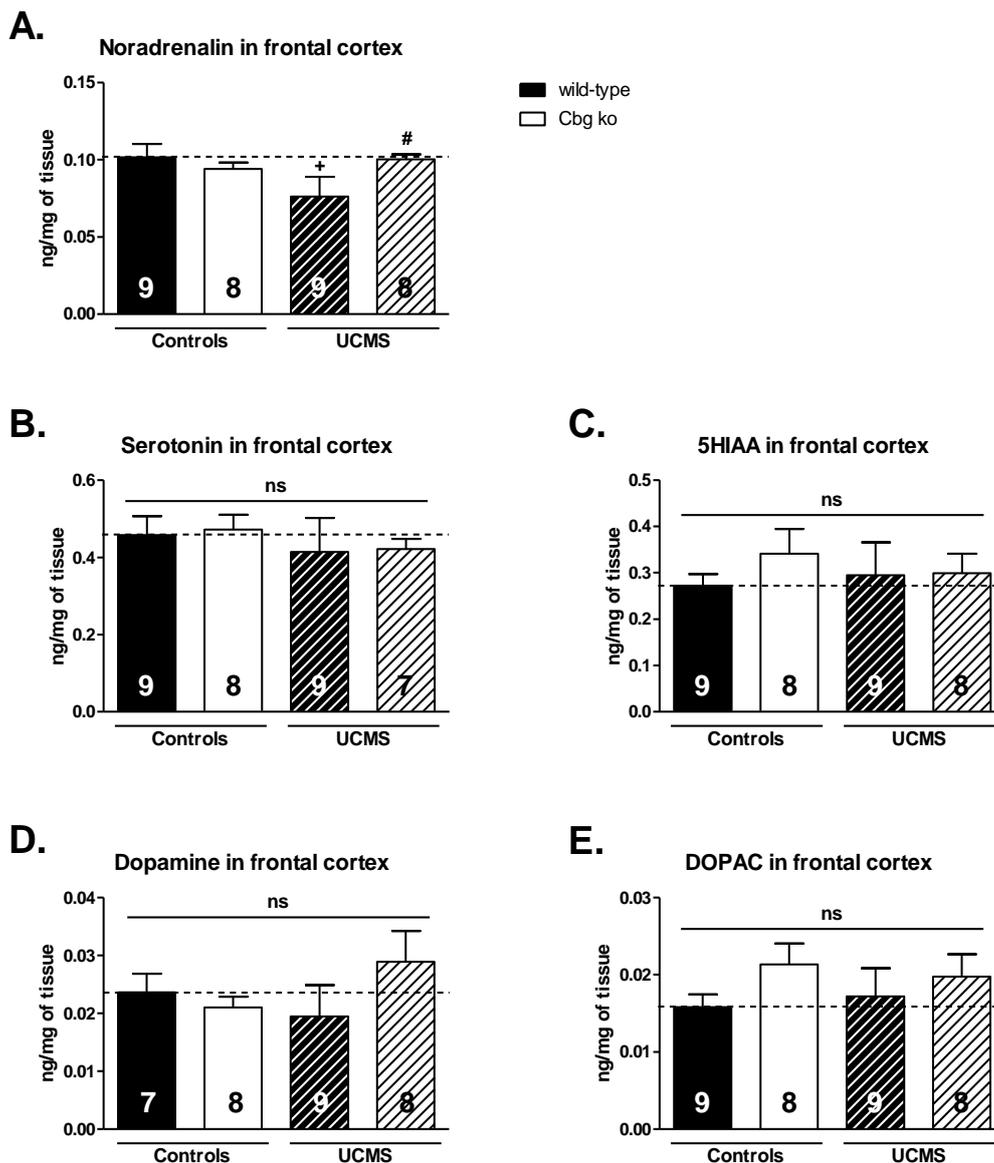


Figure 18 – Levels of the monoamines noradrenalin (A), serotonin (B) and its main metabolite 5HIAA (C), dopamine (D) and its metabolite DOPAC (E), measured in the frontal cortex by HPLC. (#) represents significant difference between genotypes within the same conditions and (+) represents significant difference from wild-type controls ($p < 0.05$ for both). Two-way ANOVA followed by Duncan posthoc test.

As a general pattern, we observe a downregulation of several genes and monoamines in the brain of wild-type mice submitted to the UCMS (Nr3c2, Nr3c1, Hsd11b, Bdnf, Cnr1 and Il1b in hippocampus; Npy in hypothalamus; and norepinephrine and dopamine in the frontal cortex). Also, Crh and its receptor Crhr1 were upregulated in the hypothalamus of these mice. Some of these genes, such as Nr3c2, Nr3c1, Bdnf and Il1b have already been found significantly decreased in hippocampus of rodents that underwent different kind of stressors, such as repeated cold swim, psychosocial stress or restraint (Lopez et al., 1998, Bartolomucci et al., 2003, Roceri et al., 2004, Alfonso et al., 2005, Hill et al., 2005). Nevertheless, these findings are not always consistent. For example, a study evaluating the expression of genes in the hippocampus of rats after 21 days of repeated restraint stress has found no difference regarding the expression of Nr3c2, Nr3c1 (Touyarot and Sandi, 2002). Regarding monoamines, the literature also reports variable profiles that seem to depend highly of the type and length of stressor, besides of the brain region investigated. In rodents, dopamine levels in the frontal cortex have been reported to be increased (Gamaro et al., 2003), decreased (Ahmad et al., 2010) and to vary according to the time after the end of stressor it was investigated (Mangiacacchi et al., 2001). On the other hand, norepinephrine levels seem to be more frequently increased after chronic stress protocols (Adell et al., 1988, 1989), despite some studies also showing no change in norepinephrine levels after chronic stress (Gresch et al., 1994). Interestingly, instead of alterations in monoamines basal levels, many studies have focused on the priming effect that chronic stress may have modulating the monoamine response to a subsequent stress (Adell et al., 1988, Gresch et al., 1994, Finlay et al., 1995, Mangiacacchi et al., 2001). This phenotype was not evaluated in the present study, but remains as an interesting perspective since it could reveal interesting aspects of the interaction between CBG and the stress response. Interestingly, the only two genes found with a trend towards an upregulation by UCMS in wild-type mice were Crh and Crhr1, coding for CRH and its high affinity receptor, respectively. Both genes have been shown to be highly implicated in the stress response (Bale and Vale, 2004), and their higher expression could be reflecting attempt to increase the corticosterone detection in this structure in response to the low plasma levels of corticosterone in found in wild-type mice submitted to the UCMS.

In Cbg ko mice we observe a significant increase in Il6 levels when compared to wild-type controls, which is not modified by UCMS. The production of IL-6 is positively or negatively regulated by a variety of stimuli. Glucocorticoids are known for inhibiting it (Akira and Kishimoto, 1992, Sehgal, 1992), and this increased expression observed in Cbg ko mice – which display suboptimal levels of these hormones both in control and chronically stressed conditions – is probably due to a lower regulation of IL6 gene transcription by glucocorticoids. When compared to wild-type control mice, Cbg ko mice also display a trend towards higher concentrations of dopamine the metabolite DOPAC and a trend towards an increase in dopamine levels only when submitted to UCMS. Some studies have shown that, at the systemic level, glucocorticoid hormones inhibit the stress-induced activation of catecholaminergic system (Lachuer et al., 1992, Kvetnansky et al., 1993), and thus Cbg ko could have an impaired inhibition of this system, which could result in a higher activation in these animals. Several genes that had their expression altered in Cbg ko mice when compared to wild-type control have been normalized by UCMS. That was the case for Hsd11b1, Crhr1 and Htr1a, which were upregulated in Cbg ko mice, and Pomc, which was downregulated. Furthermore, the same was observed for

5HIAA levels in the frontal cortex of Cbg ko controls. These processes do not seem to be mediated by corticosterone, since the levels of total and free corticosterone remained unaltered in Cbg ko mice after chronic stress. Nevertheless, corticosterone levels were always measured in the periphery, while the expression of genes and levels of catecholamines were assessed in the brain. CBG does not cross the blood brain barrier, but intrinsic has been detected in the brain at low levels (Jirikowski et al., 2007, Qian et al., 2011). The role of intracerebral CBG is still unclear, but a local regulation of glucocorticoid levels could be influencing these profiles observed in Cbg ko only.

The comparison of our studies with the literature investigating the effects of chronic stress on the brain is tricky, since there is a great variability in the methodologies of these studies that can influence their findings. Firstly, not only the duration of the chronic stressor varies (from a few days to months), but the stimuli used as stressor also change greatly within studies. Common stressors include repeated footshock, restraint or forced swim in cold water, as well as several different combinations of mild stressors administered randomly. Furthermore, the length between the end of the chronic stress protocol and the assessment of the neurobiological parameters seems to have an important impact on the findings, once the immediate stressor response does not always translates into a long-lasting effect, and even when it does, it does not necessarily follow the same pattern as the immediate response.

STUDY III – CBG DEFICIENCY SPECIFICALLY IMPAIRS HIPPOCAMPUS-DEPENDENT MEMORY CONSOLIDATION IN MALE MICE

Article under preparation

CBG deficiency specifically impairs hippocampus-dependent memory consolidation in male mice

de Medeiros, G. F.^{a,b}; Lafenêtre, P.^{a,b}; Mehta, M. M.; Janthakhin, Y.^{a,b}; Cerpa J. C.; Ducroix-Crepy, C.^{a,b}; Helbling, J. C.^{a,b}; Ferreira, G.^{a,b}; Moisan M. P.^{a,b}

^aINRA, Nutrition et neurobiologie intégrée, UMR 1286, 33076 Bordeaux, France

^bUniv. Bordeaux, Nutrition et neurobiologie intégrée, UMR 1286, 33076 Bordeaux, France

Introduction

All living organisms experience stressful events during their lifetime, which can be defined as imminent or perceived challenges to the organism's homeostasis. The stress response is an innate, adaptive response that has evolved to restore the normal homeostatic balance. This response is essential for survival; it is almost always accompanied by arousal and is most robust when the stimulus is seen as aversive and uncontrollable (Gold, 2015). The brain plays a central role in stress response and adaptation to social and physical stressors. This organ is not only responsible for regulating the physiological and behavioral responses to the stressors, but also for determining what is threatening and storing memories related to the threats (McEwen, 2006).

Survival not only depends on the individuals' immediate response to the stressor, but also on how they will memorize and integrate stress-related information to effectively respond to similar demands in the future. Glucocorticoids, hormones secreted by the adrenal cortex as the final product of the hypothalamic-pituitary-adrenal (HPA) axis activation, are the primary hormones involved in the stress response (Finsterwald and Alberini, 2014). Physical and psychological stressors trigger the secretion of these hormones, which have profound effects on cognition by influencing the limbic brain structures such as the hippocampus, amygdala, and cortex – necessary for memory formation and retrieval – to produce long-lasting changes in the central nervous system. These changes participate in the consolidation of emotional memories. Furthermore, these regions are rich in adrenergic and glucocorticoid receptors, which are known to play critical roles in encoding and retaining the information of emotional events (de Kloet et al., 2005, Lupien et al., 2007).

Although the idea that glucocorticoid hormones influence learning and memory is largely accepted in the scientific community, bidirectional effects have been reported in the literature. Whereas memory consolidation seems to be enhanced by glucocorticoids, memory retrieval is usually impaired when glucocorticoids are administered shortly before retention testing (de Quervain et al., 1998, Roozendaal, 2002). Furthermore, while mild or brief periods of stress have been reported to potentiate memory formation, strong or prolonged stress can have deleterious effects, what has been attributed to increased glucocorticoids secretion (McEwen and Sapolsky, 1995, Roozendaal et al., 1999, Radahmadi et al., 2015).

In recent decades, both human and animal research have shown that the hippocampus – a brain structure crucially involved in both memory and the neuroendocrine regulation of stress hormones – is highly susceptible to stress. These studies have highlighted the role of glucocorticoids in mediating the effects of stress on the hippocampus (Kim and Diamond, 2002, Kim et al., 2015). The consolidation of hippocampus-dependent memory is typically enhanced by stress or glucocorticoid administration after learning (Abrari et al., 2009, McReynolds et al., 2010). Several studies have reported that exposure to stress or elevated corticosterone levels impair performance on hippocampus-dependent memory tasks (McEwen and Sapolsky, 1995, Kim and Diamond, 2002). This susceptibility to the effects of glucocorticoids might be explained not only by the hippocampus being densely concentrated with receptors for corticosteroids, but also by its participation in glucocorticoid-mediated negative feedback of the HPA axis (McEwen and Sapolsky, 1995, Kim et al., 2015). Altogether, these discoveries constituted the basis for the hypothesis that glucocorticoids should have a significant and specific influence on the hippocampus, and consequently on hippocampus-dependent memory (Lupien et al., 2007).

In previous studies, we have shown that one major genetic factor influencing glucocorticoid levels variability is the gene *Serpina6*, encoding corticosteroid binding globulin (CBG) (Ousova et al., 2004). This was first described in pig models, then replicated in rats (Solberg et al., 2006) and recently in humans (Bolton et al., 2014). To estimate the role of CBG on glucocorticoid-dependent cerebral processes, our team has developed a mouse model of CBG deficiency by genetic ablation of *Serpina6* expression, the *Cbg* ko mice. These mice present with suboptimal glucocorticoid response to stress, and have been previously shown to be protected from the memory impairment induced by stress before memory retrieval (Minni et al., 2012). However, the potential role of CBG in memory formation, and in particular in memory consolidation, remains to be established. In particular, we wondered how their lower glucocorticoid response to emotionally arousing situations and stressors from different intensities would modulate their memory formation.

Methods

Animals

2-6 month old male mice (MGI ID: 4833641, *Serpina6*^{tm1.1Mmp}, produced in our laboratory in Bordeaux - France) – were used in this experiment. Wild-type and *Cbg* ko mice were littermates, obtained by breeding males and females heterozygous for the CBG deletion, and genotyped from a tail biopsy. Our mice have a C57BL/6J genetic background above 95%. Animals were maintained, from birth and throughout the whole experiment, in an animal facility with constant temperature (23 °C), under a 12 h light/dark cycle (lights on at 0700 h) and with *ad libitum* access to food and water. All the experiments were conducted in strict compliance with current European Conventions (86/609/CEE) and approved by Institutional Regional Committee for animal experimentation (agreement #5012050-A).

Behavioral testing

Variations of the novel object recognition task (NORT), the contextual and auditory fear conditioning (CFC and AFC, respectively) and the conditioned odor aversion (COA) were used to evaluate wild-type and *Cbg* ko mice's memory. Both

short-term (30 min or 60 min) and long-term (24 h) memories were evaluated for NORT and CFC, while only long-term memory (≥ 24 h) was evaluated for AFC and COA.

Novel object recognition task

This test was performed as described by Leger et al (Leger et al., 2013). Mice were first placed into an open field arena containing two identical objects (Lego[®] brick towers, lab bottles, culture flasks or cylinders) and were left to explore these objects until they reached a criterion of 20s of total exploration for both objects (training session). During the test session, performed either 60 min or 24 h after the training session, mice were placed in the same arena but one of the familiar objects was replaced by an unknown one. Time exploring these objects was again quantified until a criterion of 20s of total exploration for both objects was reached. Thereafter, the preference for the novel object was evaluated. A maximum cutoff of 10 min was established for both sessions, and animals that went beyond this criterion were excluded from the analysis. Exploration time was manually quantified by a trained experimenter outside the experimental room.

Three variations of this protocol were also performed: the first one (habituation) included 5 min habituation sessions in an empty arena for four days before the training session; in the second variation (mild stress), blood sampling from the facial vein was performed 30 min after the training session to assess the training session-induced corticosterone increase; a third variation (strong stress) involved 30 min of restraint stress (inside an adapted Falcon tube) right after the training session, preceded and followed by blood sampling from the facial vein to obtain basal and stress-induced corticosterone levels; finally, a fourth group was submitted to the training session and had blood samples collected at both 30 and 60 min post-training to estimate the increase in corticosterone levels induced by the blood sampling at 30 min.

The NORT performance depends on the action of glucocorticoids when no prior habituation to the test arena is performed. The effect of a stressor on memory consolidation should follow the same pattern as that observed for glucocorticoids. This means that the optimal stress intensity, normally obtained through the administration of a mild stressor, improves memory consolidation, while higher or lower intensities tend to either have no effect or impair memory consolidation (Roosendaal, 2002, Okuda et al., 2004). As – to our knowledge – no previous study has investigated the effect of different stressor intensities on the NORT in mice, we have chosen to test the effects of different combinations of restraint stress and blood samplings, which would act both as an additional stressor and as an indicator of the severity of the stressor (by measuring corticosterone levels in the samples).

Contextual and auditory fear conditioning

In the acquisition phase, mice were placed inside the conditioning chamber for 30 seconds before receiving two footshocks (0.5 mA, 2 s), each one paired with an auditory cue (2.000 Hz, 70 db, 20 s, starting with the footshock), and split by a 2 min interval. During the CFC, performed either 60 min or 24h after the acquisition session, mice were submitted to the same chamber for 5 min, but no footshock or auditory cue was presented. The association between the footshock and the context requires input from the hippocampus and the amygdala. Alternatively, an AFC

session was also performed 24 h after the conditioning session. In this scenario, the animals were placed in a different chamber for 5 min, where they were exposed to the same auditory cue presented during the conditioning session for the last 3 min of test, but did not receive any footshock. For both CFC and AFC sessions, freezing levels were manually quantified in the last 3 min of test by a trained experimenter outside the experimental room.

Conditioned odor aversion

For the COA, animals were first habituated to being individually caged and to only receiving drinking water 60 min per day. After 3 days of habituation, mice were presented with either a banana (isopentyl acetate, 0.05%) or an almond (benzaldehyde, 0.01%)-scented solution instead of the drinking water for 60 min (neutral stimulus – NS). On the following day (day 5), mice that were previously exposed to the banana-scented solution were now presented to the almond-scented one, and vice versa (conditioned stimuli – CS). Immediately after the end of the drinking session, each mouse was given either an intraperitoneal injection of lithium chloride (0.1ml/g of a 0.15M LiCl solution) to induce gastric distress (unconditioned stimulus – US), or a saline injection (control). After one day of recovery to re-establish the mean baseline water intake, animals were given a choice between the CS or the NS during two test sessions (days 7-8). Consumption of both solutions was estimated by weighing the bottles before and after the test sessions, and the strength of the association between the US and CS was calculated as the ratio between CS consumption and the overall (CS + NS) consumption. This kind of aversive learning depends critically on the amygdala (Chapuis et al., 2009).

Total and free corticosterone measurements

Blood was collected either from the facial vein or through a cardiac puncture into microtubes containing 10% EDTA. Samples were centrifuged at 10000 rpm for 10 min, and the supernatant (plasma) was stored at -80°C. Total corticosterone in plasma was measured with an in-house radio-immuno-assay (RIA) previously described (Richard et al., 2010). Briefly, plasma samples were extracted with absolute ethanol and total corticosterone was measured by competition between cold corticosterone and ³H-labeled corticosterone for a specific anti-corticosterone antibody provided by Dr. H Vaudry (University of Rouen, France). Free corticosterone was measured by isotopic dilution and plasma ultrafiltration using Centrifree filter device (YM membranes 30K, Millipore, France) as described in (Richard et al., 2010). The percentage of free corticosterone was calculated as the ratio of cpm filtrate (free corticosterone) / cpm retentate (bound corticosterone). Free corticosterone concentration was calculated as the percentage of free corticosterone multiplied by total corticosterone concentration.

Statistics

All results are expressed as mean ± S.E.M. Statistics were calculated with the software Prism 5 for Windows (GraphPad Software, Inc.). Unpaired Student's t-test was used to compare wild-type and Cbg ko mice or groups versus chance levels in all experiments. The level of statistical significance was set as p values <0.05 for all tests.

Results and discussion

Cbg ko mice display impairment in long-term recognition memory when trained in a novel context

The percentage of time exploring the unknown object in the test session of the NORT performed 24 hours after training was significantly higher than chance level for wild-type ($t(8) = 4.80$, $p < 0.01$), but not for the Cbg ko mice ($t(8) = 0.07$, $p = 0.50$) (Figure 1A). When the same test is performed 60 min after training, both groups spend more time exploring the unknown object ($t(12) = 3.03$, $p < 0.05$ for wild-type and $t(13) = 3.13$, $p < 0.01$ for Cbg ko), indicating intact acquisition and short-term memory (Figure 1B). This suggests that CBG deficiency specifically impairs memory consolidation. Previous habituation to the test arena has been shown to make this task less dependent on glucocorticoids modulation (Okuda et al., 2004). When training was performed after 4 days of habituation to the arena, both wild-type and Cbg ko mice show significantly greater exploration of the new object ($t(6) = 2.55$, $p < 0.05$ for wild-type and $t(7) = 5.43$, $p < 0.01$ for Cbg ko), and no significant difference in exploration between wild-type and Cbg ko animals was detected (Figure 1C). Therefore, CBG deficiency impairs memory consolidation of NORT when training is performed in a novel – but not in a familiar – context, corroborating previous data indicating that glucocorticoids specifically modulate memory consolidation of NORT when it occurs in a novel context (Okuda et al., 2004). However it is unclear whether the memory consolidation deficit in Cbg ko mice is specifically related to the novelty of the context itself or to the higher emotional arousal induced by the absence of habituation to the context.

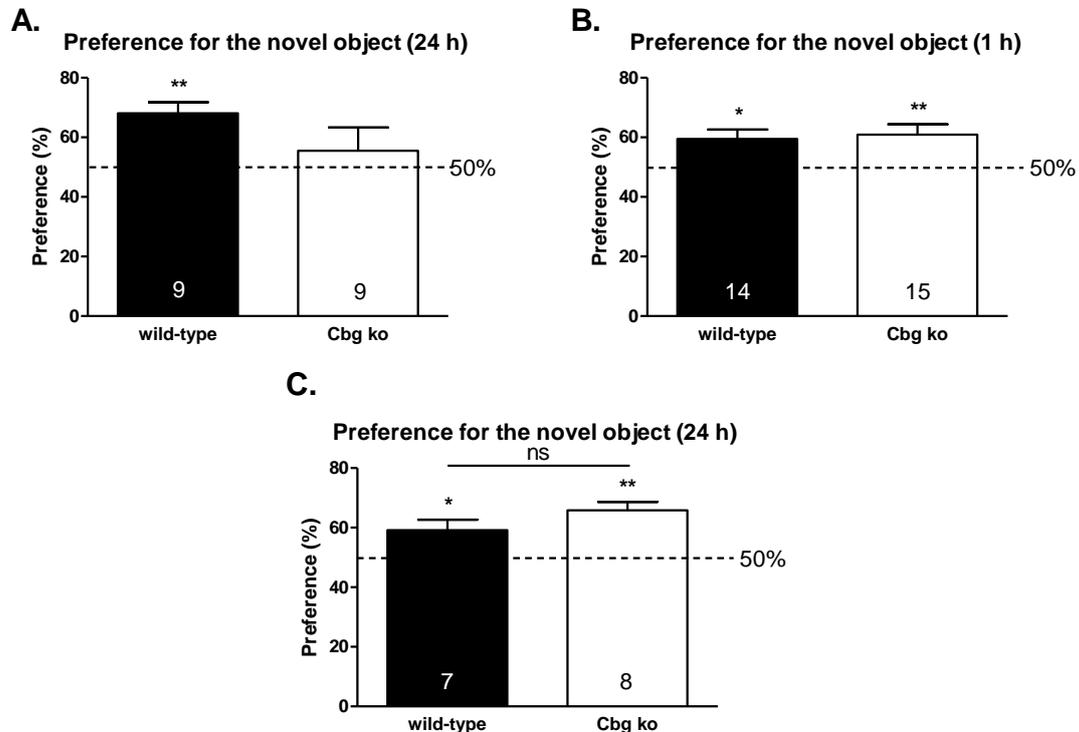


Figure 1 – Evaluation of long-term memory (A), short-term memory (B), and long-term performance after habituation (C) of wild-type and Cbg ko mice in the novel object recognition task. (*) and (**) represent significant difference from chance level (50%) ($p < 0.05$ and $p < 0.01$, respectively). One-sample t test.

Stress can modulate the corticosterone levels and the performance of wild-type and Cbg ko mice in the hippocampus-dependent long-term memory tasks

Stress is long known to modulate the performance of rodents (as well as humans) in a variety of memory-related tasks (de Kloet et al., 1999, Lupien et al., 2007, Joels et al., 2011, Finsterwald and Alberini, 2014, Kim et al., 2015). Yerkes and Dodson have proposed an empirical relationship between arousal and performance in learning tasks, which later came to be known as the Yerkes-Dodson law (Yerkes and Dodson, 1908). It states that performance increases with arousal, but only up to an optimal intensity, beyond which performance starts decreasing. This process is often illustrated as a bell-shaped curve in which the horizontal axis represents the level of emotional arousal and the vertical axis represents the performance. The levels of glucocorticoid hormones (measured in the blood, saliva, brain...), which increase with physical and emotionally arousing situations, have been associated with the influence of stress on memory performance (McIntyre and Roozendaal, 2007).

We wondered whether stress could boost the glucocorticoid response of Cbg ko mice in such a way that they would attain the necessary plasma corticosterone levels to enhance their performance. To test this hypothesis, groups of wild-type and Cbg ko mice were submitted to stressors of different intensities – mild and strong – 30 min after the training session of the novel object recognition task (in a novel context), when stress and glucocorticoid administration have already been shown to influence memory consolidation (Okuda et al., 2004, Moore et al., 2013).

As previously shown (Figure 1A and figure 2A, “no stressor”), only wild-type animals are able to recognize the novel object when NORT is performed under classic conditions (no stress, novel context). We hypothesized that in these conditions, the higher pool of free corticosterone available in the blood (and consequently in the brain, since only free corticosterone is able to cross the blood-brain barrier) of the wild-type mice after training facilitates the consolidation of the familiar object memory, and therefore allowed the recognition of the new one during the test session. Indeed, we do observe higher total ($t(29) = 6.67, p < 0.001$) and free corticosterone levels ($t(25) = 2.44, p < 0.05$) in wild-type mice after the training session (Figure 2B,C, “no stressor”). When a strong stressor is applied, we observe that Cbg ko mice improve their performance in the task, exploring the novel object significantly more than chance level ($t(12) = 3.15, p < 0.01$), while wild-type mice have their performance impaired ($t(10) = 0.54, p = 0.60$) (Figure 2A, “strong stressor”). This reversal of the animals’ performances is accompanied by an increase of total and free corticosterone levels of both groups, but still higher in wild-type mice ($t(24) = 5.05, p < 0.001$ for total and $t(18) = 0.87, p = 0.39$) (Figure 2B,C “strong stressor”). We could assume that, according to the Yerkes-Dodson law, the level of arousal and consequent glucocorticoid release in wild-type mice have reached “too high” levels after training, resulting in a consolidation impairment. Complementing these results, when submitting the mice to a mild stress we observe that all mice recognize the novel object ($t(11) = 4.19, p < 0.01$ for wild-type and $t(7) = 2.62, p < 0.05$ for Cbg ko) (Figure 2A, “mild stressor”). As expected, their levels of both free and total corticosterone are found between the high levels from wild-type mice after a strong stressor – which were strong enough to impair memory consolidation – and the low levels of Cbg ko in standard conditions (no stressor), values that seem to delimitate a range of optimal plasma concentrations for a successful performance in this task (Figure 2B,C, “mild stressor”). Interestingly, when submitted to a mild stress,

the pattern found for total corticosterone ($t(22) = 4.38, p < 0.001$, wild-type higher than Cbg ko) is the inverse of that found for the free corticosterone levels ($t(19) = 3.14, p < 0.01$, Cbg ko higher than wild-type). This is probably because the levels of total corticosterone found in wild-type submitted to a mild stressor have not exceeded the CBG binding capacity, and therefore an increase in total levels does not correspond to an increase in the free corticosterone levels. For not having a specific corticosteroid binding protein in their blood, Cbg ko mice present a linear increase of both total and free corticosterone levels.

Novel Object Recognition Task (in a novel context)

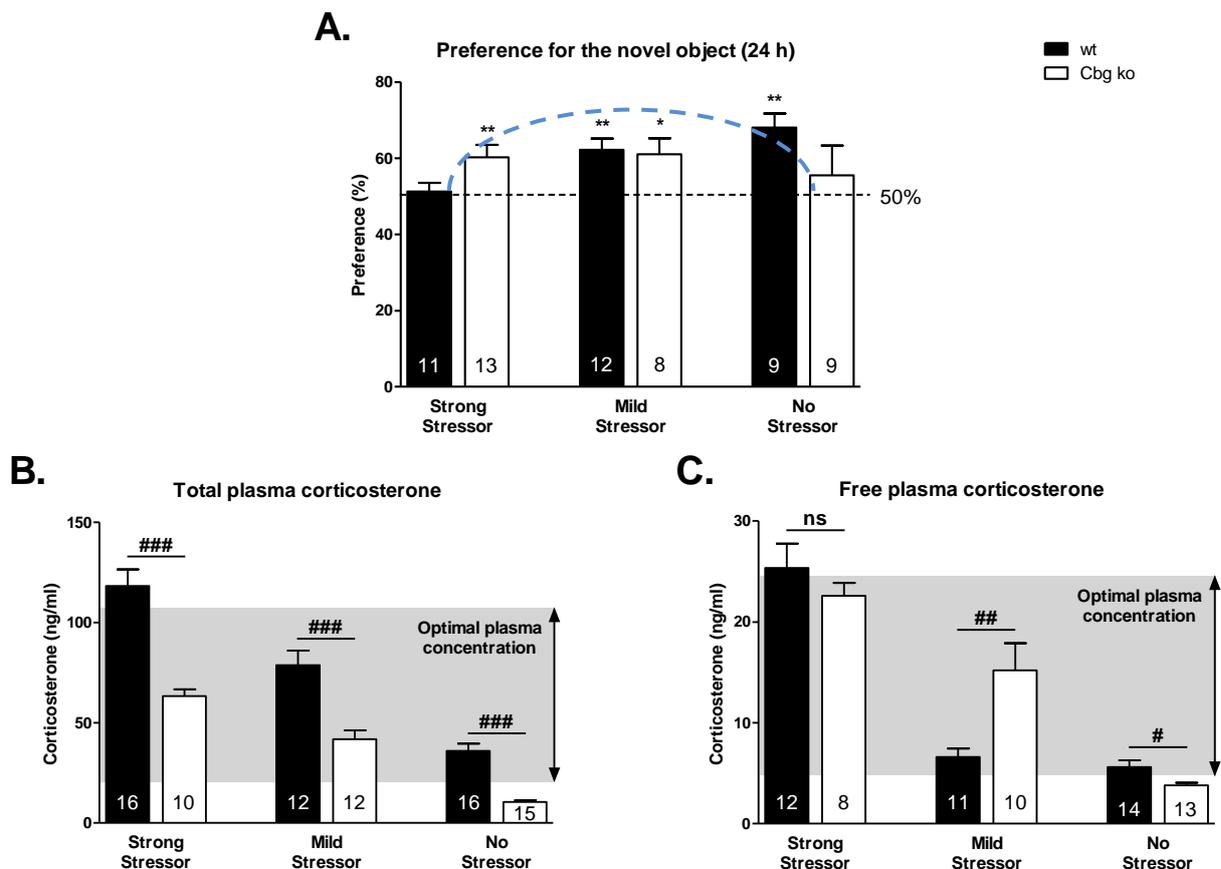


Figure 2 – Performance of wild-type and Cbg ko mice in the novel object recognition task (A), with a bell-shaped curve illustrating the pattern of Yerkes-Dodson law. Levels of total (B) and free (C) corticosterone in the plasma of wild-type and Cbg ko mice after different stressors, with optimal plasma corticosterone concentrations to perform the task estimated in grey. (*) and ()** represent significant difference from chance level (50%) ($p < 0.05$ and $p < 0.01$, respectively), while (#), (##) and (###) represent significant difference between wild-type and Cbg ko mice ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). One-sample t-test for the novel object recognition task and Student's t test for the free and total corticosterone measures.

CBG deficiency impairs contextual, but not elemental, long-term memory

We then evaluated whether the memory consolidation impairment in Cbg ko mice was specifically related to the integration of new contextual information or to tasks involving high emotional arousal in general. For this purpose, mice were

submitted to the fear conditioning paradigm, based on the association between a footshock and both a new conditioning chamber (novel context) and an auditory cue (Curzon et al., 2009, Maren et al., 2013). Cbg ko mice display a reduced freezing time when exposed to the CFC 24 hours after the acquisition phase ($t(12) = 3.58$, $p < 0.01$), confirming the previously observed impairment of hippocampus-dependent memory (Figure 3A). This reduced freezing time is not observed when the re-exposure to the context occurs 1h after the acquisition phase ($t(27) = 0.23$, $p = 0.82$), indicating once again that CBG deficiency does not have an impact on acquisition or on short-term memory, but specifically on memory consolidation (Figure 3B).

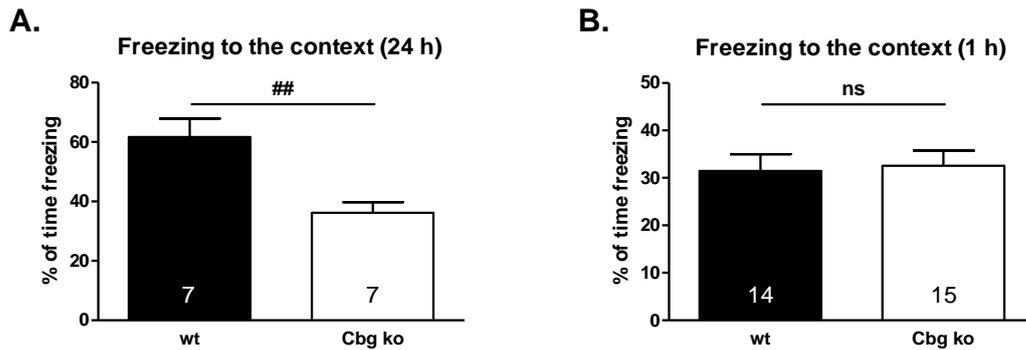


Figure 3 – Evaluation of long-term memory (A) and short-term memory (B) of wild-type and Cbg ko mice in the contextual fear conditioning. (##) represents significant difference between wild-type and Cbg ko mice ($p < 0.01$). Student's t test.

We have also investigated amygdala-dependent emotional memory, through AFC and through the COA, where both the association between the footshocks and the auditory cue (AFC) and between the odorized water and the LiCl-induced gastric discomfort induced by the LiCl injection (COA) have been shown to be amygdala-dependent (Chapuis et al., 2009, Curzon et al., 2009). The percentage of time freezing during the cued session of the fear conditioning did not differ between wild-type and Cbg ko mice ($t(11) = 0.68$, $p = 0.51$) (Figure 4A). The consumption of the odorized water, parameter used to evaluate the aversion towards the odors, was significantly lower than baseline ($t(7) = 5.29$, $p < 0.01$ for wild-type and $t(7) = 5.01$, p

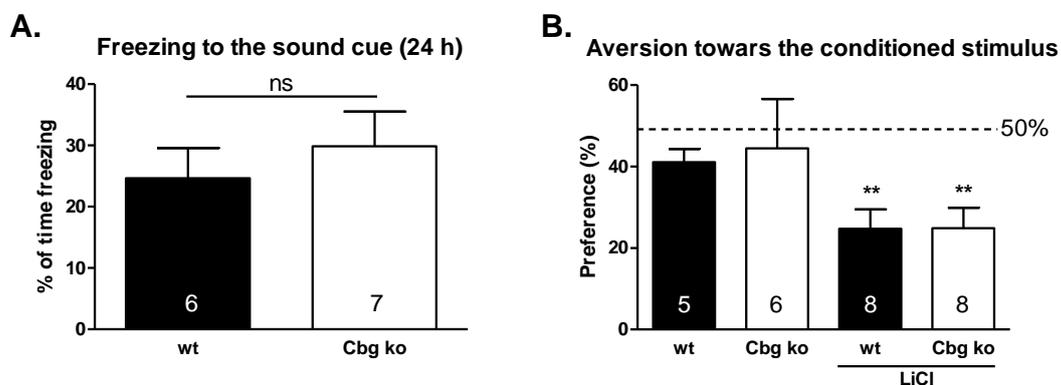


Figure 4 – Evaluation of amygdala-dependent emotional memory in wild-type and Cbg ko mice through the auditory fear conditioning (A) and in the conditioned odor aversion (B). () represents significant difference from chance level (50%) ($p < 0.01$). One-sample t test.**

< 0.01 for Cbg ko), but similar between both groups injected with LiCl ($t(14) = 0.02$, $p = 0.98$) (Figure 4B). Although both paradigms induce high levels of arousal and have been previously shown to be influenced by glucocorticoid levels (Marchand et al., 2007, Miranda et al., 2008, Marks et al., 2015), CBG deficiency does not seem to have an impact on amygdala-dependent memory.

Cbg ko mice present reduced total and free circulating corticosterone levels after the training sessions, but not in baseline conditions

Previous work from our team have shown that, in baseline conditions, the total corticosterone levels of Cbg ko mice are usually lower than their controls (Richard et al., 2010). Interestingly, free (and active) corticosterone levels are similar between Cbg ko and wild-type mice in the active phase, and only slightly higher (less than 2 nM) in the inactive phase, when levels are very low. On the other hand, levels of both total and free corticosterone differ greatly (almost 30 nM for free corticosterone) after stress (20 min of restraint stress), with Cbg ko mice presenting both lower total and free corticosterone levels. It has been proposed that although no HPA axis hyporesponse or insensitivity has been identified, CBG deficiency leads to a higher glucocorticoid clearance in the bloodstream of Cbg ko mice, leading to an insufficient pool of circulating corticosterone in the blood after stress (Minni et al., 2014).

On this context, we wanted to know if the emotional arousal induced by the memory-related behavioral tests were sufficient to induce the previously observed stress-related difference in total and free corticosterone levels. Cbg ko mice presented lower levels of both total ($t(18) = 7.44$, $p < 0.001$) and free corticosterone ($t(16) = 3.83$, $p < 0.01$) 60 min after the CFC (Figure 5A,B). This implies that the conditioning session of the fear conditioning is stressful enough to trigger an increase in the levels of corticosterone in wild-type, but not in Cbg ko animals (due to their higher clearance, as previously mentioned).

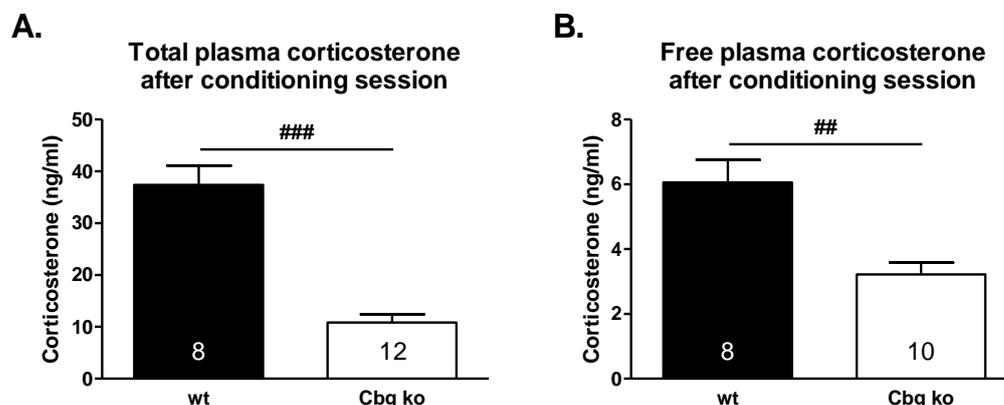


Figure 5 – Total (A) and free (B) corticosterone levels measured in wild-type and Cbg ko mice 60 min after the conditioning session of the fear conditioning. (##) and (###) represent significant difference between wild-type and Cbg ko mice ($p < 0.01$ and $p < 0.001$, respectively). Student's t test.

Final remarks

We have herein shown that CBG affects memory consolidation. We have observed that Cbg ko male mice have a long-term memory deficit, but no alteration is observed in short-term memory. Variations of the tasks influencing the levels of stress after the training session reflect in cognitive changes that follow the Yerkes-

Dodson law. The memory impairment in Cbg ko mice seems to be specific of tasks dependent on the hippocampus, since tasks that are only amygdala- and perirhinal-cortex-dependent are not affected by CBG deficiency. Our findings corroborate the numerous studies showing that insufficient glucocorticoid signaling can cause memory deficits and supports the view that memory consolidation processes depend on post-training activation of glucocorticoid-sensitive pathways. Considering the importance of CBG in modulating the effects of glucocorticoids on memory, it stands as an interesting target for drugs treating memory-related conditions known to be influenced by glucocorticoids, such as post-traumatic stress disorder or phobias. Furthermore, for being a circulating plasma protein, CBG seems to be more easily modulated by pharmacological intervention than brain glucocorticoid receptors, for example.

Complementary studies

Animals from these experiments were all sacrificed, either at: (i) baseline conditions; (ii) 60 min after the conditioning session on the fear conditioning; (iii) 60 min after the context session of the CFC; or (iv) 90 min after an injection of LiCl. Brains were immediately removed and sagittally split. One hemisphere was snap frozen in liquid nitrogen and then stored at -80°C for evaluation with mass spectrometry imaging (MSI). The other hemisphere was fixed into a 4% paraformaldehyde solution and then gradually embedded into 30% sucrose before being sliced and stored at -20°C for immunohistochemistry analysis. Both experiments are currently being performed.

MSI is a powerful new tool to investigate steroid biology within tissues that has been optimized by Prof. Brian Walker's team, in Edinburgh – SCO. It allows assessing the distribution of corticosterone directly onto mouse brain sections. This methodology differs from the approach we have used to quantify brain corticosterone in our previous study (Minni et al., 2012) for being able to reveal the distribution of corticosterone molecules simultaneously at different brain sites.

Neuronal activity in the hippocampus is being indirectly evaluated by the expression of the immediate early genes *c-Fos* and *Egr1*. Immediate early genes have been extensively used to examine the role of the hippocampus in memory processes (Kubik et al., 2007). *c-Fos* is widely believed to play a critical role in transformation of activity in neural circuits into long-term memories in the brain, and its expression in the hippocampus seems to be crucial for memory consolidation (Kubik et al., 2007). *Egr1* has been shown to play a critical role in triggering of the genetic machinery necessary for maintenance of the later phases of LTP and also for the consolidation or stabilization of long-lasting memories (Bozon et al., 2002).

Based on the previous study on the differences in memory retrieval impairments induced by stress, on which we have found lower glucocorticoid availability in the dorsal hippocampus of *Cbg* ko mice after stress (Minni et al., 2012), we also expect to find lower concentrations of glucocorticoids in the brain of *Cbg* ko mice through the MSI approach. This lower availability should be only visible or at least more pronounced in the hippocampus, since our behavioral data support that *Cbg* ko differ from wild-type mice in hippocampus-dependent tasks only. Furthermore, it should mainly be observed in the brains of animals sacrificed after the conditioning phase, when we hypothesize the insufficient glucocorticoid signaling takes place. As for the neuronal activation, which should be in part caused by the activity of the glucocorticoids in the brain, we expect to find a similar pattern to that from MSI. These differences might be even more pronounced when analyzing *Egr1*, since it has been shown to mediate the stress-related behavioral effects of glucocorticoids (Revest et al., 2005).

STUDY IV – BEHAVIORAL AND METABOLIC CONSEQUENCES OF CBG DEFICIENCY ON MALE MICE SUBMITTED TO HIGH-FAT DIET COMBINED TO CHRONIC MILD STRESS

Introduction

Obesity is one of today's most blatantly visible public health problems, which has reached epidemic proportions. It poses a major risk for serious diet-related diseases, including diabetes mellitus, cardiovascular disease, hypertension and stroke, and certain forms of cancer. Its health consequences range from increased risk of premature death to serious chronic conditions that reduce the overall quality of life. Modern lifestyle, particularly that from western countries, is usually evoked to explain this obesity epidemic. This lifestyle combines highly sedentary life, an abundance of palatable and calorically dense food, and the stressful life that leads to a chronic activation of the stress response (Dallman et al., 2006, Chida and Hamer, 2008, Doane and Adam, 2010, Uschold-Schmidt et al., 2012, Desantis et al., 2015, Vliegenthart et al., 2016).

The HPA axis is the major neuroendocrine system involved in the stress-response, and regulates the secretion and release of glucocorticoids. When acutely activated, the HPA axis triggers a battery of physiological and behavioral responses in order to reinstate the challenged body homeostasis. However, prolonged stressor exposure, called chronic stress, leads to a dysregulation (hypo- or hyper-activity) of the HPA axis which can promote detrimental effects in the whole body and constitute a base for pathophysiological consequences (Rosal et al., 2004, de Kloet et al., 2005, McEwen, 2007, Chrousos, 2009, Staufienbiel et al., 2013).

A connection between dysregulated HPA axis activity and obesity has been proposed by several authors (Dallman et al., 2006, Bose et al., 2009, Kyrou and Tsigos, 2009, Incollingo Rodriguez et al., 2015). There are several mechanisms by which dysregulation of the HPA axis and of glucocorticoid function may affect energy balance and potentially contribute to obesity. Glucocorticoids are important regulators of appetite, which is suppressed acutely after stressful situations but increased in the hours to days following a stressful event. In the case of chronic stress, this can lead to continuous elevation of glucocorticoid levels and appetite, leading to increased feeding and consequently to obesity (De Vriendt et al., 2009). Furthermore, glucocorticoids also play a complex role in fatty acid metabolism and storage. Excess glucocorticoid has been particularly associated, in both animal models and in humans, with visceral fat accumulation. It is generally seen that glucocorticoids acutely enhance lipolysis, which may restrict glucose utilization and encourage insulin resistance (Arner, 2002). Glucocorticoids have also been shown to enhance adipose lipoprotein lipase activity, promoting fat storage (Bjorntorp, 2001). Thus, it appears that glucocorticoids may enhance lipid metabolism through its effects on both the turnover and uptake of fatty acids in adipose tissue. Since lipoprotein lipase activity is greater in visceral adipose tissue than in other adipose depots (Marin et al., 1992), glucocorticoids may, in this way, contribute to a redistribution of fat such that it accumulates preferentially in visceral depots. Glucocorticoids also play a critical role in the differentiation of pre-adipocytes into mature adipocytes, a role which is insulin related, potentially further accentuating the accumulation of fat (Hauer et al., 1987, Suryawan et al., 1997). They are also known to up-regulate the NPY Y2 receptor in abdominal fat, leading to stimulation of proliferation and differentiation of adipocytes, again culminating in enhanced fat accumulation (Kuo et al., 2007). Although the

distinct molecular mechanisms by which glucocorticoids may affect adiposity remain to be determined, it is clear that active glucocorticoids are strong modulators of adipose tissue metabolism and distribution in rodents and humans, contributing to enhanced fat deposition particularly in visceral adipose depot (Spencer and Tilbrook, 2011). However, there are several factors – such as gender or baseline body mass index – that seem to influence this relationship, what adds a layer of complexity in the prediction of the effects of stress on body weight (Bose et al., 2009, Incollingo Rodriguez et al., 2015). Comorbidity of obesity and depressive disorders is often reported (Hryhorczuk et al., 2013, Preiss et al., 2013, Mansur et al., 2015). In fact, just like obesity, depressive disorders are consistently linked to chronic dysregulation of the HPA axis activity (Penninx et al., 2007, Staufenbiel et al., 2013, Hardeveld et al., 2014, Gold, 2015). Considering its role at the interface between stress and brain functioning, it is perhaps not surprising that the HPA axis has been found abnormal in psychiatric disorders, and in particular in major depression (Pariante and Lightman, 2008).

In that context, a series of studies (not yet published) have been performed by MP Moisan's team at Nutrineuro lab to investigate the complex interaction between chronic stress, calorically dense food, emotionality and corticosterone regulation by CBG. These studies have shown that when submitted to both chronic stress and HF chow, mimicking the western lifestyle, wild-type mice become obese and present more depressive-related behaviors, similarly to what is observed in humans. On the other hand, when Cbg ko mice are submitted to these same conditions, they lose weight and display an improvement in the depressive-like behavior. This metabolic and behavioral improvements presented by Cbg ko mice under these challenging circumstances draws attention to the crucial role of CBG in modulating the effects of glucocorticoids on the metabolism and on mood regulation. The results from these studies are presented in the following article, entitled *Behavioral and metabolic consequences of CBG deficiency on male mice submitted to high fat diet combined to chronic mild stress*, which is under preparation. The results presented on this article were all performed and analyzed before the beginning of this PhD thesis, but the experiments performed during my PhD aimed at further exploring and complementing the results herein presented. These complementary results will be exposed and discussed just after the article, in the Complementary studies session.

Article under preparation

Behavioral and metabolic consequences of CBG deficiency on male mice submitted to high fat diet combined to chronic mild stress

Minni A.M.^{1,2}; Cadon E.^{1,2}; de Medeiros, G.F.^{1,2}; Helbling J.C.^{1,2}; De Deurwaerdère P.^{3,4}; Grégoire S.⁵; Bretillon L.⁵; Foury A.^{1,2}; Layé S.^{1,2}; Pallet, V.^{1,2}; Castanon N.^{1,2}; Moisan M.P.^{1,2}

¹Univ.de Bordeaux, Nutrition et neurobiologie intégrée, UMR 1286, 33076, Bordeaux – France ;

²INRA, Nutrition et neurobiologie intégrée, UMR 1286, 33076, Bordeaux – France ;

³University of Bordeaux, Institut des Maladies Neurodegeneratives, UMR 5293, 33076, Bordeaux - France;

⁴CNRS, Institut des Maladies Neurodegeneratives, UMR 5293, 33076, Bordeaux – France ;

⁵Eye and Nutrition Research Group, Centre des Sciences du Goût et de l'Alimentation, UMR 1324 INRA, 6265 CNRS, Université de Bourgogne, AgroSup Dijon, 21000, Dijon - France.

Introduction

In modern Western society, obesity is becoming an important public health issue that has reached epidemic proportions. According to WHO, more than 1 European adult over 2 is considered overweight, and 15% of these are obese. Modern lifestyle is often evoked to explain this obesity epidemic, especially because of the increase of sedentary life, the palatable food environment with an abundance of calorically dense food, and the stressful everyday life. This last factor is known to lead to a chronic activation of the stress response, in particular the hypothalamic-pituitary-adrenal (HPA) axis (Dallman et al., 2003, Adam and Epel, 2007).

The HPA axis is the major neuroendocrine system involved in the stress response. It regulates the secretion and release of glucocorticoids: cortisol in humans and corticosterone in rodents. The acute activation of the HPA axis triggers a battery of physiological and behavioral responses. These responses constitute the adaptive stress response, and are rapidly orchestrated in order to reinstate the challenged body homeostasis. However, protracted stressor exposure, called chronic stress, leads to a dysregulation (hypo or hyper-activity) of the HPA axis. This dysregulation has detrimental effects for the whole body and can constitute a base for pathophysiological consequences (de Kloet et al., 2005, McEwen, 2007, Chrousos, 2009).

Growing literature indicates a connection between dysregulated HPA axis activity and obesity (Rosmond et al., 1998, Dallman et al., 2006, Kyrou and Tsigos, 2009). Cushing's syndrome and Addison's disease represent two extremes of plasma cortisolism in human pathology. While Cushing's syndrome – characterized by hypercortisolism – leads to a rapid weight gain (Carroll and Findling, 2010), Addison's disease – characterized by hypocortisolism – conducts to weight loss (Lovas and Husebye, 2007). Furthermore, it was observed that parents of children recently diagnosed with cancer reported marked weight gain associated to their chronic stress situation, although their caloric intake was lower than that from parents of healthy children (Smith et al., 2005). Yet, chronic stress present bidirectional effects on weight,

which depend on the situation and on the individual. In men, the effect of job stress can lead to weight gain or weight loss depending of baseline body mass index (BMI) (Kivimaki et al., 2006). Furthermore, it was demonstrated that the level of cortisol reactivity maybe a marker for vulnerability to stress-induced eating, with higher levels being positively correlated with food consumption during times of stress (Epel et al., 2001).

In rodents, social status has been shown to modulate the effects of chronic stress on metabolic parameters (Bartolomucci et al., 2004, Moles et al., 2006, Solomon et al., 2007). Subordination was reported to be reliably associated with increased weight gain, whereas dominance was associated with lower weight gain or weight loss. Among animals under either standard or high-fat (HF) diet, subordinate mice – reported hypophage but hypoactive – showed higher vulnerability to obesity than dominant – reported hyperphage but hyperactive (Bartolomucci et al., 2009).

Another chronic disease that has also become an important public health problem is major depression (Pariante and Lightman, 2008). As for obesity, major depression is often associated with chronic dysregulation of HPA axis activity. In particular, hypercortisolemia is associated to melancholic depression subtype, and hypocortisolemia associated to atypical depression subtype (Gold and Chrousos, 2002, Bremner et al., 2007). These pathologies frequently coexist: obesity can follow depressive episodes that occurred earlier in life, or depressed mood can be a side effect of obesity and obesity treatment. Adolescents with depression are at a greater risk to become obese and obese individuals are more likely to develop depression as non-obese individuals. It is of note that genes, environment and their interactions can lead to an inappropriate response to chronic stress embodied by a HPA axis dysregulation. In turn, this dysregulation plays an important role in the vulnerability to develop obesity and depression (Bornstein et al., 2006, Kyrkou and Tsigos, 2009).

In our previous study (Richard et al., 2010), we presented a mouse model of HPA axis dysregulation due to CBG (Corticosteroid Binding Globulin) gene deficiency: the Cbg ko mice. This model was obtained by the specific deletion of the CBG gene. CBG, or transcortin, is a high-affinity transport protein for glucocorticoids in vertebrates' blood. This plasma glycoprotein plays an essential role in glucocorticoid availability and delivery to target tissues (Gagliardi et al., 2010, Moisan, 2010).

We have reported that male Cbg ko mice present insufficient glucocorticoid signaling after stress, which is associated with increased despair-like behaviors as measured in the forced swim, tail suspension and learned helplessness tests. In the present study we investigate the influence of HPA axis hyporeactivity on weight gain and despair-like behavior induced by a western lifestyle paradigm. For this we have submitted Cbg ko mice and their wild-type controls to HF diet, combined or not to an unpredictable chronic mild stress (UCMS) procedure (Schweizer et al., 2009).

Methods

Animals and housing conditions

Cbg ko mice and their wild-type controls (MGI ID: 4833641, *Serpina6tm1.1Mmp*) were bred in our laboratory in Bordeaux from heterozygous couples (Richard et al., 2010). The experiments here presented were carried out with male Cbg ko and wild-type mice with a C57BL/6J genetic background above 95%. All mice were between 8 and 10 weeks of age by the beginning of the experiments, and weighed between 25 and 30 g.

Unless otherwise stated, animals were maintained in an animal room (23°C) with a 12 hours light/dark cycle (lights on at 0700h) and with *ad libitum* access to food and water throughout the whole experiment. All the experiments were conducted in strict compliance with current European Conventions and approved by Institutional Committee.

Experimental Design

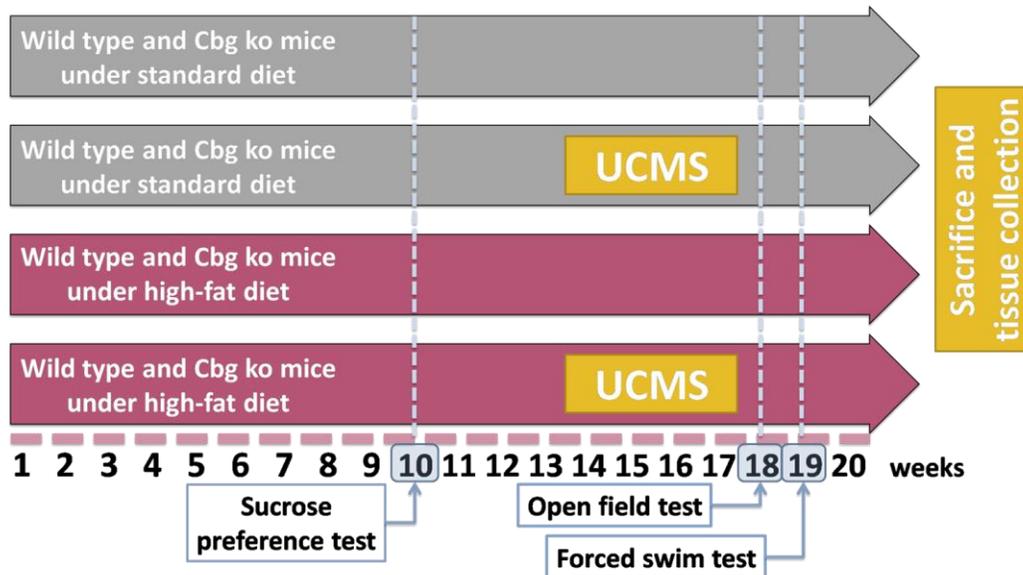
Experimental design is illustrated in Figure 1. At the beginning of the experiment, animals were group housed, with each cage containing 2 wild-type and 2 Cbg ko mice, and were allowed to habituate to their novel environment and establish the social hierarchy for at least one week. Then, animals from half of the cages started being fed with HF chow (D12451 diet, Research diet, New Brunswick, NJ, USA; 45% kcal fat, 20% kcal protein and 35% kcal carbohydrate), while the other half was kept under our standard (STD) chow (A04 diet, SAFE, Augy, France; 10% kcal fat, 20% kcal protein and 70% kcal carbohydrate). After 14 weeks of diet, a 4-week UCMS procedure was imposed to half of the cages of each diet group. Animals submitted to the UCMS procedure were housed individually until the end of the experiment, while the unstressed controls remained in their original cage distribution. The UCMS procedure consisted of random exposure to different mild stressors, twice a day, as presented in Figure 1. Thus, from the beginning of the UCMS procedure, there were 8 experimental groups: (1) wild-type standard unstressed (wild-type); (2) wild-type HF unstressed (wild-type HF); (3) wild-type standard stressed (wild-type S); (4) wild-type HF stressed (wild-type HFS); (5) Cbg ko standard unstressed (Cbg ko); (6) Cbg ko HF unstressed (Cbg ko HF); (7) Cbg ko standard stressed (Cbg ko S); (8) Cbg ko HF stressed (Cbg ko HFS). Each experimental group had between 8 and 10 mice.

Throughout the experiment, body weight of each mouse and food consumption (grams and kcal) of every cage were followed up weekly, and animals blood samples were collected periodically for endocrine measures and behavioral tests: (I) *Blood glucose*: On weeks 12 and 18, after a 24h fasting, blood glucose concentration was measured using a *Accucheck*® device (Roche Diagnostics, France) from blood samples obtained by tail nick; (II) *Plasma corticosterone*: after the 4-week UCMS period (week 18), during the resting phase (0900h) at the beginning of the active phase (1900h), blood samples were obtained by tail nick in order to measure total corticosterone. Since we aimed for basal levels of plasma corticosterone, the sampling procedure did not exceed 2 min per mouse. Blood samples were placed into tubes containing 10% EDTA. After 10 min of centrifugation at 10,000 rpm, plasma was collected and kept at -80°C.

Preference for a 1% sucrose solution was determined on week 12 – before the beginning of the UCMS procedure, and on week 18 – when the stressed animals were individually housed and the unstressed ones were still kept 4 (2 from each genotype) per cage. Animals were first habituated to two identical water bottles for 3 days before the beginning of the test. One of the bottles was then filled with 1% D-sucrose solution (Sigma, France), which remained available to the mice for two days. Liquid intake was determined by weighing the water bottles before, 24h and 48h after both solutions were offered to the animals. The preference for the sucrose solution – compared to water – was used as an index of anhedonic behavior.

Then, all mice were evaluated in the open field (week 18) and forced swim (week 19) tests, which were performed as in (Richard et al., 2010). The open field apparatus was a white square arena (40 x 40 cm) with 16-cm high walls, with its floor divided into

16 squares (10 x10 cm). Mice were placed in the center of the arena and allowed to freely explore the apparatus for 10 min (under a 600 lux illumination). The total number of squares crossed was interpreted as an index of locomotor activity, and the number of



UCMS protocol							
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Week 1	AM	Cage tilt	Wet cage	-	No bedding	-	Air puff
	PM	-	Lights on overnight	Cage switch	Cage tilt	Lights on overnight	-
Week 2	AM	Cage switch	-	Air puff	-	No bedding	Cage tilt
	PM	Lights on overnight	Cage tilt	Lights on overnight	Wet cage	-	-
Week 3	AM	Wet cage	Cage tilt	Air puff	No bedding	Cage switch	-
	PM	Air puff	Lights on overnight	-	Cage tilt	Lights on overnight	Air puff
Week 4	AM	Cage tilt	Wet cage	-	No bedding	-	Cage switch
	PM	No bedding	Lights on overnight	Air puff	Lights on overnight	-	-

Figure 1 – Experimental design. Cbg ko and wild-type male mice (2 mice from each genotype per cage) were submitted to either standard diet or high-fat diet for 20 weeks. After 14 weeks of diet, a 4-week unpredictable chronic mild stress (UCMS) procedure was imposed to half of the animals from each group. The UCMS procedure consisted of individual housing and random exposure to several different mild stressors, twice a day, as illustrated in the table. All AM stressors lasted for approximately 9 hours, while the PM stressors lasted approximately 15 hours (except for the air puff). Cage tilt: homecage inclined 45°; wet cage: bedding soaked with water; lights on overnight: lights on during the dark phase of the light/dark cycle; cage switch: mice placed in a novel cage containing another mouse’s bedding; no bedding: mouse placed directly into the acrylic cages; air puff: five puffs of compressed air at the mouse’s face.

center squares (four inner squares) crossed was used as index of anxiety. For the forced swim test, each mouse was placed into a plastic cylinder (height, 32.5cm; diameter, 15 cm) containing 3 liters of water (16 cm high) at $25\pm 0.5^{\circ}\text{C}$. During the 6 min of test, trained observers quantified immobility behaviors every 10 seconds, as described in (Bolanos et al., 2008). A mouse was judged immobile when it stopped swimming and performed only minimal movements sufficient to remain floating. Four mice were tested simultaneously in different cylinders, and the water was changed between subjects.

Animals were sacrificed in the morning (0800h - 1100h) of week 20. Mice were anesthetized with isoflurane (Aerrane, Baxter SA, Maurepas, France) and had about 1 ml of blood collected by cardiac puncture. This procedure took less than 20 s to avoid stress values. Then, animals were sacrificed by decapitation and brain, pituitary, adipose tissue, muscle and liver were collected, immediately frozen in dry ice and kept at -80°C until further use. The frontal cortex, hippocampus and hypothalamus were later dissected and punched at -20°C and stored at -80°C until their use in biochemical assays.

Total corticosterone measurement

Total corticosterone was measured by an in-house RIA (see (Richard et al., 2010) for details). Briefly, after steroid extraction of plasma samples with absolute ethanol, total corticosterone was measured by competition between cold corticosterone (B) and ^3H -B for a specific antibody anti-corticosterone provided by Dr. H. Vaudry (University of Rouen, France).

CBG binding capacity

In the CBG binding capacity assay, 10 μl of plasma were incubated into 500 μl of dextran-coated charcoal for 30 min at room temperature and under continuous shaking to remove endogenous steroids. Dextran-coated charcoal was removed by centrifugation and samples were further diluted (1:5) in PBSG buffer. 150 μl of diluted plasma were incubated with 50 μl of increasing concentrations (0.5 to 16 nM) of [^3H]-labeled corticosterone and 50 μl of PBSG buffer in duplicates for 20 min at 37°C , and then at 4°C for at least 2h. 250 μl of dextran-coated charcoal solution was added to each tube, which were then incubated at 4°C for 10 min and centrifuged for 15 min at 3400 rpm (4°C). After centrifugation, 2.5 ml of OptPhase "HiSafe" 2 (Perkin Elmer, The Netherlands) were added to the supernatant, and the amount of radioactivity present was determined with a β -scintillation counter. CBG Bmax was calculated as described in Hammond and Lahteenmaki (1983).

Chromatographic Analysis

Tissue concentrations of monoamines (norepinephrine, serotonin, dopamine, DOPAC (3,4-Dihydroxyphenylacetic acid) and 5-HIAA (5-Hydroxyindoleacetic acid)) in the frontal cortex were measured by HPLC-ECD as described previously (Navailles et al., 2010) to evaluate the impact of HF diet associated or not to the UCMS procedure. Punches of the frontal cortex were homogenized in 100 μl of 0.1 N HClO_4 , gently sonicated and centrifuged at 13,000 rpm for 30 min at 4°C . Aliquots (20 μL) of the supernatants were injected into the HPLC system.

Tissue dosages of monoamines and their metabolites were performed by a sensitive HPLC-ECD system. Samples were injected via a manual injector (Rheodyne 7725i, C.I.L. Cluzeau, Sainte-Foy-La-Grande, France) into the HPLC column (Kromasyl C8, 150 X 4.6 mm, 5 μ m; C.I.L.- Cluzeau) protected by a Brownlee–Newgard precolumn (RP-8, 15 X 3.2 mm, 7 μ m; C.I.L.-Cluzeau). The mobile phase, was delivered at 1.2 mL/min flow rate using a HPLC pump (Beckman pump 116, Beckman, Paris) as follows (in mM): 60 NaH₂PO₄, 0.1 disodium EDTA, and 2 octane sulfonic acid plus 7% methanol, adjusted to pH 3.9 with orthophosphoric acid and filtered through a 0.22 μ m Millipore filter. Detection of monoamines and their metabolites was performed with a coulometric detector (CoulochemII, ESA) coupled to a dual-electrode analytic cell (model 5011). The potential of the electrodes was set at +350 and -270 mV. Signals were recorded on a computer system and analyzed using the Chromnav system (JASCO, France). Results are expressed as ng/mg of tissue.

Plasma lipid composition and analyses

Total lipids were extracted from plasma according to the Moilanen and Nikkari method (Moilanen and Nikkari, 1981), using a mixture of chloroform and methanol (1:1, v/v).

Lipid class profiles: The distribution of lipids into phospholipids (PL), triglycerides (TG), free fatty acids (FFA), free cholesterol (CH), and cholesterol esters (CE) in the plasma was determined using a combination of thin-layer chromatography on silica gel-coated quartz rods and flame ionization detection (Iatroscan® system, Iatron, Tokyo, Japan), according to Ackman's technique (Ackman, 1981). The values obtained for each compound were corrected according to their response factor using specific calibration curves, as published by (Sébédio and Juanéda, 1991). Data were reported as a percentage of the total lipids in the sample.

Preparation of cholesterol esters, phospholipids and triglycerides: Total lipids from the plasma were submitted to a thin-layer chromatography separation on precoated silica gel plates (Merck, Darmstadt, Germany) using a solvent mixture of hexane/diethyl ether/acetic acid (80:20:1, v/v/v). The silica gel plates were visualized under UV light (360 nm) after spraying with 2',7'-dichlorofluorescein. The spots corresponding to CEs, PL and TG were scraped off the plate and the samples were transesterified following the fatty acid analysis procedure.

Fatty acid analysis: Lipids were transesterified using boron trifluoride in methanol according to (Morrison and Smith, 1964). The fatty acid methyl esters were analyzed by gas chromatography on a Hewlett Packard Model 5890 gas chromatograph (Palo Alto, CA, USA) fitted with a CPSIL-88 column (100 m x 0.25 mm i.d., film thickness 0.20 μ m; Varian, Les Ulis, France) equipped with a flame ionization detector. Hydrogen was used as a carrier gas (inlet pressure 210 kPa). The oven temperature was held at 60°C for 5 min, increased to 165°C at 15°C /min and held for 1 min, and then to 225°C at 2°C /min and finally held at 225°C for 17 min. The injector and the flame ionization detector were maintained at 250°C and 250°C, respectively. Fatty acid methyl esters were identified by comparison with standards. The data were processed using the EZChrom Elite software (Agilent Technologies, Massy, France).

Gene expression by real-time qPCR

Total RNA was extracted of hippocampus with TRIzol reagent (Invitrogen, Cergy Pontoise, France) according to the manufacturer's protocol. RNA quality was evaluated with RNA Nano chips onto a Bioanalyser 2100 (Agilent, Boeblingen, Germany). 2 µg of total RNA was reverse-transcribed with Superscript III (Invitrogen, Cergy Pontoise, France) and random hexamers, according to the manufacturers' protocol. 5 µL of cDNA diluted 1:20 was PCR amplified using 10 µL of Mesagreen QPCR MasterMix (Eurogentec, Seraing, Belgium) and 5 µL of primer mix at 300 nM in a total volume of 20 µL onto an ABI7500 thermocycler. PCR program consisted of 40 cycles of 95°C for 15 s and 60°C for 1 min. All primer pairs were designed using Primer Express software (PE APPLIED Biosystems, Courtaboeuf, France) in a way avoiding genomic DNA amplification. Sequences of primers used are provided in Table 1 and presented a 100% precision. The specificity of the PCR reaction was validated following MIQE (Minimum Information for publication of Quantitative realtime PCR Experiments) guidelines (Bustin et al., 2009). mRNA levels of target genes were normalized using 18S RNA (Rn18s) expression for each sample (cDNA diluted 1:2000), as Rn18s proved not to vary between our experimental conditions. The quantification of target mRNA levels was calculated with the SDS2.1 software (Perkin Elmer, Courtaboeuf, France) and expressed as relative mRNA expression.

Table 1 – Sequences of primers used for gene expression analyses by RT-qPCR

Protein	Gene		Primers' Sequences 5' → 3'
18S	Rn18s	Forward	ACCGCAGCTAGGAATAATGGA
		Reverse	GCCTCAGTTCGAAAACCAA
Mineralocorticoid receptor	Nr3c2	Forward	GCCGTGGAAGGACAACACA
		Reverse	CCTAAGTTCATGCCGGCTTG
Glucocorticoid receptor	Nr3c1	Forward	GTGGAAGGACAGCACAATTACCT
		Reverse	GCGGCATGCTGGACAGTT
Corticotropin-releasing hormone	Crh	Forward	CAGCCCTTGAATTTCTTGCA
		Reverse	TCACCCATGCGGATCAGA
Corticotropin-releasing hormone receptor 1	Crhr1	Forward	GGAGCATCCGGTGCCTG
		Reverse	AAAGCCGAGATGAGGTTCCA
Pro-opiomelanocortin	Pomc	Forward	ACGGAGAGCAACCTGCTGG
		Reverse	GCGAGAGGTCGAGTTTGCA
11β-hydroxysteroid dehydrogenase type 1	Hsd11b1	Forward	GGAAGGTCTCCAGAAGGTAGTGTC
		Reverse	GAGGCTGCTCCGAGTTCAAG
Interleukin-6	Il6	Forward	ACAAGTCGGAGGCTTAATTACACAT
		Reverse	AATCAGAATTGCCATTGCACAA
Interleukin-1β	Il1b	Forward	AACCAACAAGTGATATTCTCCATGAG
		Reverse	GCCCAAGGCCACAGGTATT
Serotonin receptor 1A	Htr1a	Forward	TCACTTGGCTCATTGGCTTTC
		Reverse	GCGCCAGCCCAGCAT
Brain-derived neurotrophic factor	Bdnf	Forward	CACAATGTTCCACCAGGTGAGA
		Reverse	GCCTTCATGCAACCGAAGT

Cannabinoid receptor 1	Cnr1	Forward	GTGCTGTTGCTGTTCAATTGTG
		Reverse	CTTGCCATCTTCTGAGGTGTG
Neuropeptide Y	Npy	Forward	TCCAGCCCTGAGACACTGATT
		Reverse	CACCACATGGAAGGGTCTTCA
Glucose transporter type 4	Slc2a4	Forward	TCCCTTCAGTTTGGCTATAACATTG
		Reverse	CTACCCAGCCACGTTGCATT
Peroxisome proliferator-activated receptor gamma	Pparg	Forward	CACAATGCCATCAGGTTTGG
		Reverse	GCTGGTCGATATCACTGGAGATC
Peroxisomal acyl-coenzyme A oxidase 1	Acox1	Forward	CAGCCAGATTGGTAGAAATTGCT
		Reverse	CCACGCCACTTCCTTGCT
Peroxisome proliferator-activated receptor alfa	Ppara	Forward	GCAGAGGTCCGATTCTTCCA
		Reverse	GGTCGTTCAAGTCCAAGTTTGC
Carnitine palmitoyltransferase I	Cpt1b	Forward	CCAAACGTCACTGCCTAAGCT
		Reverse	GGCCGCACAGAATCCAAGTA
Beta-3 adrenergic receptor	Adrb3	Forward	CCCAGCCAGCCCTGTTG
		Reverse	GCACCTTCATAGCCATCAAACC
Beta-2 adrenergic receptor	Adrb2	Forward	TGGGAACGGCTACTCTAGCAA
		Reverse	TGTTTGGCTCCCCTGTGTAGT
Hormone-sensitive lipase	Lipe	Forward	CTCCTATGACCTACGGGAAGGA
		Reverse	TCAGATTTTGCCAGGCTGTTG
Monoacylglycerol lipase	Mgl1	Forward	CGGAACAAGTCGGAGGTTGA
		Reverse	GCATTGCTCGTCCACTCTT
Adipose triglyceride lipase	Pnpla2	Forward	TCACCATCCGCTTGTTGGA
		Reverse	TGCTACCCGTCTGCTCTTTCA
Phosphoenolpyruvate carboxykinase 1	Pck1	Forward	GTGTCATCCGCAAGCTGAAG
		Reverse	CAGTGAGAGCCAGCCAACAGT
Tyrosine aminotransferase	Tat	Forward	TAGGTTTGGCTGCTTGGAGAC
		Reverse	TCACAAAGCCTTCCTCGTCC
Glucose-6-phosphatase	G6pc	Forward	TCAACCTCGTCTTCAAGTGGATT
		Reverse	CACGGAGCTGTTGCTGTAGTAGTC

Leptin measurement

The levels of leptin were assessed through an enzyme-linked immunosorbent assay (ELISA), following the manufacturers' protocol (Millipore rat/mouse ELISA insulin kit (EZRFI-13K) and Millipore mouse ELISA leptin kit (EZML-82K)). Briefly, plasmatic leptin will bind an antiserum and the resulting complexes will be immobilized in the wells of a microtiter plate. After washing, a purified biotinylated detection antibody is added and allowed to bind to the immobilized leptin. After a second wash, horseradish peroxidase is added and binds the biotinylated detection antibody. Then after the wash away of free enzyme conjugates, the quantification of immobilized antibody-enzyme conjugates is made by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'- tetramethylbenzidine. The enzyme activity is measured spectrophotometrically. Since the increase in absorbance is directly proportional to the amount of captured leptin in the sample, the latter can be derived by interpolation from a

reference curve generated in the same assay with reference standards of known concentrations of mouse leptin.

Statistical analyses

All results were expressed as mean \pm S.E.M. Outliers with value that differed from the mean more than twice the S.D. were excluded from analysis. Statistics were calculated with the software Statistica 8. For body weight evolution, statistical analyses were performed in three stages: (I) at day 0, an unpaired Student test was performed to compare wild-type and Cbg ko mice; (II) between weeks 0 to 14, a three-way repeated measures analysis of variance (ANOVA) was carried out for the factors genotype, diet and week; (III) a four-way repeated measures ANOVA was performed between weeks 14 and 20 to assess the effects of genotype, diet, UCMS and week on body weight. Despite being presented in different graphs, the weight evolution of wild-type and Cbg ko mice were always analyzed within the same ANOVA. All ANOVA analyses were followed by Duncan posthoc test when needed. For corticosterone concentration analysis, a four-way repeated measures ANOVA was carried out for the factors genotype, diet, UCMS and time of the day, also followed by Duncan posthoc test when needed. For all the other experiments, three-way ANOVAs were performed to assess the effects of genotype, diet and UCMS, followed by Duncan posthoc tests when needed. Since 8 groups of mice were being simultaneously tested, if a statistical difference did not appear through the three-way ANOVA but still the means of some groups seemed different, a separate two-way ANOVA or unpaired t test was performed to evidence these differences. The level of statistical significance was set as p value $<$ 0.05. On graphs, ns indicates no significant difference, one symbol p $<$ 0.05, two symbols p $<$ 0.01 and three symbols p $<$ 0.001.

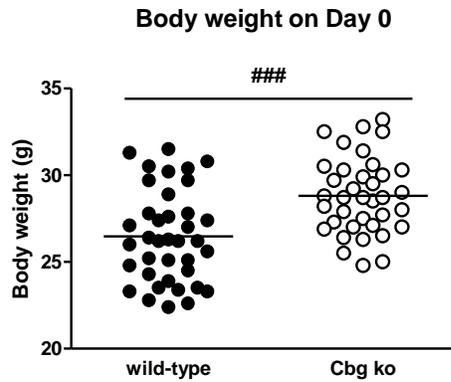
Results

Consequences of high-fat diet and UCMS on weight gain

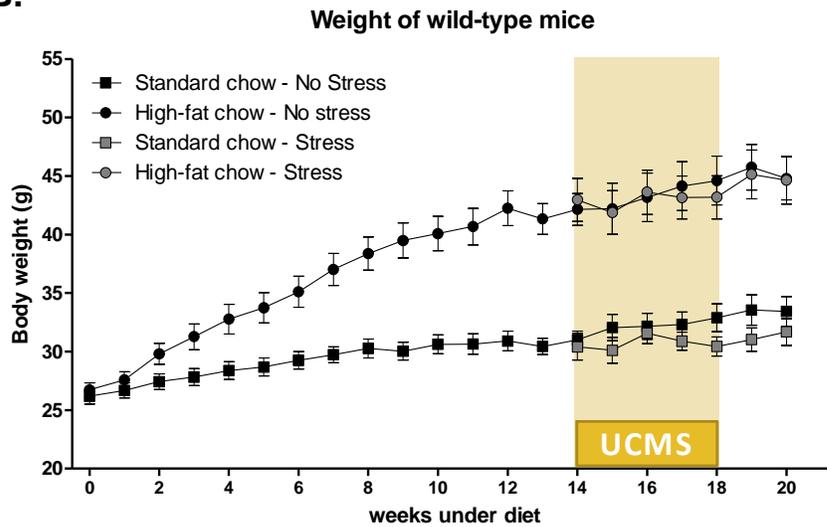
Body weight evolution was followed each week throughout the experiment. At the beginning of the experiment, Cbg ko mice were significantly heavier (8.8%) than wild-type mice (28.8 ± 0.4 g vs. 26.5 ± 0.4 g; $t(73) = 4.14$, p $<$ 0.001; Figure 2A). Then, during the HF diet only period (weeks 0 to 14) the 3-way repeated measures ANOVA showed a diet effect ($F(1,70) = 58.85$, p $<$ 0.001, HF $>$ STD), a genotype effect ($F(1,70) = 5.19$, p $<$ 0.05, Cbg ko $>$ wild-type), a week effect ($F(14,980) = 365.19$, p $<$ 0.0001) and a diet and week interaction ($F(14,980) = 98.21$, p $<$ 0.001) (Figure 2B,C). Duncan posthoc test has not revealed any significant difference between wild-type and Cbg ko groups within the same diet nor between STD and HF groups within the same genotype. Nevertheless, it is possible to observe a clear trend towards an increase in weight for HF diet fed mice, regardless of the genotype, and this trend is accompanied by decreasing p values on Duncan posthoc.

When UCMS was associated to the HF diet (weeks 15 to 20), a diet effect ($F(1,66) = 133.92$, p $<$ 0.001), a UCMS effect ($F(1,66) = 8.27$, p $<$ 0.01) and a week effect ($F(5,330) = 67.94$, p $<$ 0.001) were detected (Figure 2B,C). Significant interactions were found for the factors week and stress ($F(5,330) = 11.96$, p $<$ 0.001), week and diet ($F(5,330) = 12.80$, p $<$ 0.001), week, genotype and stress ($F(5,330) = 4.07$, p $<$ 0.01) and week, diet and stress ($F(5,330) = 3.60$, p $<$ 0.01). No significant effects were

A.



B.



C.

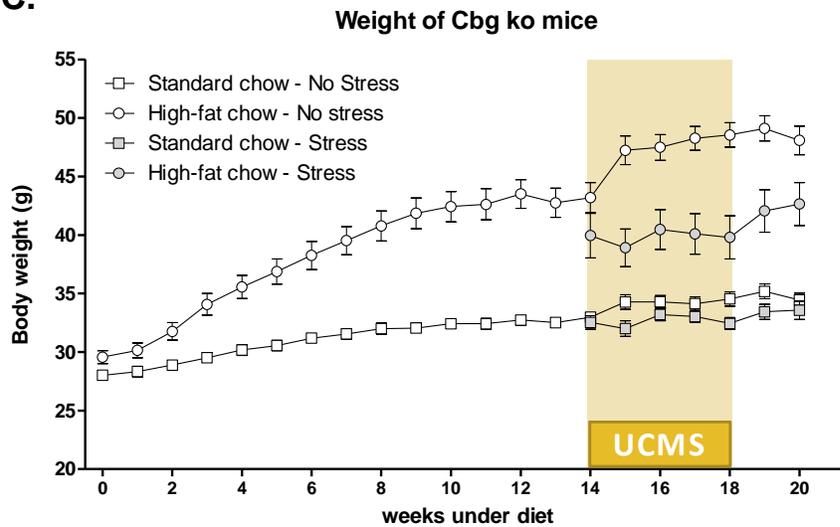


Figure 2 – Body weight of wild-type and Cbg ko mice at the beginning of the experiment (A). Body weight evolution during the 20 weeks of experiment of wild-type (B) and Cbg ko mice (C). (#) represents significant difference between genotypes.

detected in the posthoc test. Nevertheless, it is possible to conclude that: (I) mice under HF diet only (HF groups) were significantly heavier than mice under standard diet (33.5% for wild-type HF and 40.7% for Cbg ko HF on week 20); (II) UCMS procedure under standard diet (STD groups) did not induce significant changes in body weight, regardless of the genotype; and (III) the body weight of wild-type HF mice was similar to that of Cbg ko HFS mice but Cbg ko HFS mice presented a significant 18.0% weight loss when compared to Cbg ko HF ($p < 0.0001$). These HFS mice remain 23.8% heavier than Cbg ko mice under standard diet. Given this last observation, we decided to perform additional analyses. A repeated measures one-way ANOVA, performed separately for both genotypes and including HF fed animals only, has revealed an overall UCMS effect for the Cbg ko mice ($F(1,17) = 13.76$, $p < 0.01$), but not for the wild-type mice group.

From weeks 12 to 14, mice were first fasted for 24h to measure blood glucose levels and then submitted to a series of tests (as already described), resulting in a short period of stress. Interestingly, we observed that this stressful period affected differently the body weight of wild-type and Cbg ko mice. Indeed, between weeks 14 (the end of this stressful period) and 15 (when animals returned to resting condition), while wild-type HF mice presented a similar weight (-0.5%), Cbg ko HF mice had a rapid and important weight gain (+9.3%). Besides the fact that Cbg ko HF mice were heavier than wild-type HF, this new observation suggested a different weight regulation and metabolism.

Consequences of high-fat diet and UCMS on emotional behavior

To examine the impact of HF diet and stress on the emotional reactivity of CBG-deficient mice, Cbg ko and wild-type mice were evaluated in the sucrose preference test (twice, on weeks 12 and 18) to measure anhedonic behavior, then in the open field test (week 18) to measure locomotor activity and anxiety-like behavior, and finally in the forced swim test (week 19) to measure despair-like behavior.

At both week 12 (Figure 3A) and 18 (Figure 3B), unstressed mice were housed 4 per cage with mixed genotypes; thus, we could only measure the effect of the HF diet in the sucrose preference test. No effect of diet was detected at week 12 or at week 18. As for the stressed animals (Figure 3C), evaluated at week 18 in individual cages, no genotype effect, diet effect or interaction between genotype and diet were detected. For all conditions, sucrose consumption was around 60%.

Effects of genotype ($F(1,60) = 17.64$, $p < 0.001$) and UCMS ($F(1,60) = 15.32$, $p < 0.001$), as well as interactions between genotype and diet ($F(1,60) = 4.10$, $p < 0.05$), genotype and UCMS ($F(1,60) = 25.75$, $p < 0.001$), and genotype, diet and UCMS ($F(1,60) = 14.61$, $p < 0.001$), were found for despair-like behavior in the forced swim test (Figure 3D). A posthoc test revealed that control Cbg ko mice displayed greater immobility scores than wild-type controls ($p < 0.001$). Furthermore, in wild-type mice, immobility behavior was increased by the UCMS procedure ($p < 0.05$ wild-type vs. wild-type S) and by the association of HF and UCMS ($p < 0.001$ wild-type vs. wild-type HFS). For Cbg ko mice, immobility behavior was not significantly affected by HF diet or UCMS conditions (despite a tendency to increase with UCMS), and was always higher than that from wild-type mice for every corresponding condition ($p < 0.001$ for standard condition, $p < 0.001$ for HF diet and $p < 0.05$ for UCMS). However, Cbg ko HFS mice showed decreased immobility when compared to control Cbg ko ($p < 0.05$), and to wild-type HFS mice ($p < 0.001$), but no difference from that from control wild-type mice. Thus, in

addition to the body weight loss observed in Cbg ko HFS mice, we also observe reduced despair-like behavior in this same group.

Locomotor activity was evaluated by measuring the total number of squares crossed during the 6 min of the open field test (Figure 3E). Effects of genotype ($F(1,52) = 5.62, p < 0.05$), diet ($F(1,52) = 13.02, p < 0.001$) and UCMS ($F(1,52) = 6.02, p < 0.05$) on total locomotion were detected, as well as an interaction between genotype, diet and UCMS ($F(1,52) = 5.47, p < 0.05$). Posthoc analyses showed that wild-type HF displayed lower (29.2%) locomotor activity than control wild-type mice ($p < 0.01$). Cbg ko controls also displayed a reduced (27.2%) locomotor activity when compared to wild-type

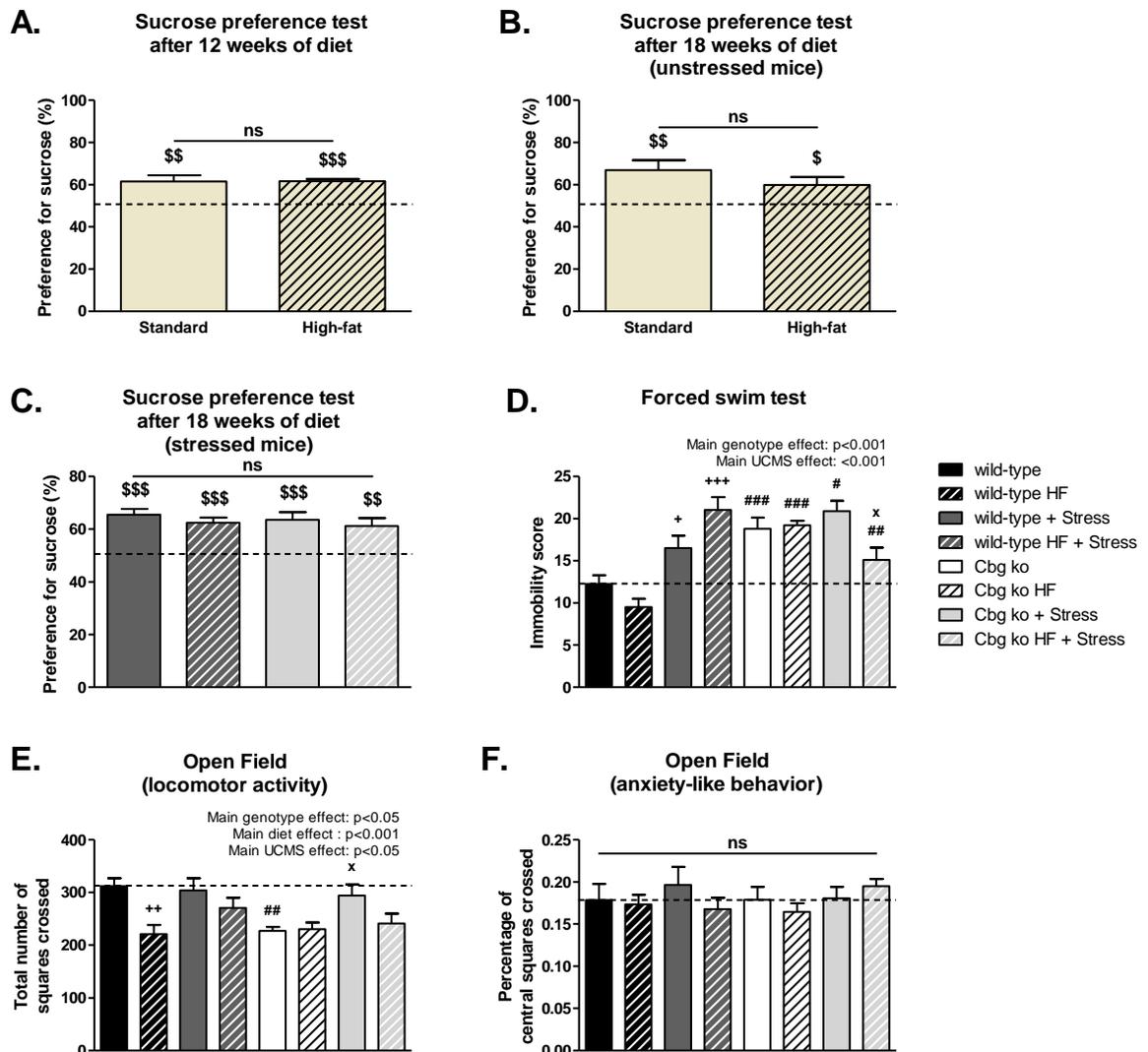


Figure 3 – Consequences of high-fat diet and UCMS on emotional behavior. Sucrose preference test, performed before the UCMS and while animals were all collectively housed with mixed genotypes per cage (A), or performed after the UCMS while unstressed animals were still collectively housed with mixed genotypes per cage (B) and stressed animals were individually housed (C). Despair-like behavior displayed in the forced swim test after the UCMS (D). Locomotor (E) and anxiety-like (F) behaviors displayed in the open field test after the UCMS. (\$) represents significant difference from chance level, while (#) represents significant difference between genotypes within the same conditions, and (+) and (x) represent significant difference from the control group from the same genotype (wild type and Cbg ko, respectively).

controls ($p < 0.01$). Furthermore, compared to Cbg ko controls, Cbg ko S mice presented a 29.5% higher locomotor activity ($p < 0.05$). No difference was observed regarding anxiety-like behavior (Figure 3F), regardless of the genotype, diet or UCMS conditions.

Consequences of high-fat diet and UCMS on HPA axis

Plasma corticosterone concentrations were measured in the morning (Figure 4A) and in the evening (beginning of the active phase) at week 18 (Figure 4B). Analyses revealed genotype ($F(1,55) = 105.18$, $p < 0.001$), diet ($F(1,55) = 28.22$, $p < 0.001$), UCMS ($F(1,55) = 12.01$, $p < 0.01$) and time of the day ($F(1,55) = 4.51$, $p < 0.05$) effects. Interactions were found between genotype and diet ($F(1,55) = 4.48$, $p < 0.05$), genotype and UCMS ($F(1,55) = 11.63$, $p < 0.01$), time of the day and genotype ($F(1,55) = 23.90$, $p < 0.001$), time of the day and UCMS ($F(1,55) = 5.89$, $p < 0.05$) and time of the day, genotype and UCMS ($F(1,55) = 5.93$, $p < 0.05$).

The posthoc test revealed that under standard diet, as observed in our previous study (Richard et al., 2010), corticosterone levels were lower in Cbg ko when compared to wild-type mice. This difference was smaller in the morning (36.3 ± 3.7 nM vs. 58.1 ± 7.8 nM; $p < 0.05$) and highly significant in the evening (25.7 ± 2.5 nM vs. 94.6 ± 8.6 nM; $p < 0.001$). HF diet, UCMS or the combination of both conditions did not change this profile. However, we noticed that when under UCMS conditions, no significant effect of the genotype for morning or evening values is observed. Then, regarding HF diet effect, while no statistical difference was observed between wild-type and wild-type HF mice in the morning (despite an apparent increase in the wild-type HF group), corticosterone levels were significantly increased in the evening (+44%, $p < 0.001$ wild-type HF vs. wild-type). A similar profile can be observed among Cbg ko mice, but no statistical significance was detected. Conversely, UCMS conditions had no significant effect on morning corticosterone levels of wild-type S and Cbg ko S mice. In the evening, however, UCMS induced a significant decrease in corticosterone levels of wild-type S mice (-39%, $p < 0.01$ wild-type S vs. wild-type). Interestingly, no difference was observed between Cbg ko and Cbg ko S mice. Finally, when HF diet was combined to UCMS, no significant difference was observed (despite slightly increase values for HFS mice) for morning or evening values, regardless of the genotype. Thus, most differences were observed for evening corticosterone levels, at the circadian peak. We found that wild-type mice were more susceptible to the opposed effects of HF diet and UCMS, and when both conditions were combined, a compensatory effect appeared. These phenomena were not observed in Cbg ko mice, which presented low corticosterone levels and seemed to be insensitive or at least less impacted by these conditions. Furthermore, the circadian rhythm of corticosterone levels was found blunted in Cbg ko mice, regardless of the diet and stress conditions. CBG binding capacity was also evaluated in wild type mice (Figure 4C). Only an overall effect of diet ($F(1,20) = 14.51$, $p < 0.01$) was detected, with HF fed mice displaying a higher binding capacity than wild-type mice under standard diet, regardless of the stress status.

The expression of genes involved in the HPA axis activity was also evaluated (Figure 5). No genotype, diet or UCMS effects were found for mineralocorticoid receptor (GR, Nr3c2) or glucocorticoid receptor (GR, Nr3c1) mRNA expression in the hippocampus (Figure 5A,B). However, within wild-type mice, both Nr3c2 and Nr3c1 expression showed a non-significant trend towards a decrease in the UCMS condition. Cbg ko, on the other hand, seem to display a discrete non-significant increase in Nr3c1

expression when under HF diet. A marginally significant genotype effect ($F(1,56) = 3.59$, $p = 0.06$) and a significant UCMS effect ($F(1,56) = 6.29$, $p < 0.01$) were found for the expression of Hsd11b1 (11 β -hydroxysteroid dehydrogenase type 1, 11 β HSD1) (Figure 5C) mRNA in the hippocampus, but no interaction between factors was detected. Still, Cbg ko controls display a trend towards an increased expression when compared to wild-type controls, and UCMS seems to decrease the expression of Hsd11b1, regardless of the genotype.

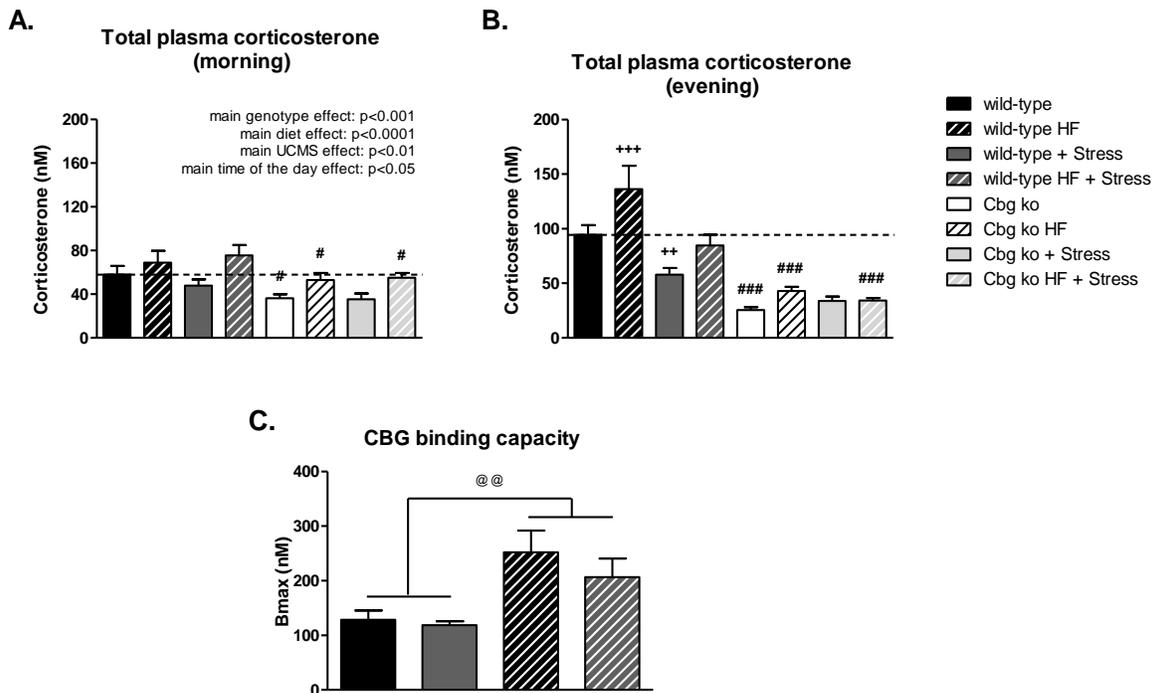


Figure 4 – Total plasma corticosterone concentration in the morning (A) and evening (B) of week 18. (#) represents significant difference between genotypes within the same conditions, while (+) and (x) represent significant difference from the control group from the same genotype (wild type and Cbg ko, respectively) and (@) represents a significant overall effect of diet.

Corticotropin-releasing hormone (Crh) and corticotropin-releasing hormone receptor 1 (Crhr1) mRNA expression were measured in the hypothalamus (Figure 5D,E). A genotype effect ($F(1,57) = 6.14$, $p < 0.05$) and an interaction between genotype, diet and stress ($F(1,57) = 5.74$, $p < 0.05$) were found for Crh expression. Posthoc analysis showed a significant effect of genotype under UCMS conditions (wild-type S vs. Cbg ko S, $p < 0.01$), with Crh mRNA expression being lower in Cbg ko mice. Moreover, while only non-significant trends towards higher expression in HF and HFS groups were observed among Cbg ko mice, the wild-type HF group displayed a trend and wild-type UCMS group displayed a significant elevation of Crh gene expression when compared to wild-type controls ($p < 0.05$). For Crhr1, an interaction between genotype, diet and UCMS was detected ($F(1,60) = 5.95$, $p = 0.02$). The posthoc analysis showed that Crhr1 mRNA levels were lower in Cbg ko HF compared to wild-type HF ($p < 0.05$). Moreover, among wild-type mice, we observed a higher expression of Crhr1 in the HF fed group ($p < 0.01$) and a non-significant trend in the same direction for wild-type S and wild-type HFS groups. In Cbg ko mice, the expression levels of Crhr1 were not altered by the different conditions, despite a non-significant tendency towards a lower expression in

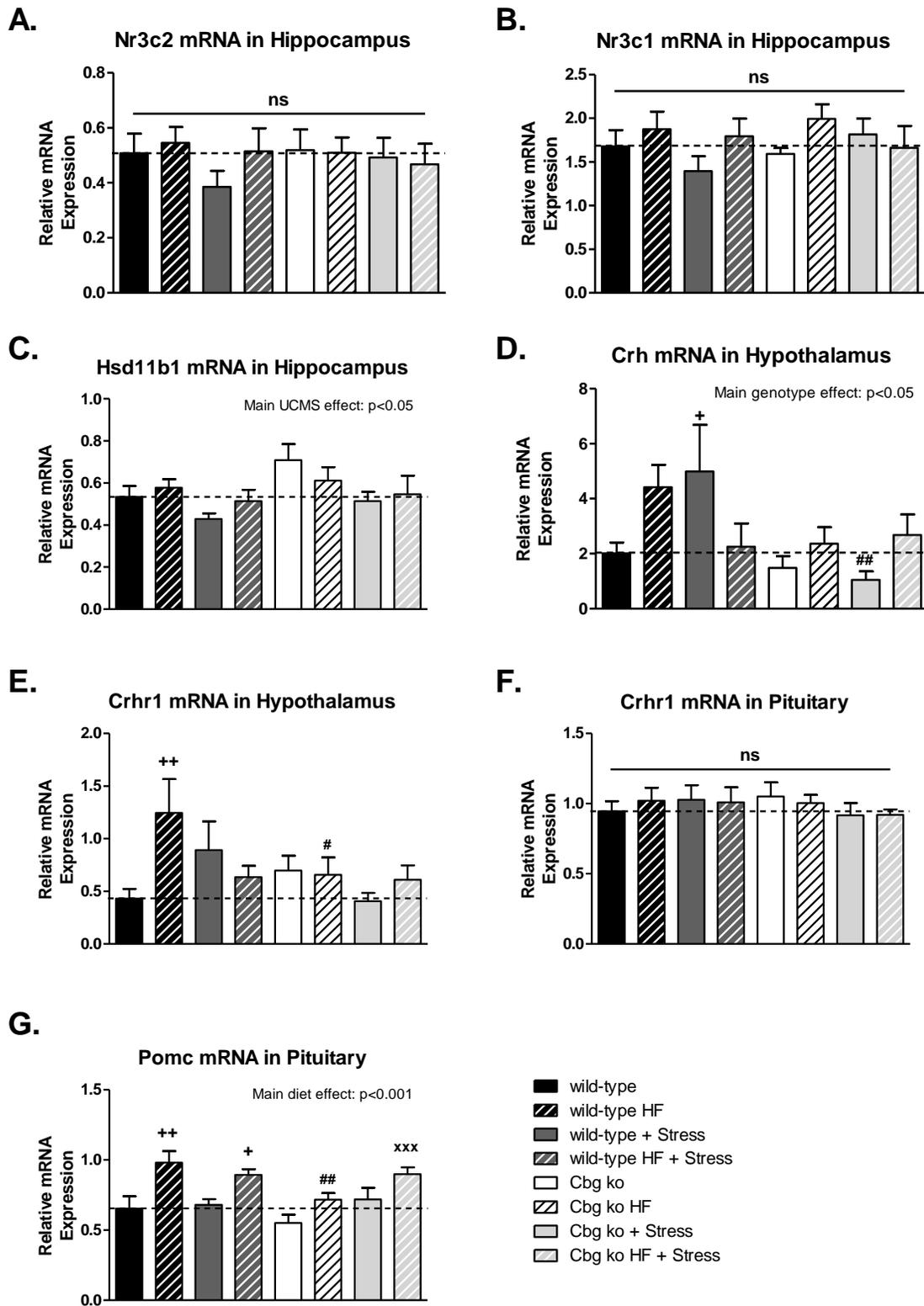


Figure 5 – Expression of genes from the HPA axis. Gene expression evaluated by RT-qPCR for MR (Nr3c2, A), GR (Nr3c1, B) and Hsd11b1 (C) in hippocampus, of Crh (D) and Crhr1 (E) in hypothalamus, and of Crhr1 (F) and Pomc (G) in pituitary. (#) represents significant difference between genotypes within the same conditions, while (+) and (x) represent significant difference from the control group from the same genotype (wild type and Cbg ko, respectively).

Cbg ko S group. Also, Cbg ko controls tend to express more *Crhr1* in the hypothalamus than wild-type controls. *Crhr1* mRNA was also measured in the pituitary but no difference between groups was detected (Figure 5F). Pro-opiomelanocortin (*Pomc*) mRNA was measured in pituitary (Figure 5G). The three-way ANOVA showed a strong diet effect ($F(1,58) = 23.96$, $p < 0.001$) and an interaction between genotype and stress ($F(1,58) = 5.15$, $p < 0.05$). The post hoc test revealed that, when under HF diet, wild-type mice display a higher expression of *Pomc* mRNA in the pituitary than Cbg ko mice. Furthermore, wild-type HF present an increase in *Pomc* expression when compared to wild-type controls ($p < 0.01$), and a trend in the same direction is observed for Cbg ko mice. The combination of HF and UCMS increases the *Pomc* expression in mice from both genotypes ($p < 0.05$ for wild-type and $p < 0.001$ for Cbg ko).

Consequences of high-fat diet and UCMS on energy metabolism

As a possible factor explaining the important weight regulation difference between wild-type and Cbg ko mice induced by HF diet combined to UCMS procedure, food intake was monitored for both quantitative (grams) and qualitative (kcal) consumption. Concerning unstressed mice, (Figure 6A,B) (for which genotype effect could not be examined because mice from both genotypes were housed in the same cages), a t test showed that animals under HF diet consumed significantly less grams of food (29.8%) than animals under standard diet ($t(8) = 39.6$, $p < 0.001$), but had a 14.5% higher caloric intake ($t(8) = 12.35$, $p < 0.001$), suggesting that animals regulated their food intake according to the calorie density of the diet. Regarding animals submitted to the UCMS (Figure 6C,D), identical effects of diet were also detected (HF animals ate 23.4% less but consumed 24.8% more calories than mice under standard diet, $F(1,31) = 55.94$, $p < 0.001$ for grams consumed and $F(1,31) = 32.45$, $p < 0.001$ for kcal intake), but neither genotype effect nor genotype and diet interaction were observed. Thus, we conclude that the weight loss observed in Cbg ko HFS mice was not due to differences in food consumption or calorie intake. The levels of the anorexigenic hormone leptin was also measured in the plasma of mice from HFS groups only. A t-test did not reveal any significant difference in leptin levels between both genotypes, but a trend towards lower levels is observed for Cbg ko HFS mice.

Blood glucose was measured at two phases of this experiment: after 12 weeks of diet (Figure 7A) and after 18 weeks of diet – combined or not to UCMS procedure (Figure 7B). At 12 weeks, only a diet effect was detected ($F(1,63) = 33.62$, $p < 0.001$). Wild-type and Cbg ko mice presented similar blood glucose levels under standard diet (82.4 mg/dL and 85.9 mg/mL respectively), and under HF diet (113.9 mg/dL and 112.1 mg/dL respectively), and HF diet increased the levels of both by 37%. At week 18, we observed that glucose levels were 40.7% higher for wild-type and 30.3% higher for Cbg ko mice under standard diet when compared to week 12. An interaction between diet and stress ($F(1,65) = 4.68$, $p = 0.03$), with posthoc analyses showing a trend towards higher glucose levels in wild-type submitted to HF diet when compared to those on standard diet (+18.5% wild-type vs. wild-type HF, $p = 0.09$), but this trend was not observed for Cbg ko mice (Cbg ko vs. Cbg ko HF, $p = 0.29$). Furthermore, the UCMS procedure induced a similar trend to that from HF: glucose levels were 19.8% higher in wild-type mice only (wild-type vs. wild-type S, $p = 0.09$), and not in Cbg ko (Cbg ko vs. Cbg ko S, $p = 0.17$). Finally, when HF diet was associated to UCMS, no change was observed in blood glucose levels when compared to unstressed standard diet groups for each

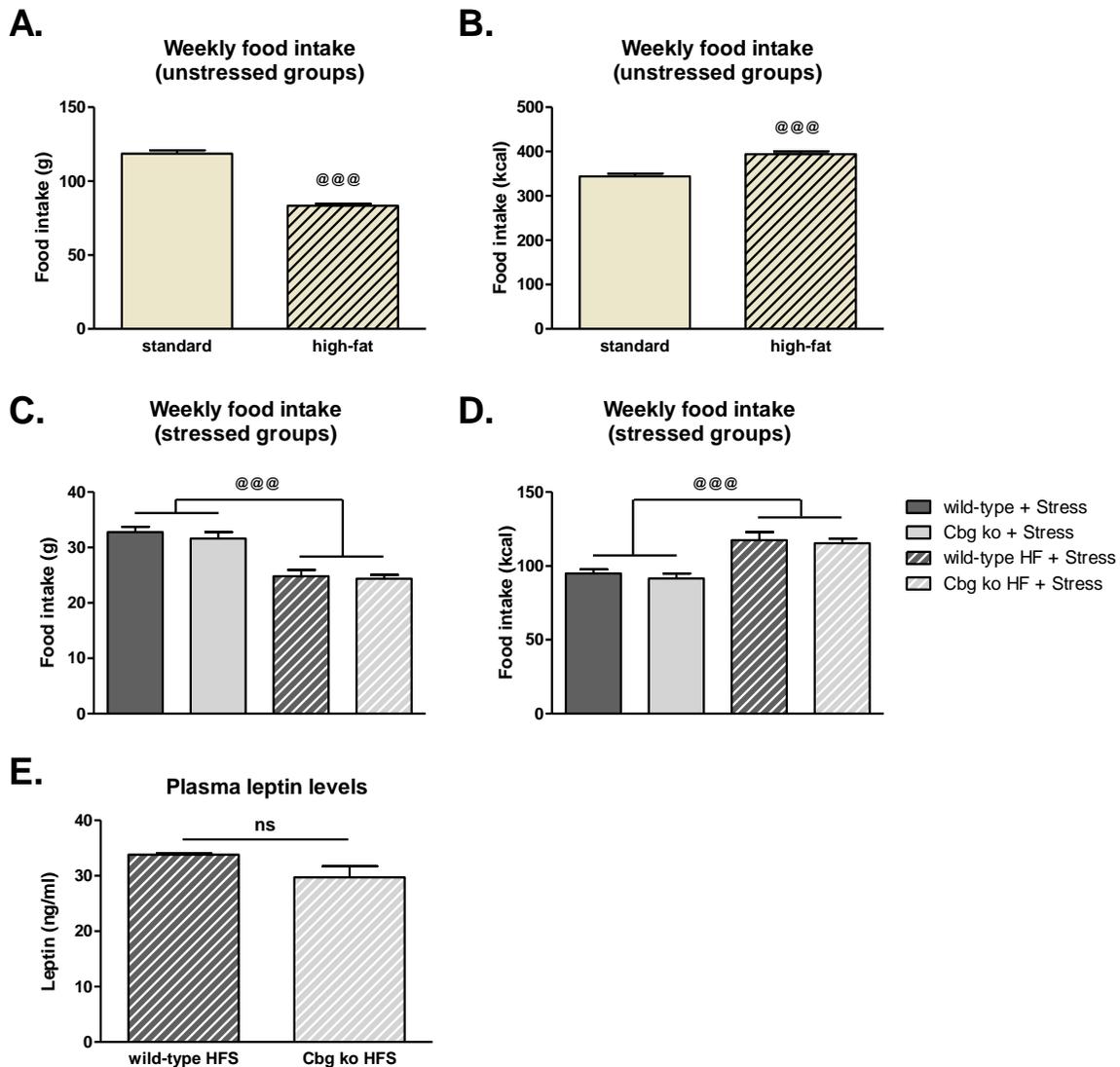


Figure 6 – Quantitative (grams) and qualitative (kcal) assessment of food intake. Food intake for unstressed mice, which were collectively housed with mixed genotypes per cage (A,B), and for stressed mice, which were individually housed (C,D). Leptin levels measured in HFS groups only (E). (@) represents a significant overall effect of diet.

genotype. Furthermore, wild-type HFS and Cbg ko HFS presented similar blood glucose levels (mean of 129.9 mg/dL and 125.4 mg/dL, respectively). Thus, Cbg ko mice seem less sensitive to the isolated effects of HF diet and UCMS on glucose regulation after 18 weeks of the experiment when compared to wild-type mice.

At sacrifice, we observed that the liver of mice under HF diet, regardless of the genotype and UCMS treatment, presented steatosis. This observation, in addition to the body weight loss observed in Cbg ko HFS, prompted us to evaluate plasma lipid profiles, including CE, TG, PL, FFA and CH. No genotype or diet effects were found for CH levels (Figure 8A), but a significant UCMS effect reducing CH levels was found ($F(1,35) = 4.18$, $p < 0.05$, ~15% in wild-type S vs. wild-type and ~20% in Cbg ko S vs. Cbg ko mice). Regarding plasma CE (Figure 8B), a strong diet effect ($F(1,35) = 71.32$, $p < 0.001$) was detected, as well as an interaction between diet and UCMS ($F(1,35) = 4.59$, $p < 0.05$). In unstressed condition, the HF diet led to a similar increase of CE in

wild-type and Cbg ko (40% and 43%, respectively; $p < 0.001$ for both when compared to the respective controls), whereas in UCMS condition the HF diet induced a slightly less important increase in Cbg ko when compared to wild-type mice (Cbg ko vs. Cbg ko HFS +22%, $p < 0.01$ and wild-type vs. wild-type HFS +33%, $p < 0.001$). For TG levels (Figure 8C), only an effect of diet was found ($F(1,35) = 77.36$, $p < 0.001$), which was opposed to that from cholesterol esters. Levels were decreased by 46% in wild-type (wild-type vs. wild-type HF) and by 68% in Cbg ko (Cbg ko vs. Cbg ko HF). While UCMS alone had no effect on TG levels, the association of UCMS and HF produced a similar decrease in TG levels as HF diet alone (57% between wild-type vs. wild-type HFS, $p < 0.001$ and 58% between Cbg ko vs. Cbg ko HFS, $p < 0.001$).

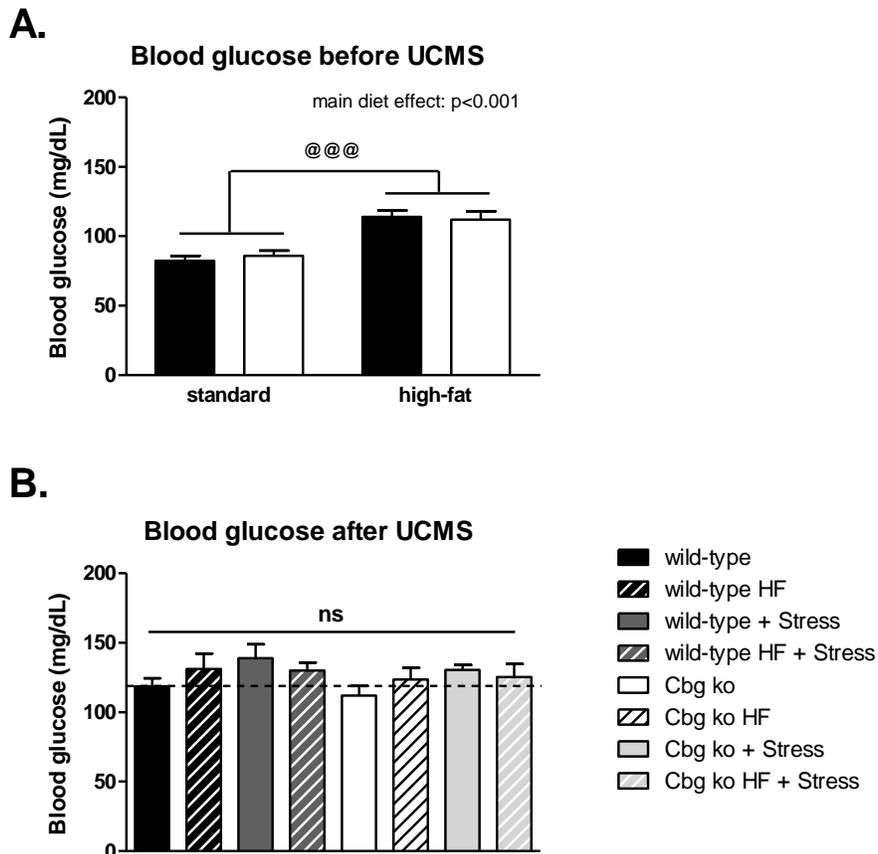


Figure 7 – Blood glucose concentration after 12 (before the UCMS, A) or 18 weeks of diet (after the UCMS, B). (@) represents a significant overall effect of diet.

For plasma FFA (Figure 8D) we observed genotype ($F(1,35) = 11.98$, $p < 0.01$) and diet effects ($F(1,35) = 6.67$, $p < 0.05$). We noticed that profiles of plasma FFA levels induced by each treatment were similar between wild-type and Cbg ko (HF decreased, UCMS induced no change and HFS decreased the levels), but once again, this profile was blunted in Cbg ko mice. The post hoc test revealed only a significant difference between wild-type S and Cbg ko S groups ($p < 0.05$), despite a clear profile of higher levels in wild-type mice from all groups being observed. Finally, genotype ($F(1,35) = 4.60$, $p < 0.05$) and diet effects ($F(1,35) = 6.24$, $p < 0.05$) were found for plasma PL levels (Figure 8E). Regardless of the conditions, levels of PL were slightly higher in Cbg ko mice. Moreover, in both wild-type and Cbg ko mice, HF diet combined to UCMS tended to increase plasma PL levels. Plasma fatty acids were then measured in CE, PL and TG classes (Tables 2, 3 and 4).

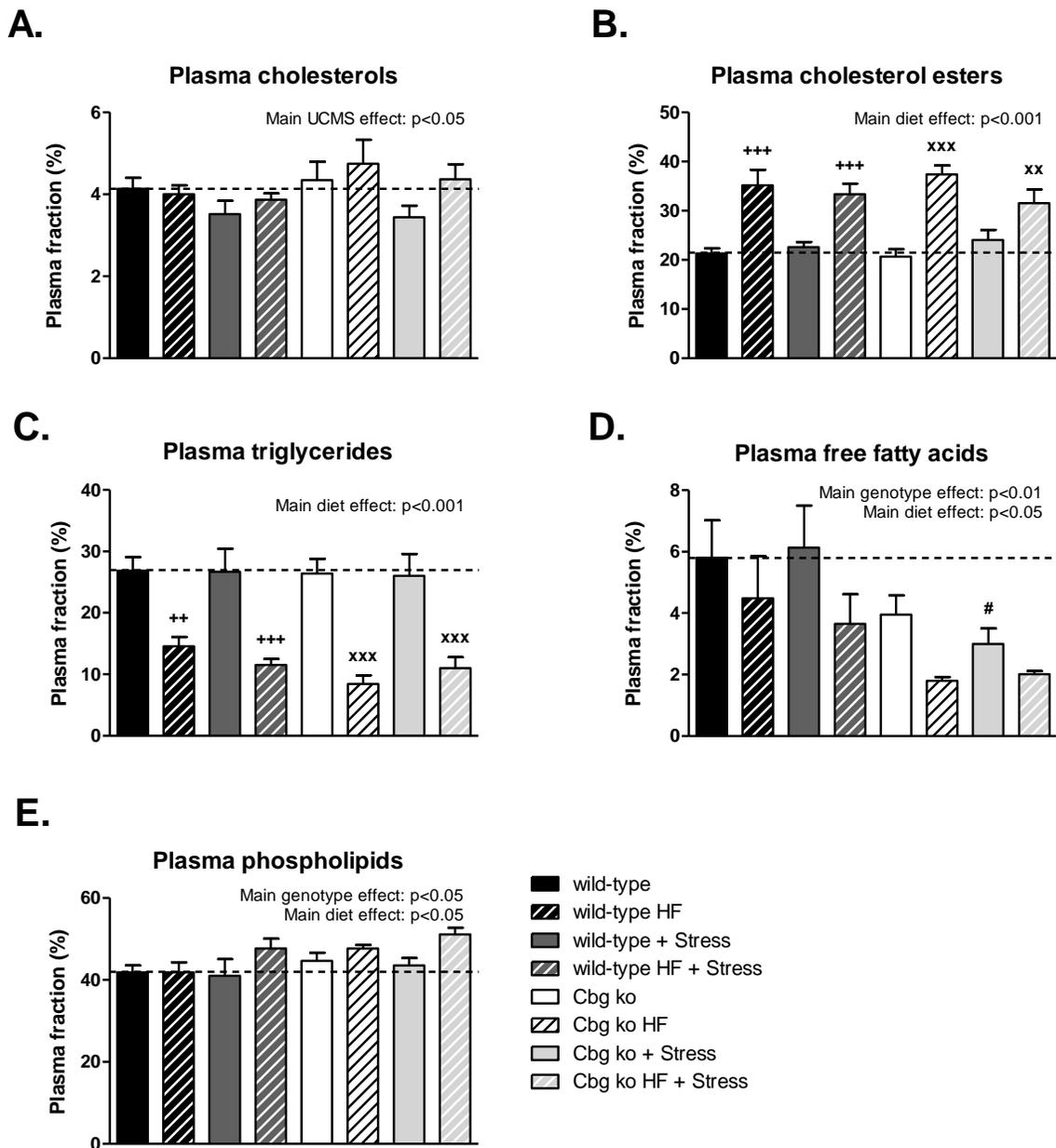


Figure 8 – Cholesterol (A), cholesterol esters (B), triglycerides (C), free fatty acids (D) and phospholipids (E) profiles measured at sacrifice, after 20 weeks of diet. (#) represents significant difference between genotypes within the same conditions, while (+) and (x) represent significant difference from the control group from the same genotype (wild type and Cbg ko, respectively).

To further study the metabolic status of these mice, we investigated the expression of several genes involved either in lipid (Figure 9) or glucose metabolism (Figure 10). Regarding lipid metabolism, we analyzed the expression of transcription factors (peroxisome proliferator-activated receptor alpha and gamma – Ppara and Pparg) regulating lipid metabolism, of genes coding for enzymes (peroxisomal acyl-coenzyme A oxidase 1 - Acox1, and carnitine palmitoyltransferase I - Cpt1b) implicated in beta-oxidation, of receptors (beta-3 adrenergic receptor - Adrb3) and enzymes (hormone-sensitive lipase – Lipe, monoacylglycerol lipase – Mgl1, and adipose triglyceride lipase – Pnpla2) involved in lipolysis in the adipose tissue (Table 1).

Table 2 –Analysis of plasma fatty acids composition of cholesterol ester class.

PLASMA FATTY ACIDS COMPOSITION OF CHOLESTEROL ESTERS CLASS

fatty acids:	genotype Cbg+/+		Cbg+/+ Cbg+/+		Cbg+/+ Cbg-/-		Cbg-/- Cbg-/-		Cbg-/- Cbg-/-		genotype =		Statistical analysis; p value				genotype x diet x UCMS
	diet UCMS	standard no	standard S	HF no	HF S	standard no	standard S	HF no	HF S	diet	diet	UCMS	genotype x diet	genotype x UCMS	diet x UCMS	genotype x diet x UCMS	
12:0	0,36	0,28	0,09	0,14	0,38	0,26	0,08	0,12	0,71	<0,0001	0,14	0,34	0,92	0,005	0,62		
14:0	0,73	1,10	0,44	0,76	1,41	0,63	0,24	0,51	0,58	0,008	0,65	0,24	0,049	0,14			
15:0	1,36	1,24	0,55	0,55	1,02	0,34	0,08	0,16	0,003	0,0006	0,14	0,95	0,65	0,62			
16:0	6,96	5,73	4,93	6,44	6,90	4,67	3,40	4,34	0,02	0,04	0,53	0,06	0,48	0,71			
16:1n-9	0,72	0,69	0,46	0,42	0,72	0,64	0,45	0,45	0,72	<0,0001	0,24	0,65	0,89	0,60			
16:1n-7	10,79	9,61	2,82	1,84	11,20	10,23	2,80	2,21	0,50	<0,0001	0,04	0,80	0,86	0,85			
17:0	0,56	0,66	0,15	0,17	0,36	0,28	0,08	0,21	0,09	0,0002	0,97	0,14	0,69	0,24			
18:0	1,15	1,20	1,83	3,22	1,24	0,66	0,28	0,85	0,005	0,04	0,35	0,003	0,34	0,016			
18:1n-9	11,20	10,43	5,74	5,72	11,62	10,80	6,04	6,49	0,27	<0,0001	0,48	0,89	0,77	0,25			
18:1n-7	1,60	1,19	0,51	0,51	1,33	1,35	0,48	0,46	0,43	<0,0001	0,13	0,93	0,12	0,12			
18:2n-6	38,36	38,31	34,85	33,67	37,21	37,14	34,44	38,15	0,54	0,007	0,055	0,17	0,82	0,07			
18:3n-6	0,77	0,79	0,75	0,96	0,69	0,94	0,76	0,80	0,76	0,78	0,22	0,56	0,99	0,11			
18:3n-3	0,54	0,56	0,30	0,33	0,87	0,47	0,24	0,38	0,36	<0,0001	0,39	0,26	0,21	0,94			
20:3n-9	0,60	0,63	0,22	0,18	0,41	0,80	0,22	0,19	0,70	<0,0001	0,03	0,83	0,06	0,02			
20:3n-6	0,99	1,27	1,18	1,01	1,07	1,31	1,28	0,99	0,28	0,27	0,69	0,97	0,38	0,007			
20:4n-6	16,84	19,50	40,56	39,76	16,88	21,94	44,85	39,70	0,16	<0,0001	0,46	0,54	0,43	<0,0001			
20:5n-3	2,35	2,38	0,68	0,58	2,31	2,42	0,61	0,67	0,97	<0,0001	0,74	0,74	0,45	0,0007			
22:6n-3	4,12	4,42	3,79	3,75	4,39	5,12	3,67	3,35	0,37	<0,0001	0,31	0,07	0,71	0,41			
															0,005		

Table 4 – Analysis of plasma fatty acids composition of triglycerides class.

PLASMA FATTY ACIDS COMPOSITION OF TRIGLYCERIDES CLASS

genotype	Cb _g +/+		Cb _g +/-		Cb _g -/-		Cb _g -/-		Cb _g -/-		Cb _g -/-		genotype =		Statistical analysis; p value		diet x UCMS	geno x diet x UCMS	
	diet UCMS	standard no	standard S	HF no	HF S	standard no	standard S	HF no	HF S	standard no	standard S	HF no	HF S	genotype =	diet UCMS	genotype =			genotype =
fatty acids:																			
14:0	1,08	1,00	0,68	1,32	1,59	1,41	0,69	1,02	0,26	0,02	0,20	0,03	0,45	0,03	0,11	0,62	0,99	0,79	
15:0	0,61	0,62	0,46	0,40	0,82	0,90	0,14	0,29	0,93	0,01	0,77	0,11	0,62	0,01	0,51	0,55	0,86	0,44	
16:0	21,79	22,58	20,58	20,13	25,48	27,41	22,11	18,83	0,005	<0,0001	0,73	0,01	0,57	0,01	0,30	0,91	0,03	0,18	
16:1n-9	0,93	0,91	1,11	1,09	0,92	1,29	1,49	0,93	0,01	0,02	0,30	0,51	0,55	0,01	0,07	0,86	0,0003	0,0003	
16:1n-7	7,27	6,33	2,74	2,28	8,95	8,54	3,26	2,08	0,01	<0,0001	0,07	0,03	0,91	0,01	0,39	0,44	0,0003	0,0003	
17:0	0,33	0,51	0,23	0,57	0,53	0,47	0,32	0,23	0,88	0,25	0,39	0,33	0,11	0,03	0,11	0,75	0,86	0,44	
18:0	2,22	2,28	5,23	5,64	2,14	1,92	5,01	6,66	0,68	<0,0001	0,03	0,15	0,26	0,01	0,15	0,08	0,01	0,08	
18:1n-9	31,30	32,71	35,05	34,26	30,51	32,91	36,50	37,14	0,23	<0,0001	0,24	0,11	0,43	0,20	0,11	0,89	0,20	0,89	
18:1n-7	6,91	5,81	3,00	2,70	6,33	7,06	3,28	2,93	0,17	<0,0001	0,23	0,85	0,04	0,73	0,85	0,03	0,73	0,03	
18:2n-6	17,24	17,16	20,40	20,34	14,08	11,77	17,40	19,72	<0,0001	<0,0001	0,96	0,07	0,95	0,08	0,07	0,08	0,08	0,08	
20:0	0,27	0,36	0,23	0,24	0,37	0,30	0,21	0,28	0,48	0,001	0,76	0,80	0,37	0,54	0,80	0,03	0,54	0,03	
18:3n-6	0,27	0,29	0,29	0,28	0,18	0,20	0,32	0,25	0,17	0,16	0,76	0,20	0,69	0,40	0,20	0,69	0,40	0,69	
20:1n-9	1,33	1,49	0,94	0,96	1,43	1,17	0,86	1,08	0,36	<0,0001	0,50	0,21	0,27	0,09	0,21	0,27	0,09	0,69	
18:3n-3	3,65	3,37	2,99	3,06	1,94	1,07	1,05	1,64	<0,0001	0,29	0,69	0,59	0,96	0,14	0,59	0,96	0,14	0,36	
20:2n-6	0,23	0,18	0,44	0,50	0,21	0,19	0,42	0,57	0,66	<0,0001	0,15	0,56	0,20	0,009	0,56	0,20	0,009	0,51	
20:3n-9	0,26	0,26	0,27	0,33	0,33	0,33	0,43	0,31	0,04	0,28	0,65	0,89	0,22	0,66	0,89	0,22	0,66	0,19	
22:0	0,14	0,16	0,09	0,12	0,17	0,13	0,08	0,08	0,57	0,005	0,97	0,66	0,27	0,47	0,66	0,27	0,47	0,66	
20:3n-6	0,35	0,32	0,48	0,53	0,41	0,36	0,59	0,53	0,24	0,0003	0,62	0,93	0,44	0,71	0,93	0,44	0,71	0,58	
22:1n-9	0,77	0,68	0,52	0,47	0,56	0,19	0,19	0,17	0,003	0,058	0,23	0,85	0,59	0,38	0,85	0,59	0,38	0,50	
20:4n-6	0,72	0,79	2,13	2,38	0,81	0,66	2,86	2,43	0,14	<0,0001	0,61	0,11	0,08	0,82	0,11	0,08	0,82	0,36	
20:5n-3	0,39	0,46	0,25	0,29	0,42	0,34	0,40	0,34	0,5	0,048	0,88	0,08	0,12	0,89	0,08	0,12	0,89	0,74	
22:4n-6	0,15	0,15	0,36	0,36	0,16	0,18	0,51	0,52	0,005	<0,0001	0,83	0,02	0,87	0,98	0,02	0,87	0,98	0,88	
22:5n-6	0,17	0,28	0,27	0,32	0,16	0,10	0,34	0,38	0,76	0,003	0,41	0,07	0,38	0,82	0,07	0,38	0,82	0,39	
22:5n-3	0,30	0,24	0,26	0,27	0,31	0,25	0,33	0,31	0,15	0,47	0,17	0,30	0,75	0,31	0,30	0,75	0,31	0,71	
22:6n-3	1,30	1,07	0,99	1,17	1,22	0,86	1,19	1,30	0,96	0,61	0,45	0,13	0,62	0,03	0,13	0,62	0,03	0,88	

We observe a significant main effect of UCMS on *Acox1* expression in the liver ($F(1,44) = 5.44, p < 0.05$) and an interaction between genotype and diet ($F(1,44) = 4.09, p < 0.05$) (Figure 9A). The posthoc analysis revealed that UCMS increases the expression of this gene in the liver of *Cbg ko* mice when compared to *Cbg ko* controls, but this effect is not significant among wild-type mice (despite a clear trend). The expression of *Acox1* in *Cbg ko* mice also presented a trend towards higher expression when compared to wild-type controls, and curiously the combination of HF diet and UCMS induced non-significant changes in opposite directions, with an increase in wild-type mice and a decrease in *Cbg ko*. No significant effect of genotype, diet or UCMS on the expression of *Ppara* in liver (Figure 9B). Nevertheless, HF diet displayed a tendency to increase the expression of *Ppara* in wild-type mice only, while UCMS presented a trend in the opposite direction for animals from both genotypes. In animals from both genotypes, the association of HF and UCMS led to an expression of *Ppara* between that from controls and UCMS mice. No statistically significant differences for *Cpt1b* expression in muscle was detected (Figure 9C). Still clear trends towards increased *Cpt1b* expression for all wild-type groups when compared to wild-type controls, and for *Cbg ko* HF and HFS groups when compared to *Cbg ko* controls, were observed.

For *Pparg* expression in fat tissue (Figure 9D), effects of genotype ($F(1,30) = 5.82, p < 0.05$) and diet ($F(1,30) = 21.12, p < 0.001$), besides interactions between genotype and diet ($F(1,30) = 7.57, p < 0.01$) and genotype and UCMS ($F(1,30) = 4.94, p < 0.05$) were detected. Furthermore, a marginally significant interaction between genotype, diet and UCMS ($F(1,30) = 3.78, p = 0.06$) was observed. Posthoc analyses revealed higher expression of *Pparg* in *Cbg ko* HF ($p < 0.05$) and *Cbg ko* HFS ($p < 0.001$) when compared to their controls. *Cbg ko* HFS also displayed higher expression than wild-type HFS ($p < 0.001$) and wild-type animals under HF diet presented a non-significant trend towards higher expression of *Pparg*. Both genotype ($F(1,29) = 9.15, p < 0.01$) and diet ($F(1,29) = 5.03, p < 0.05$) effects were found for *Adrb3* expression (Figure 9E) in fat tissue, besides interactions between genotype and diet ($F(1,29) = 8.76, p < 0.01$), genotype and UCMS ($F(1,29) = 10.67, p < 0.01$) and diet and UCMS ($F(1,29) = 7.43, p < 0.05$). The posthoc test revealed differences between the expression in *Cbg ko* HFS mice and their *Cbg ko* ($p < 0.001$) controls and wild-type HFS ($p < 0.001$). A tendency towards decreased expression of *Adrb3* is observed for all wild-type groups when compared to control, but none has reached statistical significance. *Lipe* expression in fat was also influenced by genotype ($F(1,32) = 12.75, p < 0.01$) and diet ($F(1,32) = 23.37, p < 0.001$) (Figure 9F). An interaction between these two factors ($F(1,32) = 15.48, p < 0.001$) and marginally significant interactions between genotype and UCMS ($F(1,32) = 4.10, p = 0.05$) and genotype, diet and UCMS ($F(1,32) = 3.97, p = 0.06$) were also observed. The posthoc test revealed that HF diet increases the expression of *Lipe* in the fat tissue of *Cbg ko* ($p < 0.05$), and this increase is even more pronounced when HF is associated with UCMS ($p < 0.001$). *Cbg ko* groups under HF diet also present higher *Lipe* expression than their correspondent wild-type groups, but this difference is only significant for the groups where HF was combined to UCMS ($p < 0.001$). *Mgll* expression presented highly significant effects of genotype ($F(1,31) = 44.50, p < 0.001$), diet ($F(1,31) = 74.64, p < 0.001$) and UCMS ($F(1,31) = 15.52, p < 0.001$) (Figure 9G). Furthermore, interactions between genotype and diet ($F(1,31) = 44.10, p < 0.001$), genotype and UCMS ($F(1,31) = 23.19, p < 0.001$), diet and UCMS ($F(1,31) = 17.90, p < 0.001$) and genotype, diet and UCMS ($F(1,31) = 18.23, p < 0.001$) were detected. The posthoc test revealed that HF diet increases the expression of *Mgll* in the fat tissue of *Cbg ko* ($p < 0.01$), and this increase is again more pronounced when HF is

Furthermore, Cbg ko HFS groups display significantly higher expression of this gene than wild-type HFS group ($p < 0.05$).

It is interesting to notice that the expression of genes involved in lipolysis in the adipose tissue is particularly affected by the combination of HF and UCMS in Cbg ko mice only (Figure 9D,E,F,G,H). This same group presented an important weight loss from the moment both conditions were combined. This puts increased lipolysis as an interesting mechanism to explain the weight loss observed in these animals.

The expression of genes involved in glucose metabolism included genes coding for glucose transporter type 4 (Slc2a4), for a receptor mediating glycogenolysis and gluconeogenesis (beta-2 adrenergic receptor – Adrb2) and for enzymes involved in these same processes (phosphoenolpyruvate carboxykinase 1 – Pck1, glucose 6-phosphatase alpha – G6pc, and tyrosine aminotransferase – Tat) (Table 1). A highly significant main effect of diet ($F(1,55) = 36.4$, $p < 0.001$) and an interaction between the

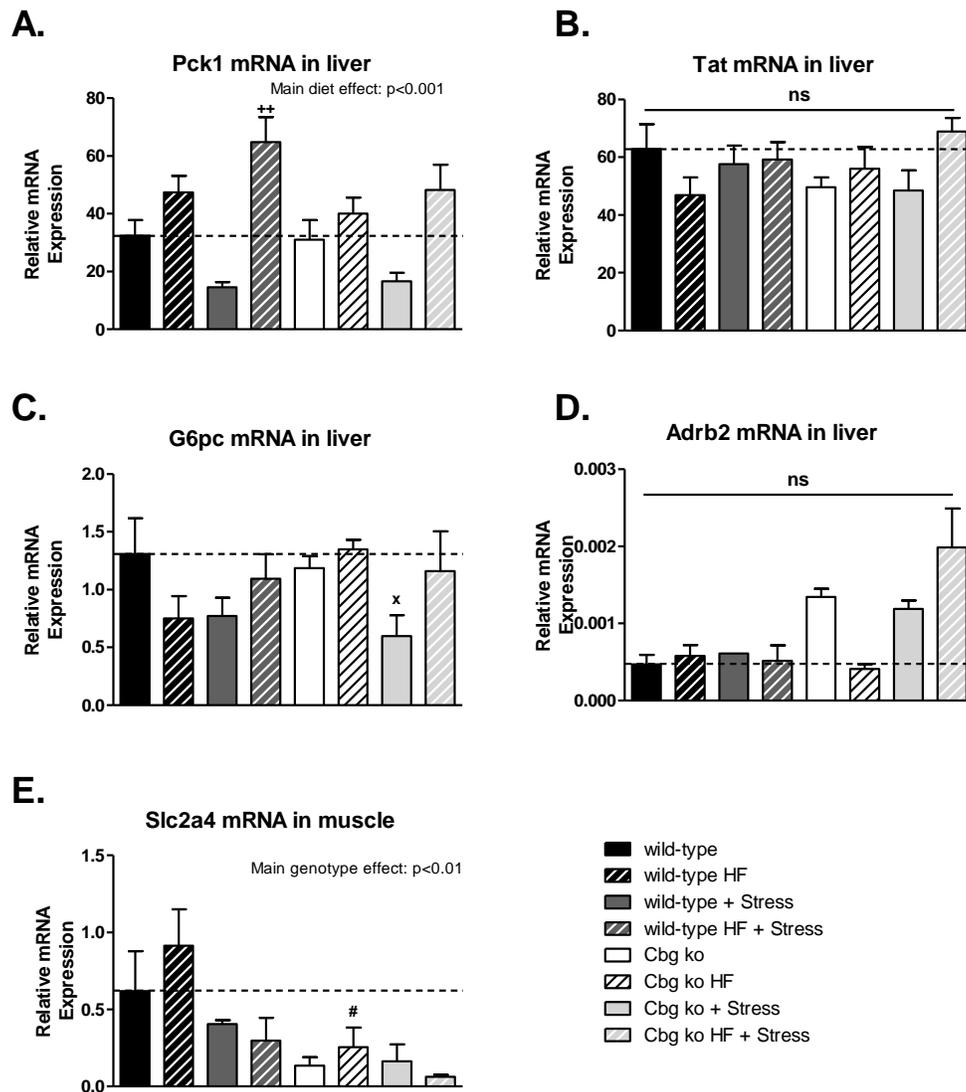


Figure 10 – Expression of genes implicated in glucose metabolism. Gene expression evaluated by RT-qPCR for Pck1 (A), Tat (B), G6pc (C) and Adrb2 (D) in liver and Slc2a4 (E) in muscle. (#) represents significant difference between genotypes within the same conditions, while (+) and (x) represent significant difference from the control group from the same genotype (wild type and Cbg ko, respectively).

factors diet and UCMS ($F(1,55) = 10.93$, $p < 0.01$) were detected for the expression Pck1 in liver (Figure 10A). The posthoc test revealed only a significant higher expression in wild-type animals submitted to the combination of HF diet and UCMS when compared to their wild-type controls ($p < 0.01$), although a general tendency of UCMS to decrease Pck1 expression and of HF diet and HF diet associated or not to UCMS to increase its expression was observed in mice from both genotypes. No significant effects of genotype, diet or their combination was found for Tat expression in the liver (Figure 10B). A small trend towards lower expression is observed in Cbg ko controls when compared to wild-type controls, and trends towards lower expression in wild-type S and higher expression in Cbg ko HFS when compared to their respective controls were also seen. As for G6pc expression in liver (Figure 10C), the three-way ANOVA detected no main effects, but a posthoc test revealed a significant decrease in the expression in Cbg ko mice submitted to UCMS when compared to their Cbg ko controls ($p < 0.05$). Despite not reaching statistical significance, a general pattern towards a lower expression in UCMS groups and a normalization of this expression in animals under HF diet combined with UCMS can be observed. Furthermore, HF diet alone seems to decrease G6pc expression in wild-type mice only. Finally, no statistically significant differences were detected for the expression Adrb2 in liver (Figure 10D). However, all Cbg ko displayed non-significant trends towards higher expression than their respective wild-type group, except for animals submitted to HF only. Furthermore, HF seems to induce a non-significant decrease and the combination of HF and UCMS an increase in the expression of Adrb2 in Cbg ko mice. Finally, regarding the expression of Slc2a4 in muscle (Figure 10E), only a main genotype effect ($F(1,38) = 9.36$, $p < 0.01$) and a marginally significant UCMS effect ($F(1,38) = 3.55$, $p = 0.07$) were detected. The posthoc revealed a significant decrease in the expression of Slc2a4 in the muscle of Cbg ko mice submitted to HF diet when compared to wild-type mice under HF diet. A clear trend towards lower expression levels is observed in Cbg ko controls when compared to wild-type controls. In wild-type mice, non-significant trends towards increase in expression Slc2a4 with HF, decrease with UCMS and further decrease with HF associated to UCMS were observed. Interestingly, the expression profile of Cbg ko mice for this gene seems to be similar to that from wild-type mice, but blunted.

Consequences of HF diet and UCMS on mood neurobiological parameters

The monoamines NA (Figure 11A), serotonin (Figure 11B) – and its main metabolite 5HIAA (Figure 11C), and dopamine (Figure 11D) – and its metabolite DOPAC (Figure 11E), were measured in frontal cortex of each group by HPLC. An interaction between genotype and UCMS was detected for the neurotransmitter norepinephrine ($F(1,64) = 4.19$, $p < 0.05$). Norepinephrine levels were decreased by UCMS in the frontal cortex of wild-type mice (wild-type vs. wild-type S, $p < 0.05$) but not of Cbg ko S mice, which had expression levels similar to those from Cbg ko, but higher than wild-type S ($p < 0.05$). No effects of genotype, HF or UCMS procedure were detected for serotonin, 5-HT1A, dopamine or DOPAC. However, we observed by t test analysis that dopamine levels in Cbg ko HFS mice were higher than in Cbg ko mice ($p = 0,009$). Furthermore, non-significant trends towards increased serotonin and dopamine metabolites are observed in control Cbg ko mice when compared to wild-type controls.

Gene expression analyses were also performed for some genes that are known to be regulated by glucocorticoids or diet, as well as involved in mood modulation

(Figure 12). No significant effect of diet, genotype or UCMS was detected by the ANOVA for the serotonin receptor (Htr1a) mRNA expression measured in hippocampus (Figure 12A). However, a higher expression of this gene was found in Cbg ko mice when compared to wild-type mice controls ($p = 0.03$ by t test). Bdnf gene expression in hippocampus (Figure 12B) was not different between groups. However UCMS had a tendency to modulate gene expression in different ways between genotypes: expression was decreased in wild-type and increased in Cbg ko mice. A posthoc analysis revealed a trend of wild-type S mice towards a lower expression than the control wild-type ($p = 0.07$). Interestingly, cannabinoid receptor type 1 (Cnr1) mRNA in the hippocampus (Figure 12C) presented the same profile of expression from Bdnf, but no statistically significant effects were detected. Npy gene expression in hypothalamus (Figure 12D) showed a robust diet effect ($F(1,28) = 25.20, p < 0.001$), with HF diet increasing Npy expression – as expected, despite no increase in food intake being observed. The posthoc test revealed a significant difference between wild-type controls and wild-type S groups ($p < 0.01$) only, despite a clear increase in Npy expression being observed in all HF groups, regardless of the stress status.

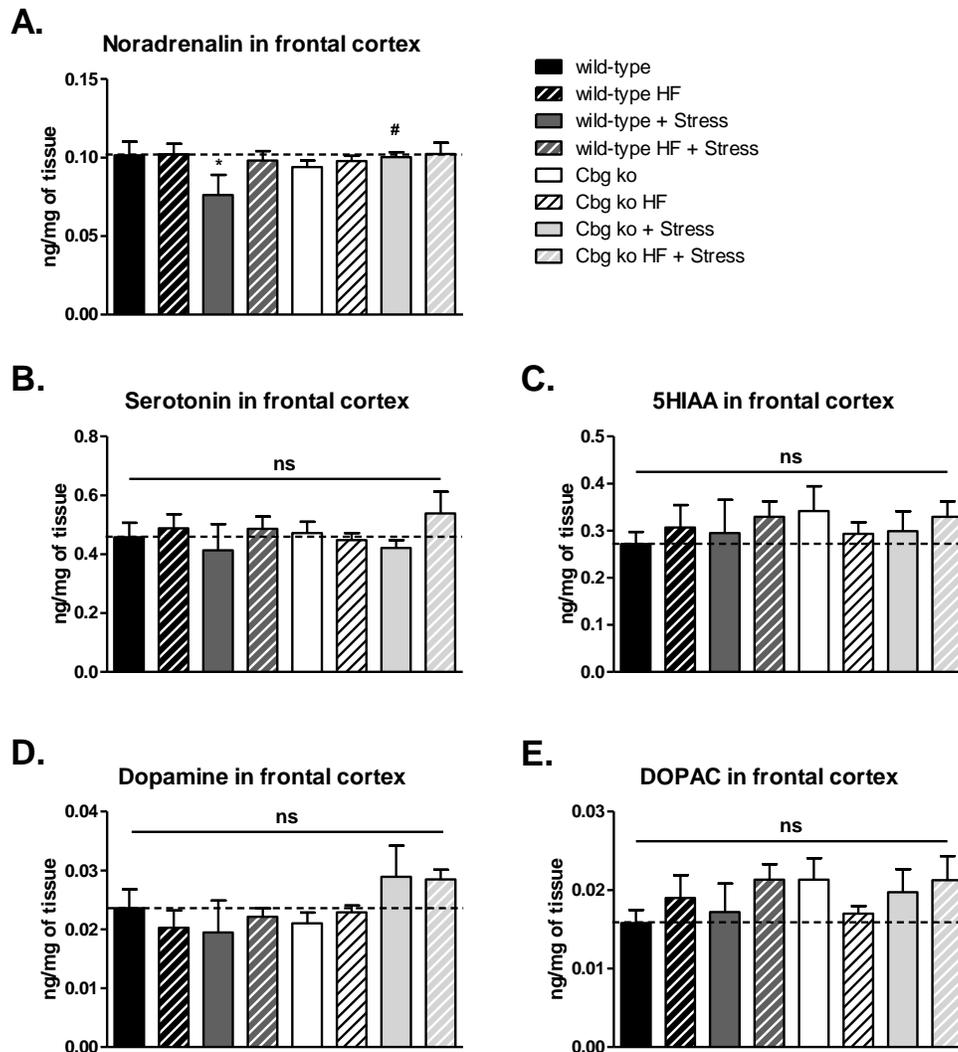


Figure 11 – Levels of the monoamines noradrenalin (A), serotonin (B) and its main metabolite 5-HIAA (C), dopamine (D) and its metabolite DOPAC (E), measured in the frontal cortex by HPLC. (#) represents significant difference between genotypes within the same conditions.

Finally, the cerebral cytokines interleukin 6 (Il6) (Figure 12E) and Interleukin 1 beta (Il1b) (Figure 12F) were examined. The three-way ANOVA detected only a genotype effect ($F(1,60) = 4.07, p < 0.05$) for Il6 mRNA expression in the hippocampus, which was higher in Cbg ko mice. No significant effect of diet, genotype or UCMS was detected for Il1b gene expression. However, Il1b expression seemed lower in wild-type S when compared to wild-type, resembling the profile observed for Il6. Cbg ko displayed similar expression of Il1b to those from wild-type mice under STD diet, and their profile was not influenced by HF, UCMS, or their combination.

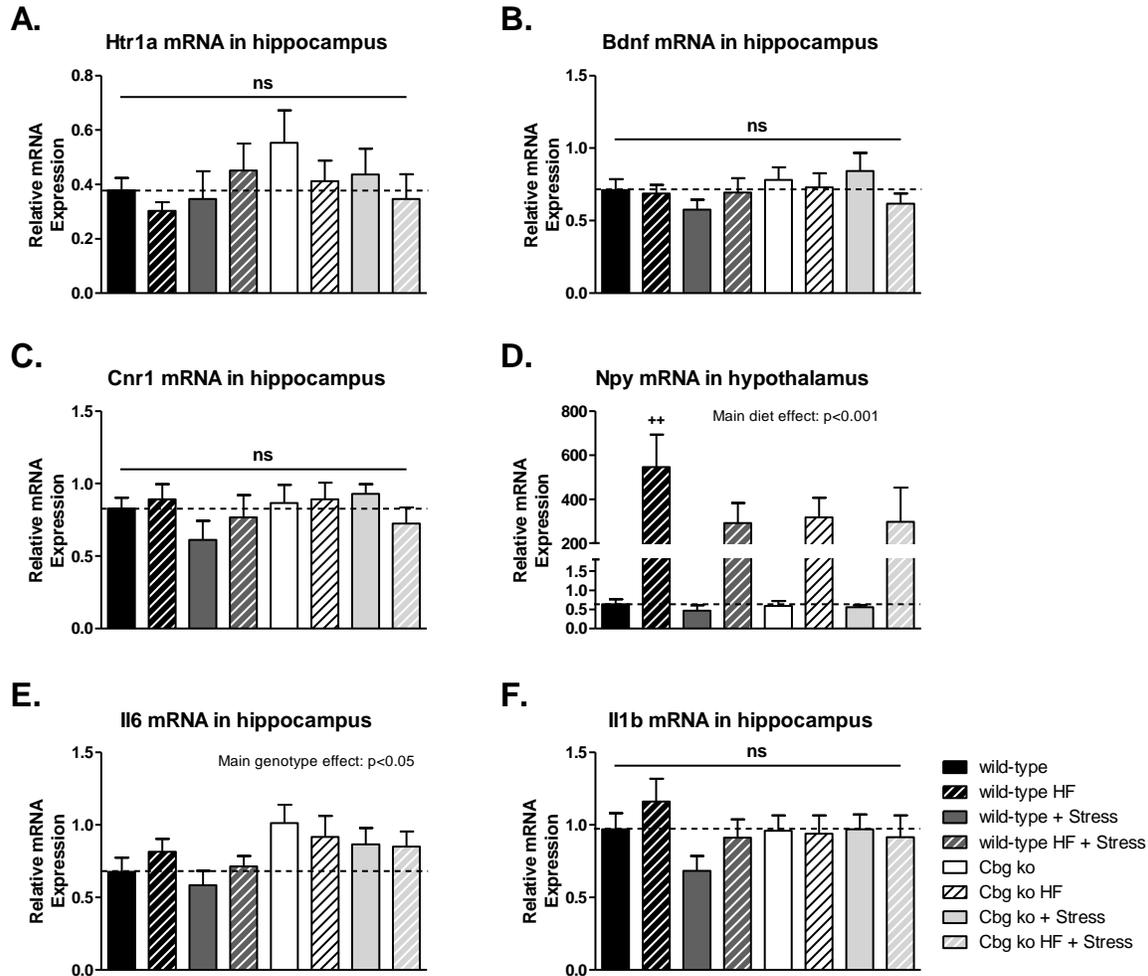


Figure 12 – Expression of genes involved in mood modulation and regulated by glucocorticoids and diet. Gene expression evaluated by RT-qPCR of Htr1a (A), Bdnf (B), Cnr1, Il6 (E) and Il1b (F) in hippocampus and of Npy (D) in hypothalamus. (+) represents significant difference from wild-type control group.

Discussion

Obesity and depression are two important public health issues in modern Western society. The repeated exposure to stressful environments and the easy access to food – notably enriched in fat and sugar – are important factors predisposing the development of these conditions. Glucocorticoids have been long known to be implicated in both metabolic and emotional processes, and interindividual differences in glucocorticoid activity could be one of the factors explaining why some people are more

susceptible to the deleterious effects of the western lifestyle than others. Since CBG has been extensively pointed out as an important factor regulating glucocorticoid availability and action, this study aimed at characterizing the role of CBG in modulating the physiological and emotional alterations induced by high fat diet, UCMS and the combination of both.

Impact of CBG deficiency under standard diet and "no stress" conditions

The comparison of wild-type and Cbg ko mice under STD diet provides new interesting data. Indeed, Cbg ko animals hold an endogenous decreased capacity to cope with stressors. This is particularly evident in their increased immobility behavior in the forced swim test, as previously described (Richard et al., 2010), and decreased locomotion in the open-field test when performed under aversive conditions. The origin of these behavioral differences stems from a lower circulating corticosterone pool in Cbg ko plasma compared to wild-type mice. Although free corticosterone could not be measured in this study, we know from previous analyses that the low corticosterone pool in Cbg ko mice translates into equivalent free corticosterone at rest but suboptimal free corticosterone levels in stressful conditions. Furthermore, we have previously shown that free corticosterone levels were inversely correlated with immobility scores in the forced swim test (Richard et al., 2010). Thus, the altered behavior in the forced swim test and in the aversive open-field test is probably the result of these suboptimal free corticosterone levels.

Several indices suggest that Cbg ko mice under standard rearing conditions display a slight hypocorticism, which might be a product of the accumulation of mild stressors that are naturally present in the lab's housing and breeding conditions. First, several glucocorticoid target genes are more weakly regulated in Cbg ko when compared to wild-type mice. Il6 and Htr1a mRNA, which are known to be negatively regulated by glucocorticoids (Huang and Cidlowski, 2002, Lanfumey et al., 2008), are overexpressed in the hippocampus of Cbg ko mutants. As these genes have an influence on mood regulation, their overexpression may partially explain the behavior of Cbg ko in the forced swim and open-field tests. Slc2a4 is another well described glucocorticoid target gene (Dinneen et al., 1995) that was found under-induced in the muscle of Cbg ko mice. The consequence of this suboptimal expression of Slc2a4 will be lower glucose utilization by tissues, which may explain an altered adaptation to stressors. Also, free fatty acid levels were found lower in Cbg ko plasma when compared to wild-type mice, just like the expression of genes coding for an enzyme involved in beta-oxidation in liver (Acox1), and for a receptor and an enzyme implicated in lipolysis in fat tissue (Adrb3 and Pnpla2). These findings can be explained by the lower availability of glucocorticoids in CBG deficient mice, as glucocorticoids are known to increase lipid metabolism in basal non-pathogenic states (de Guia and Herzig, 2015). Finally, the expression of Hsd11b1 in the hippocampus and of Crhr1 in the hypothalamus was also slightly increased in Cbg ko mice. Although not directly regulated by glucocorticoids, their overexpression might have been induced over time in order to compensate the low circulating corticosterone levels in Cbg ko mice.

As for body weight, Cbg ko mice were found ~9% heavier than wild-type. This was a highly significant effect ($p < 0.001$) found using a large number of mice ($n = 40$ per group). We have measured fat mass percentage through an X-ray scan in a previous experiment, but no difference was found between wild-type and Cbg ko mice. Rather, bone mineral density – another physiologic parameter downregulated by

glucocorticoid – was found elevated in Cbg ko animals (unpublished data). Overall, our data suggest that Cbg ko mice, when kept under standard diet and with limited stress, show signs of hypocorticism associated with behavioral and glucocorticoid-related alterations.

Impact of high-fat diet in CBG deficient mice

Our data show that Cbg ko and wild-type under a high fat diet presented the same weight gain after 12 weeks of diet. Food intake was also similar between genotypes, with HF diet increasing calorie intake but decreasing quantity. Consequences of high fat diet on behavioral reactivity were observed in wild-type mice, which showed significant decrease in locomotion in the open field test and a trend towards a decreased immobility in the forced swim test. However, HF diet did not further decrease the locomotion of Cbg ko mice in the open field, nor further increased their immobility behavior in the forced swim test. The impact of HF diet on the HPA axis was absent or much weaker in Cbg ko when compared to wild-type mice: elevation of corticosterone levels was observed in both genotypes, but the expected increases in *Crh* (Dallman et al., 1995), *Crhr1* and *Pomc* mRNAs expression were observed in wild-type only. CBG binding capacity in wild-type mice was significantly increased by HF diet. Interestingly, the increased expression of *Hsd11b1* observed in control Cbg ko mice (but not that from *Crhr1* in the hypothalamus) shows a trend towards normalization in Cbg ko mice, probably an effect of the increase in plasma corticosterone concentrations (Cbg ko HF display similar values to wild-type controls in the morning measurement).

Npy mRNA expression was greatly induced in HF groups, regardless of the genotype, corroborating other studies (Dryden et al., 1995, Wang et al., 2007). Still, *Npy* gene expression was found to be lower in Cbg ko in comparison to wild-type mice. An increased *Npy* mRNA expression has been described to contribute to energy and fat storage, favoring obesity development. It was observed in studies performed in rats that adrenalectomy led to decrease food intake, weight loss and was associated to decreased NPY efficiency (Castonguay et al., 1986). These effects were then reversed by exogenous corticosterone administration (King et al., 1983). Moreover, high levels of corticosterone can lead to excessive food intake resulting in important weight gain and fat store (Campfield et al., 1995). In our experiment, whereas *Npy* expression tended to be lower in Cbg ko HF than in wild-type HF group, mice from these groups were collectively housed through the whole experiment hence food intake per genotype could not be measured. HF diet induced hyperglycemia in both genotypes – although this blood glucose increase in Cbg ko was no longer significant after 18 weeks of diet.

Furthermore, the differences between genotypes in free fatty acid levels, as well as in *Slc2a4* mRNA levels, were exacerbated in animals under HF diet. On the other hand, the non-significant differences that were observed between wild-type and Cbg ko controls regarding the expression of some genes implicated in lipid and glucose metabolism (*Acox1*, *Adrb3*, *Pnpla*, *Tat* and *Adrb2*) are less evident when both groups are submitted to HF diet. The same is observed for *Hrt1a* and *Il6* in the hippocampus. Finally, no effect of HF diet was detected on the levels of neurotransmitters in cortex.

Thus, the effects of HF diet were reflected mainly on metabolic changes in both wild-type and Cbg ko mice, and an effect of HF diet on behavior was only observed in wild-type mice. At the biological level, these differences between genotypes may originate from the abnormally increased corticosterone levels in wild-type HF mice, which remain low in Cbg ko HF due to their CBG deficiency. In both genotypes, the

observed physiological alterations produced by HF diet were consistent with the effects of increased corticosterone. Regarding the behavior, the influence of elevated corticosterone is not so clear, since corticosterone usually leads to enhanced locomotor activity, and the opposite was observed in the open field test. Nevertheless, the decreased mobility might have been influenced by the increased weight, which could be physically limiting the movements of HF fed mice. Immobility scores in the FST have been previously shown to be increased by HF diet intake, which were also associated with elevated corticosterone levels (Sharma and Fulton, 2013, Abildgaard et al., 2014). Again, the apparent insensitivity of Cbg ko to the effects of HF diet might stem from their inability to maintain an increased plasma corticosterone pool.

Impact of UCMS in CBG deficient mice

Some authors have observed that chronic mild stress exposure can lead to weight loss associated to hypophagia and reduction of body fat mass, both in rodents (stress procedure consisting in chronic intermittent immobilization stress, 2 hours / day) and in humans (stress procedure consisting in one application of the *Life Events and Difficulties Schedule* – LEDS – interview) (Marti et al., 1993, Rojo et al., 2006). However, in our study, no effect of UCMS (consisting in most of the commonly used “mild” stressors (Mineur et al., 2006, Schweizer et al., 2009)) was observed on body weight, food intake, lipid metabolism, or sucrose preference, regardless of the mice’s genotype. The expected effect of UCMS increasing immobility in the forced swim test was detected for wild-type mice, although the locomotor activity assessed the open field test was not affected. This result indicates that the UCMS performed in our conditions had a selective impact on emotional reactivity in wild-type animals. Again, Cbg ko mice seem not to be further affected by UCMS, and maintain their high basal immobility levels.

Plasma CH levels were found slightly lower after UCMS, presenting a similar effect on both wild-type and Cbg ko. A tendency towards a lower expression of genes related to glucose (notably for Pck1, G6pc and Slc2a4) and lipid metabolism (Ppara) was also found in UCMS groups, regardless of the genotype. A tendency of UCMS to increase the expression of Acox 1 in the liver was observed, regardless of the genotype. Interestingly, for some genes involved in lipid metabolism, such as Cpt1b, Adrb3, Mgll and Pnpla2, UCMS tended to cause a decrease in expression, but in wild-type mice only. Some of these same genes (Adrb3 and Pnpla2) were originally downregulated in Cbg ko mice when compared with wild-type controls, and are now downregulated in the wild-type S group, which presents lower glucocorticoid levels (significantly lower in the evening) than their wild-type controls.

The decreased corticosterone levels wild-type mice submitted to the UCMS was also associated with alterations in genes related to HPA axis activity. Trends towards decreased Nr3c2 and Nr3c1 expression in hippocampus (as expected (van Haarst et al., 1996, Furay et al., 2008)), and with increased Crh and Crhr1 expression in hypothalamus were observed. Although the most common assumption is that glucocorticoid levels increase after stressful conditions, a recent review on the effects of chronic stress on corticosterone profiles (Dickens and Romero, 2013) shows that in about half of the studies performed on lab animals analyzed the corticosterone levels are either decreased or unaltered. These same authors propose that a change in glucocorticoid levels might be a better marker for stress conditions than an increase itself. Furthermore, blunted corticosterone levels after chronic stress could be interpreted as a disengagement of the individual following repeated and uncontrollable

stress exposure. Norepinephrine levels in frontal cortex was significantly reduced by UCMS in wild type mice only, and Bdnf and Cnr1 expression in hippocampus of wild-type S mice also displayed trends towards this same direction. Some of these alterations, notably the decrease in Bdnf and Cnr1 levels which have been associated in the literature with increases in depressive-like behavior (McLaughlin et al., 2007, Beyer et al., 2010, Wang et al., 2013, Borsoi et al., 2015), and may contribute to the altered behavior observed for wild-type S mice in the forced swim test. Interestingly, no changes in the expression of these genes were found in Cbg ko mice submitted to the UCMS. In these mutants, corticosterone levels are not different from controls after the UCMS procedure, remaining at low levels. The immobility behavior remained high and did not increase further. Overall, Cbg ko mice seem not to be further impacted by the effects of UCMS on behavior and on the HPA axis, maintaining the endogenous helplessness trait and hypocorticosteronism observed in baseline conditions.

Impact of high-fat diet combined to UCMS in CBG deficient mice

The combination of HF diet and UCMS provided some unexpected results. In wild-type HFS mice, the UCMS treatment did not affect the weight gained by the HF diet, as already observed in a study conducted on C57BL/6J male mice (Petro et al., 2004). It also has not affected locomotor or anxiety parameter in the open field test. The immobility behavior in the forced swim test was higher in wild-type HFS than in wild-type controls, and thus not further modified by the association of HF diet to UCMS. These results are in discrepancy with another study investigating a similar procedure, evaluating the effects of a long term HF diet combined to chronic stress exposure on male mice. On that study, authors showed a protective role of long term exposure to HF diet on the development of stress-induced depressive-like symptoms, evaluated in the forced swim test and through body weight. Interestingly, they observed the opposite consequences on mice under a low-fat diet, which showed no improvement of the behavioral consequences of chronic stress exposure (Finger et al., 2011). These divergences between studies might be explained by differences between the chronic stress protocols, with the chronic social defeat performed in that study being psychologically and physically more intense than our UCMS procedure.

However, at the biological levels, we observed normalization of the evening corticosterone levels in wild-type mice, together with many genes for which significant alterations or trends towards higher or lower expression have been observed in either HF or UCMS conditions. Among these genes are N3cr2, N3cr1, Hsd11b1, Bdnf, Cnr1, Il6 and Il1b in hippocampus, Crh and Crhr1 in the hypothalamus, Ppara, Tat and G6pc in liver, Pparg, and Mgl1 and Pnpla2 in fat tissue. Furthermore, the levels of plasma CH and norepinephrine in the frontal cortex were also normalized. The expression of Pck1 in the liver displayed a significant increase when compared to wild-type controls when HF and UCMS were associated. Furthermore, Slc2a4 expression in muscle remained identical to that from wild-type S groups, displaying a lower trend than in HF and control groups, suggesting that the alterations in glucose metabolism induced by UCMS could not be reversed by the association of HF diet. These findings suggest that, despite the normalization in expression of some genes, wild-type mice still display alterations on glucose metabolism when under HF diet and UCMS combined.

A very different picture was observed for Cbg ko mice. Cbg ko HFS showed an 18% weight loss compared to Cbg ko HF mice, indicating an influence of UCMS on the effects of HF diet. The same Cbg ko HFS group presented reduced immobility behavior

in the forced swim test, reaching levels similar to that from wild-type controls, reflecting an improvement in their despair-like behavior. Their locomotor activity in the open field was not affected by the combination of HF and UCMS, being similar to that from Cbg ko controls or under HF diet. The corticosterone levels of the Cbg ko HFS also remained low, particularly in the evening, and HPA genes were not greatly affected (Chr expression was slightly increased, and Hsd11b1 was slightly decreased, probably as a result of the slight increase in corticosterone levels). Pomc gene expression in pituitary was found elevated in Cbg ko HFS when compared to Cbg ko controls, possibly matching their increasing trends for plasma corticosterone levels. In humans, POMC levels were negatively correlated with adiposity and plasma leptin levels (Page-Wilson et al., 2015), what corroborates the greater weight loss and trend towards lower plasma leptin found for Cbg ko HFS mice in our study. Also associated to the specific weight loss observed in the Cbg HFS group is the pronounced increase in expression of mRNA for a receptor implicated in the activation of lipolysis (Adrb3), of a transcription factor that regulates lipolysis (Pparg), and of enzymes involved in lipolysis in the adipose tissue (Lipe, Mgl1 and Pnpla2).

Serotonin levels in cortex were also slightly increased in Cbg ko HFS, while Htr1a, Bdnf, Cnr1 and Il6 gene expression displayed a discrete decrease when compared to Cbg ko controls. Besides its key role in depression, where reduced levels have been associated with depressive phenotypes, serotonin is also a regulator of HPA axis activity. The 5HT1a receptor, product of the Htr1a gene, is also involved in this regulatory mechanism. The activation of 5HT1a by serotonin has been shown to increase the expression of Crh in the hypothalamus and of Pomc in the pituitary, culminating in increased secretion of ACTH (Jorgensen et al., 2002). As previously mentioned, Pomc mRNA expression was increased and a trend towards the same direction was observed for Crh mRNA, being possibly the consequence of a higher serotonergic activation. Low levels of brain Bdnf and Cnr1 are associated with increased depressive-like behavior, and controversially we observe a decrease in this specific behavior in Cbg ko HFS. However, low expression of these genes have also been associated with weight loss (Xu et al., 2009) and increased beta-oxidation (Tam et al., 2014), corroborating the specific reduction in body weight as a result of the combination of HF and UCMS in Cbg ko mice. Finally, the trend towards a lower expression of Il6, which is classically associated with increased inflammation as well as elevated adiposity and depressive-like behavior, corroborates the aforementioned results and are consistent with the amelioration of the depressive-like phenotype and obesity in Cbg ko submitted to both HF and UCMS.

Taken together, these data suggest that, in wild-type animals, the effects of HF diet and of UCMS on most physiological parameters evaluated are normalized when HF diet and UCMS are combined, except for glucose utilization. As the weight gain and the helplessness trait remained altered in the wild-type HFS group compared to HF alone, it is tempting to conclude that glucose utilization is involved in the maintenance of the obese and despair-like phenotypes. Nevertheless, this hypothesis is not supported by the fact that Slc2a4 mRNA levels were also found very low in the Cbg ko HFS group, which showed weight loss and decreased in immobility behavior in the forced swim test – indicating a reduction on their endogenous despair-like behavior. Interestingly, no increase in Npy expression was detected for HFS mice when compared to HF only, regardless of the genotype. This contradicts the findings of (Kuo et al., 2008) who suggested that the combination of chronic stress and a HF/high-sugar diet (but not

stress or diet alone) amplifies and accelerates diet-induced obesity and the metabolic syndrome by releasing NPY directly into the adipose tissue.

Some of the physiological alterations identified in Cbg ko HFS mice may explain the phenotype of metabolic and behavioral recovery observed in this group: (I) the increase in the levels of Serotonin in cortex and of the expression of Htr1a in hippocampus, Crh in hypothalamus and Pomc in pituitary when compared to Cbg ko controls, suggesting that the glucocorticoid activity is enhanced in CBG HFS (free corticosterone levels remain unmeasured, but may eventually reveal an increased glucocorticoid signaling in the Cbg ko HFS); (II) the corticosterone levels of Cbg ko HFS, which reached normal values in the morning (comparable to those from wild-type mice) but remained low in the evening; (III) and the downregulation of Bdnf and Cnr1 expression in the hippocampus, as well as the upregulation of several proteins involved in lipolysis in fat tissue, suggesting an increased lipid metabolism.

Overall, these results show that CBG deficiency influences the effects of HF, UCMS and combined HF and UCMS conditions. On the latter, Cbg ko mice seemed to be paradoxically more “adapted” to this highly challenging situation. This study demonstrates a larger and more important role of CBG than those previously described. Despite the direct influence of CBG on the pool size of glucocorticoids in plasma, we have also showed that it presents important roles in the metabolism, notably on lipids composition and synthesis and on glucose metabolism. Further analyses of these systems are necessary to precisely determine how CBG influences these parameters.

Complementary studies

Behavioral and metabolic consequences of CBG deficiency on male mice submitted to high fat diet combined to chronic mild stress – Replicating the original Western lifestyle experiment

When I joined the team, we decided to replicate the experiments presented in this article in order to explore some important aspects missing in the first experiment and to further extend and understand the molecular pathways involved. In particular, we were interested in evaluating the fat distribution on the animals from all groups, and also run some classical metabolic tests, such as the insulin resistance and the glucose tolerance tests. We also wanted to evaluate if there were any changes in the free corticosterone values throughout the HF diet and HF combined to the unpredictable chronic mild stress (UCMS) periods. Based on the previously presented results, we hypothesized that the increase in despair-like behaviors observed in the mice from the wild-type HF + UCMS group stems from an overexposure to glucocorticoids – secreted in response to both the UCMS procedure and to the obesity onset – and that the Cbg ko mice would be protected from these effects due to their glucocorticoid hyporesponse. We also expected that fat mass was reduced in Cbg ko HFS due to the increased lipolysis that seem to occur in this group.

Methods

Cbg ko and wild-type mice (MGI ID: 4833641, *Serpina6tm1.1Mmp*, produced in our laboratory in Bordeaux - France) used in this experiments were littermates, obtained by breeding males and females heterozygous for the CBG mutation, and genotyped from a tail biopsy. Our mice have a C57BL/6J genetic background above 95%. Animals were maintained, from birth and throughout the whole experiment, in an animal facility with constant temperature (23 °C), under a 12 h light/dark cycle (lights on at 0700 h) and with ad libitum access to food and water.

40 male mice – 20 Cbg ko and 20 wild-types – of about 15 weeks old were selected and split by age into 10 collective cages (4 mice/cage, mixed genotypes). They were then put under either HF chow (D12451, from Research Diets) or standard chow (STD, A04, from SAFE - Scientific Animal Food & Engineering) (5 cages/diet) and had their weight and food intake evaluated once a week. Blood samples were taken right in the beginning of the experiment (morning and evening) to obtain basal corticosterone and CBG levels (before the beginning of the HF). On the 14th week of diet, blood samples were again collected (morning and evening), and animals were then put in fasting for 24h for a blood glucose measurement. From the 16th to the 20th week (4 weeks), animals from 3 cages of each dietary group (6 mice/diet/genotype) were placed into individual cages and submitted to an UCMS procedure, while the remaining four cages (4 mice/diet/genotype) were left undisturbed. On the 20th week, after the end of the UCMS, blood samples were collected from all animals (morning and evening). On the 21st week animals had their body composition measured through magnetic resonance imaging (nuclear magnetic resonance) and then went through a battery of behavioral tests (open field, splash test and forced swim test). On the 22nd week we intended to evaluate these animals into calorimetric cages, but unfortunately the equipment had some technical problems and we could not perform this evaluation. Instead, animals were submitted to the glucose tolerance and the insulin resistance

tests. The sucrose preference test was repeated and an evaluation of food consumption after fasting was performed on the 23rd week. Finally, animals were sacrificed on the 24th week of test, and samples of blood, brain (1/2 brain, pituitary, hippocampus, hypothalamus and cortex), liver, spleen, muscle, fat (brown, visceral and subcutaneous), adrenals and tail tip (for genotype confirmation) were collected. The experimental design is illustrated on Figure 19 and the detailed description of all procedures can be found below.

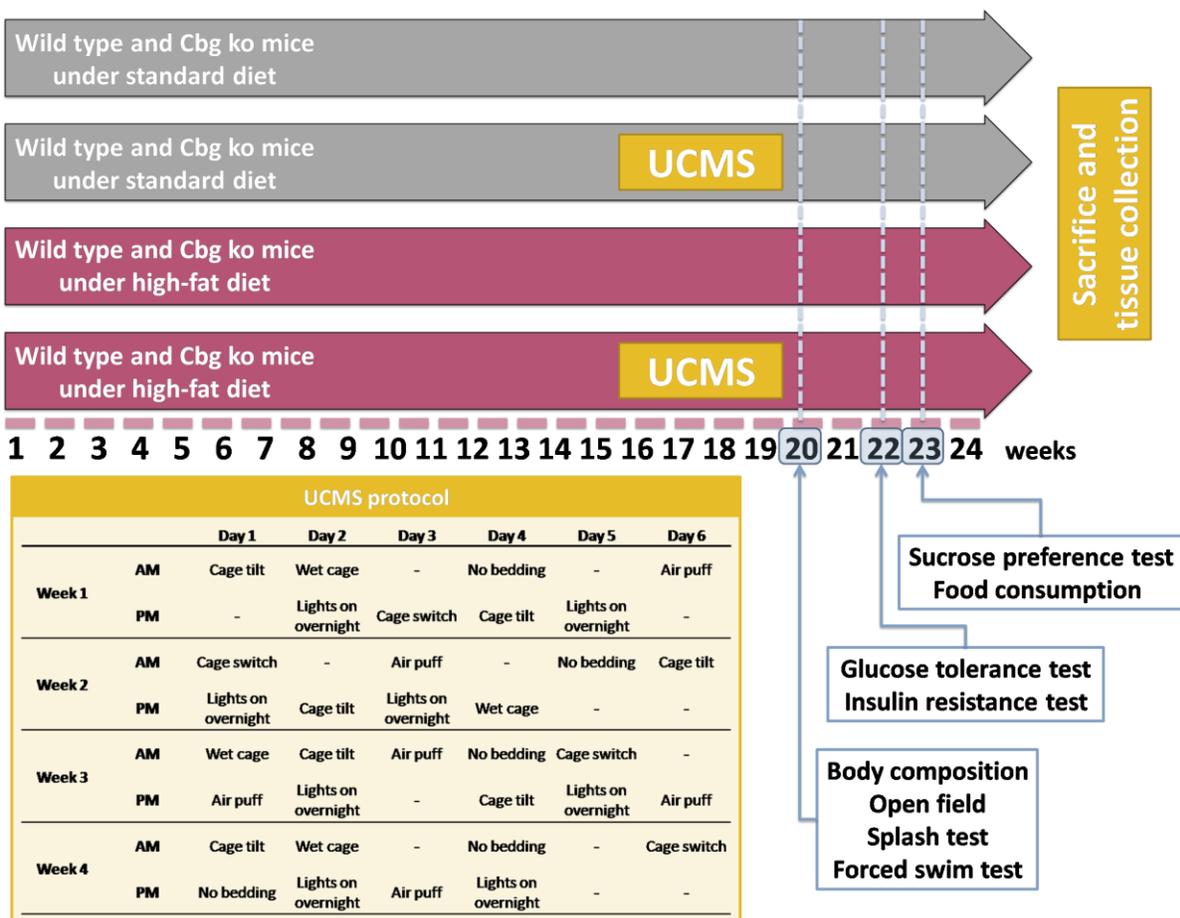


Figure 19 – The unpredictable chronic mild stress protocol.

Blood sampling

Blood samples were obtained from unstressed animals both in the morning (0900 h) and in the evening (1900 h), and after sacrifice (afternoon). The time between taking each cage from the animal facility to the experimental room and collecting the blood from all animals in that cage did not exceed 3 minutes to avoid stress values. Blood was collected from the facial vein or by cardiac puncture (sacrifice) into tubes containing 10% EDTA. Samples were centrifuged at 10000 rpm for 10 min, and the supernatant (plasma) was stored at -80°C.

Corticosterone, free corticosterone and CBG dosing

Total corticosterone and CBG binding capacity were measured as previously described (page 81). Free corticosterone measurements, 5 μ l of diluted [³H]-labeled corticosterone were added to 50 μ l of plasma and incubated at 37°C for 30 min. Then, 50 μ l of the solution were loaded onto a Centrifree filter device (YM membranes 30K, Millipore, France) and centrifuged at room temperature for 30 min at 3400 rpm. 5 μ l of the filtrate was collected and counted in scintillation vials into 2.5 ml OptPhase “HiSafe” 2, as well as the 5 μ l of the initial solution. Free corticosterone percentage was calculated as the ratio of filtrate cpm (free corticosterone)/ total cpm (free + bound corticosterone). Free corticosterone concentration was obtained by multiplying the percentage of free corticosterone by the total corticosterone concentration in the same sample, which was previously measured by RIA.

Blood glucose measurement

On week 14, after a 24h food deprivation, and on week 22, during the glucose tolerance and the insulin resistance tests, blood glucose concentration was measured by tail nick using Accucheck-Performa ® device (Roche Diagnostics, France). After a small incision in the tip of the tail, tails were gently rubbed to stimulate blood flow. The first blood drop was always discarded, and a second one was placed on the device’s strip to determine the blood glucose concentration.

Unpredictable chronic mild stress (UCMS)

After 16 weeks of diet and during 4 weeks, an UCMS procedure was imposed to half of the animals. All stressed groups were placed into individual cages and exposed to different and random mild stressors, usually twice a day, as presented in Figure 19.

Nuclear magnetic resonance

Body composition was evaluated with Minispec body composition analyzer (Bruker – Massachusetts, USA), which provides analysis of fat tissue, lean tissue and free body fluid by the same physical selection rules that give rise to contrast in MRI. It acquires and analyzes signals from all protons in the entire sample volume and provides 3 components of interest: Fat, Free Body Fluid and Lean Tissue values. For this procedure, animals were first weighted and then physically restrained inside plastic tubes, then placed inside the nuclear magnetic resonance machine. Measurement took less than 2 min per mouse.

Open field test

The open field test was performed as previously described (page 98).

Forced swim test

The forced swim test was performed as previously described (page 80).

Splash test

Animals were splashed on the back with a sucrose 10% solution and then placed inside a clean individual cage. The latency to start grooming and the time spent grooming were registered during 5 min.

Sucrose preference test

Preference for a sucrose solution was determined at week 23, when mice from stressed groups were individually housed and those from non-stressed groups were then divided per genotype (2 mice/cage) for the duration of the test. On the first three days of test, two water bottles were placed in each cage to familiarize mice with the bottles. Then, one of them had the water changed for a 1% D-sucrose solution (Sigma, France). Liquid intake was evaluated during three days, by weighting the bottles every 24 hours. The percentage of sucrose solution drunk during these 2 days was measured as an index of anhedonic behavior.

Glucose tolerance and the insulin resistance tests

For the glucose tolerance and the insulin resistance tests, all animals were individually housed and food deprived. After 5 hours of fasting, mice had their baseline blood glucose level measured, as described above. Then, animals were either given 2 g/kg of a D-glucose solution (0,2 g/ml) by oral gavage – for the glucose tolerance test, or 0,75 IU/kg of fast-acting insulin (Actrapid, Novo Nordisk - Denmark) intraperitoneally – for the insulin resistance test. The dose of insulin was adjusted according to the animal's fat mass (measured on the week before through nuclear magnetic resonance), with an animal that has the mean fat mass of the control groups receiving 0,075 UI/kg of body weight and the others receiving proportional doses of insulin according to their lean mass. As lean mass is the principal site of insulin action, this adjustment would avoid that mice with increased fat mass receive larger doses of insulin than lean mice, even though the mass of insulin-sensitive tissue (lean mass) might not differ significantly or even be lower in fat mice (Ayala et al., 2010). Blood glucose levels were measured at 15, 30, 45, 60, 90 and 120 min. Both tests were performed with the same mice with a 5 days interval.

Sacrifice and tissue collection

Animals were food restricted for about 3-4 hours before sacrifice. Mice were anesthetized with isoflurane (Aerrane, Baxter SA, Maurepas, France) and had about 1mL of blood taken by cardiac puncture. Then, animals were sacrificed by decapitation. Adipose tissue (representative samples of brown (interscapular), visceral (perirenal) and subcutaneous (flank)), muscle, liver, spleen and adrenal glands were collected and immediately frozen into dry ice and kept at -80°C. Brain was immediately dissected and samples of hippocampus, hypothalamus, pituitary and cortex were also stored at -80°C.

Statistical analyses

Analyses of experiments involving a single time-point (percentage of body fat/lean mass, percentage of brown/visceral/subcutaneous adipose tissue, food intake

after fasting, forced swim test, splash test and open field test) were performed with a one-way ANOVA followed by Dunnett's multiple comparison test, comparing HF, stress, and HF + stress groups from each genotype to their respective STD control group. Analyses involving repeated measures (weight, percentage of weight gain and food intake throughout the experiment, oral glucose tolerance and insulin resistance tests) were performed with a repeated measures ANOVA followed by Duncan post-test. Finally, the sucrose preference test was analyzed through a one-sample t test, comparing means to a hypothetical value of 50% (chance level). All analyzes were performed with the software Prism 5 for Windows (GraphPad Software, Inc.), except for the repeated measures ANOVA which was performed using Statistica 6.1 (Statsoft, Inc.).

Results and discussion

Analyzing the weight gain from weeks 0 to 15 (Figure 20A,B), when no stress was associated to the diet, we observe a main effect of the diet ($F(1,315) = 5.30$, $p < 0.05$ for wild-type and $F(1,345) = 8.94$, $p < 0.01$ for Cbg ko mice, STD < HF), of time ($F(15,315) = 78.00$, $p < 0.001$ for wild-type and $F(15,345) = 85.06$, $p < 0.001$ for Cbg ko mice) and interactions between diet and time ($F(15,315) = 3.70$, $p < 0.001$ for wild-type and $F(15,345) = 7.04$, $p < 0.001$). The posthoc tests revealed only significant effect of the HF diet on the weight of wild-type mice on weeks 11, 12 and 15. As for the weight after the association of UCMS, we observe main effects of diet ($F(1,152) = 20.90$, $p < 0.001$ for wild-type and $F(1,168) = 52.27$, $p < 0.001$ for Cbg ko mice, STD < HF), UCMS ($F(1,152) = 8.74$, $p < 0.01$ for wild-type mice only, no stress > UCMS), and time ($F(8,168) = 3.77$, $p < 0.001$ Cbg ko mice only). Interactions were found between diet and UCMS ($F(1,152) = 4.73$, $p < 0.05$ for wild-type mice only), diet and time ($F(8,152) = 3.80$, $p < 0.001$ for wild-type and $F(8,168) = 10.47$, $p < 0.001$ for Cbg ko mice), time and UCMS ($F(8,168) = 10.20$, $p < 0.001$ for Cbg ko mice only) and between time, UCMS and diet ($F(8,168) = 3.88$, $p < 0.001$ for Cbg ko mice). The posthoc tests did not reveal trends or significant differences between the STD and HF groups submitted or not to UCMS throughout the weeks.

When analyzing the percentage of weight gain from weeks 1 to 16 (Figure 20C,D), also observe a main effect of time ($F(15,315) = 99.51$, $p < 0.001$ for wild-type and $F(15,345) = 70.15$, $p < 0.001$ for Cbg ko mice) and interactions between diet and time ($F(15,315) = 4.09$, $p < 0.001$ for wild-type and $F(15,345) = 5.83$, $p < 0.001$). A marginally significant effect of diet was observed in Cbg ko mice only ($F(1,345) = 3.97$, $p = 0.06$, STD < HF). The posthoc tests did not reveal any significant difference between STD and HF groups throughout the weeks. After the UCMS was combined to the diet, the ANOVA detected main effects of diet ($F(1,114) = 10.94$, $p < 0.01$ for wild-type and $F(1,126) = 19.63$, $p < 0.001$ for Cbg ko mice, STD < HF) and time ($F(6,126) = 4.88$, $p < 0.001$ for Cbg ko mice only) only. Interactions were found between diet and time ($F(6,114) = 4.21$, $p < 0.001$ for wild-type and $F(6,126) = 10.83$, $p < 0.001$ for Cbg ko mice), time and UCMS ($F(6,126) = 7.14$, $p < 0.001$ for Cbg ko mice only) and time, diet and UCMS ($F(6,126) = 4.07$, $p < 0.001$ for Cbg ko mice only). Once again, the posthoc tests did not reveal trends or significant differences between the STD and HF groups submitted or not to UCMS throughout the weeks.

A more detailed analysis of the weight of each group split from the first week of test (Figure 20E,F) has revealed that the wild-type group under HF diet that was later submitted to the UCMS was heavier than the other wild-type groups from the first week

of experiment. This has clearly biased the results of this experiment in terms of weight gain under HF diet, and on the influence of the UCMS on this phenotype. As for the Cbg ko mice, we do in fact observe a non-significant trend towards the same profile observed in the 2009 experiment: while no effect of UCMS is observed in mice under STD diet, those under HF diet lose weight when UCMS is combined.

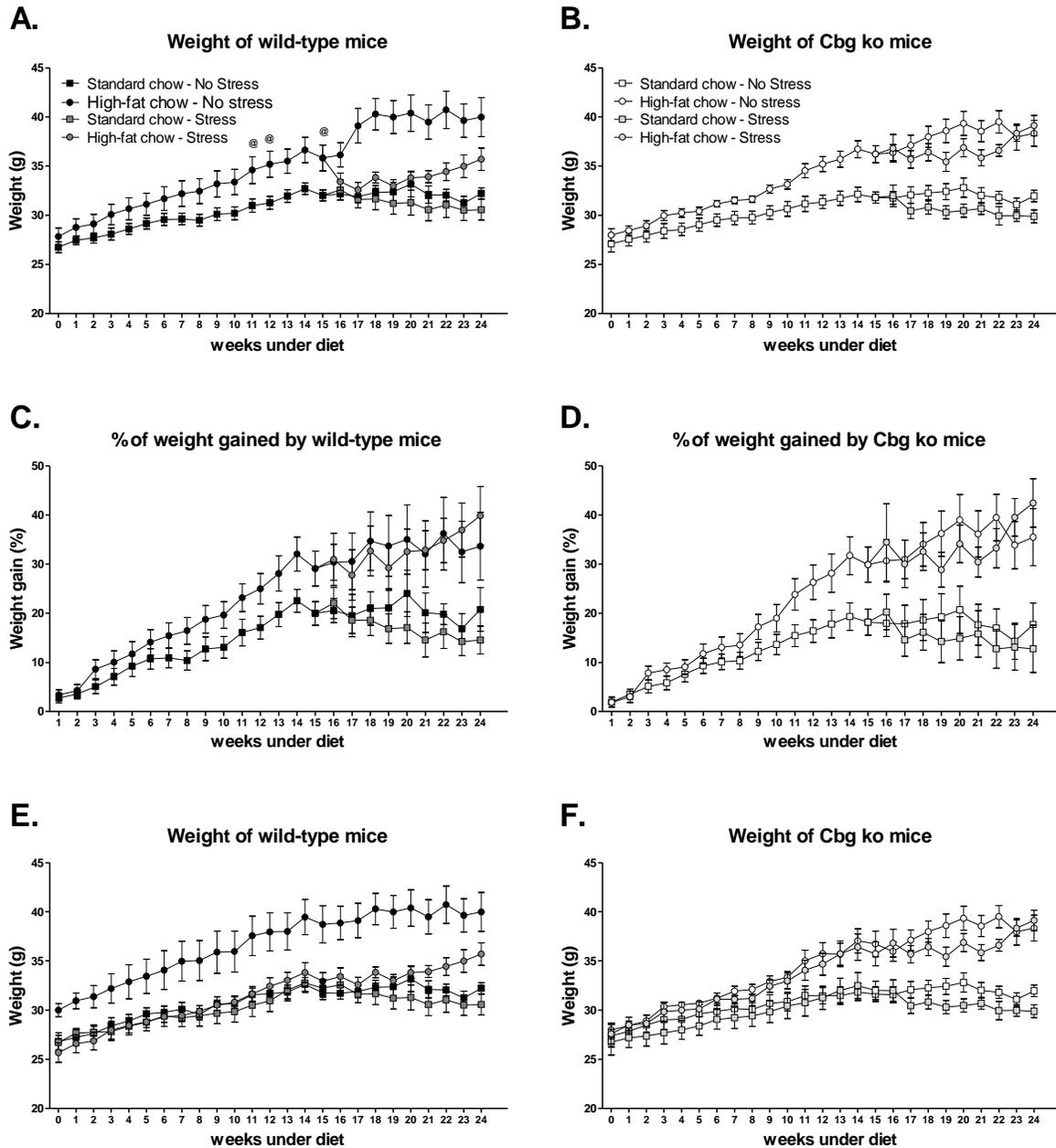


Figure 20 – Weight (A,B/E,F) and percentage of weight gained (C,D) by wild-type (A,C,E black) and Cbg ko (B,D,F white) mice. Circles represent animals under high-fat diet (HF), while squares represent animals under standard diet (STD). Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice, respectively. (@) represents significant difference ($p < 0.05$) between HF and STD groups. Analyses were performed separately for each genotype through repeated measures ANOVA followed by Duncan post-test.

When comparing the data from wild-type mice in this experiment and those from the experiment performed in 2009, we observe that wild-type HF animals from this experiment gained less weight and started gaining weight much later than those from the original western lifestyle experiment (main effects of diet ($F(1,540) = 16.00$, $p < 0.001$ $STD < HF$), time ($F(15,540) = 67.00$, $p < 0.001$), and interaction between diet and time ($F(15,540) = 13.00$, $p < 0.001$) for the first experiment; and effects of diet ($F(1,315) = 5.30$, $p < 0.05$, $STD < HF$), time ($F(15,315) = 78.00$, $p < 0.001$) and interaction between diet and time ($F(15,315) = 3.70$, $p < 0.001$) for this experiment). Regarding the percentage of weight gained from the beginning of the diet, we observe a similar profile to that from the weight gain, except that no main effect of diet was detected in the present experiment (main effects of diet ($F(1,540) = 36.00$, $p < 0.001$), time ($F(15,540) = 51.00$, $p < 0.001$), and interaction between diet and time ($F(15,540) = 9.00$, $p < 0.001$) for the first experiment; and effects of time ($F(15,315) = 79.00$, $p < 0.001$) and interaction between diet and time ($F(15,315) = 3.30$, $p < 0.001$) for this experiment). The effects detected on the posthoc test are illustrated in Figure 21. It is possible to observe that the difference in weight between STD and HF groups was much more pronounced and appears way earlier in time in the 2009 experiment, and maybe the fact that the mice from the 2014 experiment had their body weight less impacted by HF diet might have influenced the effect of UCMS on weight.

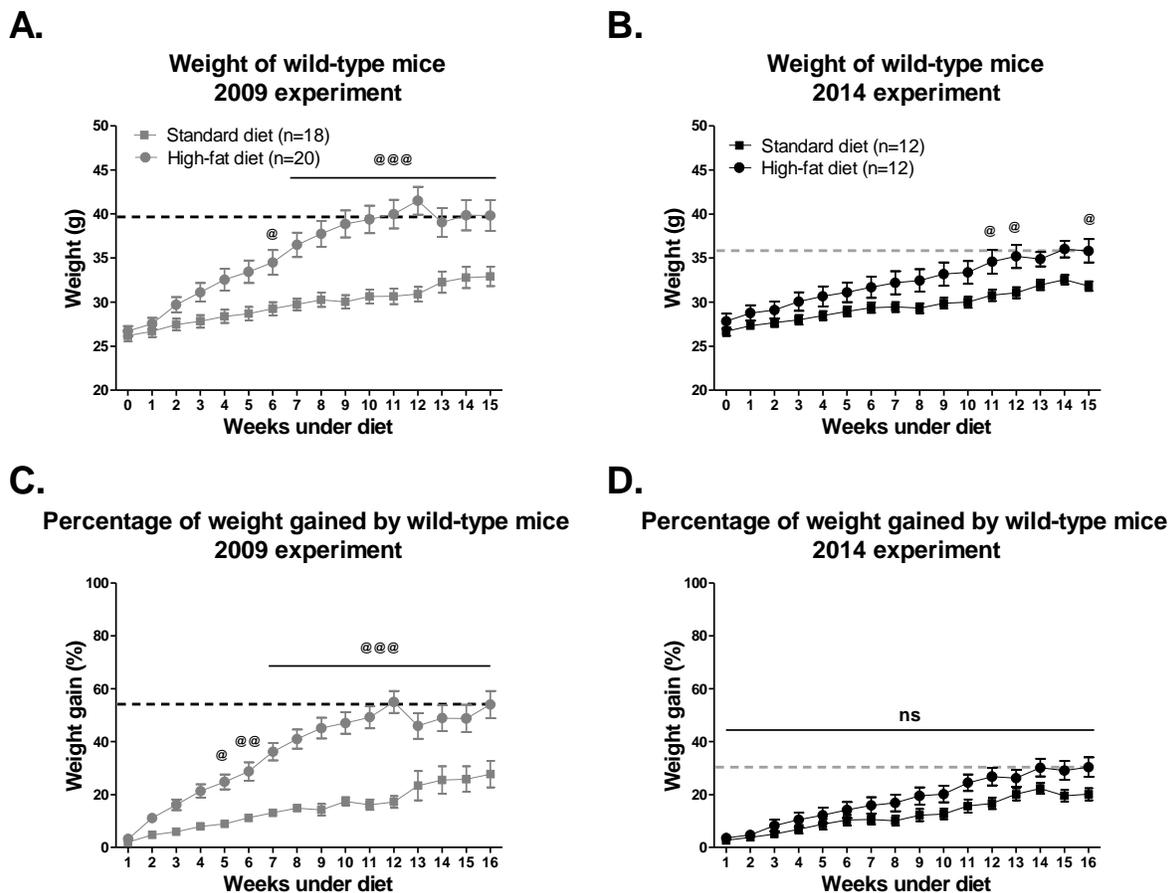


Figure 21- Comparison of absolute weight (A-B) and percentage of weight gained (C-D) between wild-type animals from the western lifestyle experiments conducted in 2009 (original) and 2014 (replicate). Circles represent animals under high-fat diet (HF), while squares represent animals under standard (STD) diet. (@), (@@) and (@@@) represent significant differences ($p < 0.05$,

p<0.01 and p<0.001, respectively) between HF and STD groups. Analyses were performed with repeated measures ANOVA followed by Duncan posthoc test.

We have also analyzed body composition through nuclear magnetic resonance on the week following the end of the UCMS period, and found no difference between genotypes regarding the percentage of fat or lean mass (Figure 21). For both genotypes, HF fed groups - as expected - had greater percentage of fat and, inversely, lower percentage of lean mass ($F(1,39) = 84.70$, $p < 0.001$ for the percentage of fat mass; $F(1,39) = 54.31$, $p < 0.001$ for percentage of lean mass).

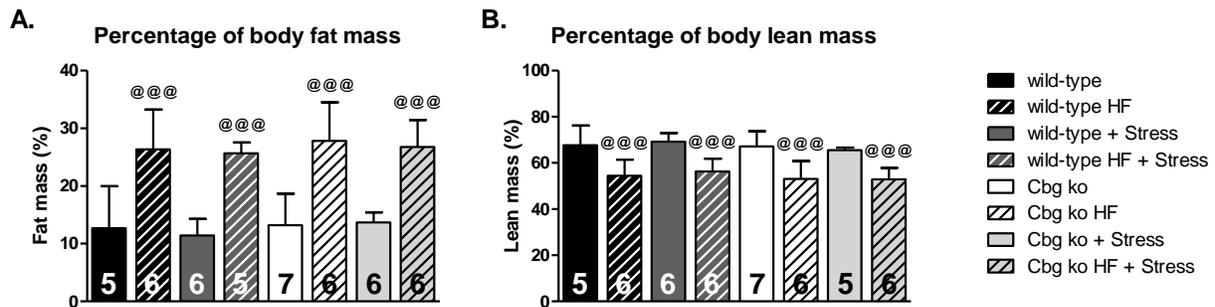


Figure 21 – Percentage of fat (A) and lean (B) mass of wild-type (black) and Cbg ko (white) mice after 21 weeks of diet. Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice, respectively. (@@@) represents significant ($p < 0.001$) overall effect of diet. Analyses were performed with two-way ANOVA.

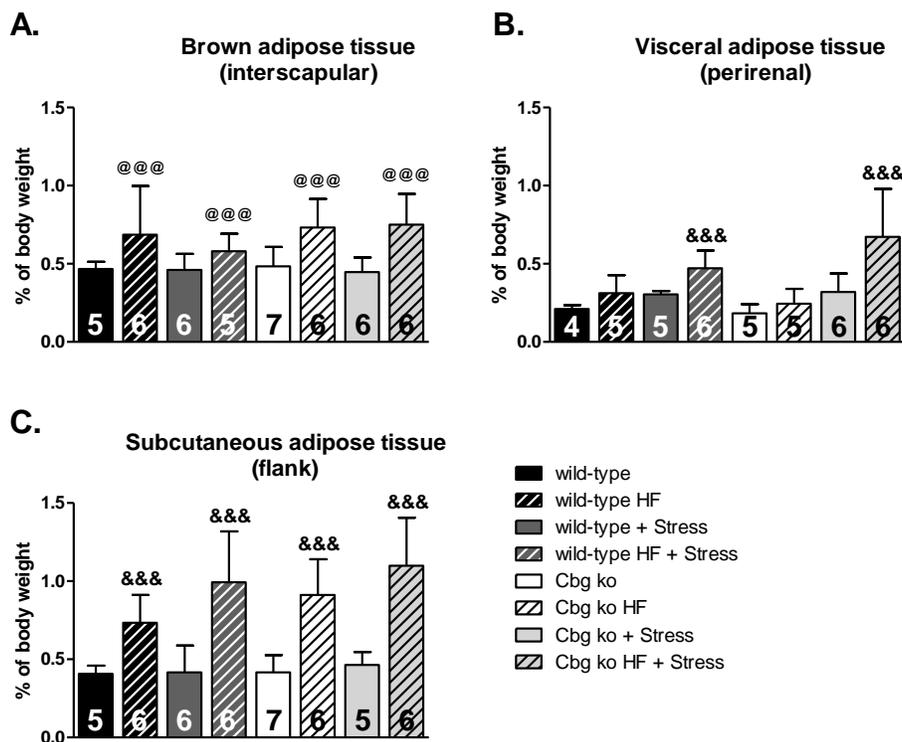


Figure 22 – Percentage of brown (interscapular, A), visceral (perirenal, B) and subcutaneous (flank, C) adipose tissue in wild-type (black) and Cbg ko (white) mice after 21 weeks of diet. Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice, respectively. (@@@) represents a main effect of diet ($p < 0.001$), while (&&&) represents the posthoc analysis of an interaction between diet and UCMS. Analyses were performed with two-way ANOVA followed by Duncan’s posthoc test.

Regarding the distribution of the fat mass, an overall effect of diet was detected for brown (interscapular) adipose tissue, with HF groups presenting higher accumulation of this type of fat than STD fed animals ($F(1,39) = 20.02$, $p < 0.001$) (Figure 22A). As for visceral (perirenal) adipose tissue (Figure 22B), main effects of diet ($F(1,34) = 14.26$, $p < 0.001$, STD < HF) and UCMS ($F(1,34) = 20.55$, $p < 0.001$, no stress < UCMS) were detected. Furthermore, a marginally significant interaction between diet and UCMS was revealed ($F(1,34) = 3.93$, $p = 0.06$), with animals submitted to both HF and UCMS combined displaying higher accumulation of visceral fat than all other groups ($p < 0.001$ when compared to all). For the subcutaneous (flank) adipose tissue (Figure 22C) we also observe effects of diet ($F(1,39) = 67.60$, $p < 0.001$, STD < HF) and UCMS ($F(1,39) = 4.12$, $p < 0.05$, no stress < UCMS). Although no significant interaction was detected between diet and UCMS ($F(1,39) = 2.50$, $p = 0.12$), the profile observed in the graph has led us to perform a posthoc test for this interaction. The result has revealed that only HF and HFS ($p < 0.001$ for all HF vs. STD comparisons) groups displayed a significant increase in subcutaneous fat accumulation when compared to the other groups.

Since mice wild-type and Cbg ko mice were housed together in the same cages, food intake was only measured regarding the diet and the stress treatment (Figure 23A). From the beginning of the experiment until week 16, main effects of diet ($F(1,150) = 33.88$, $p < 0.001$, with STD > HF) and time ($F(15,150) = 8.60$, $p < 0.001$) were detected. After the UCMS was combined to the diet, besides the diet ($F(1,48) = 60.57$, $p < 0.001$, STD > HF) and time ($F(6,48) = 7.95$, $p < 0.001$) effects, we also observe a marginally significant effect of UCMS ($F(1,48) = 4.52$, $p = 0.07$, no stress < UCMS). We have also evaluated the food intake during two hours after 15 hours of fasting (Figure 23B). This experiment was performed 23 weeks after the beginning of the HF diet, and two weeks after the end of the UCMS. Both effects of diet ($F(1,36) = 30.00$, $p < 0.001$, STD > HF) and UCMS ($F(1,36) = 17.33$, $p < 0.001$, no stress < UCMS) were detected. Again, despite no interaction between these factors was detected, the profile observed in the graph has led us to perform a posthoc test for this interaction. The posthoc results revealed that HF alone decreases ($p < 0.001$) while UCMS alone increases ($p < 0.05$ when compared to unstressed controls and $p < 0.001$ for the other comparisons) food intake after fasting. The combination of HF and UCMS seems to normalize food intake. No difference regarding food intake after fasting was observed between wild-type and Cbg ko mice.

Metabolic parameters were evaluated after 22 weeks of HF diet (one week after the end of the UCMS period). Animals submitted to both HF and UCMS had higher levels of blood sugar after the oral administrations of glucose (2g/kg) when compared to their respective controls (from 15 min until 90 min for wild-type and from 15 min until 120 min for Cbg ko mice), indicating lower glucose tolerance (interaction between time and groups, $F(42,40) = 2.08$, $p < 0.001$) (Figure 24A,B). Cbg ko mice submitted to HF diet only also had higher blood glucose levels than their control group (from 15 min until 120 min, except for T60), and the same trend is observed in wild-type groups (although statistical significance is only reached at 15 min). No differences were observed between the stressed groups and their controls. When analyzing the area under the curves (two-way ANOVA performed separately for each genotype) has revealed main effects of both diet ($F(1,20) = 15.72$, $p < 0.01$), with a posthoc analysis showing that, under unstressed conditions, HF fed mice present higher AUC – hence higher blood glucose levels – than STD fed mice. In Cbg ko mice both effects of diet ($F(1,22) = 15.10$, $p < 0.05$) and stress ($F(1,22) = 4.73$, $p < 0.05$) were observed. The posthoc revealed that, both in stressed and unstressed conditions, animals fed with HF diet display higher

glucose levels than mice fed with STD diet. Regarding the insulin resistance test (Figure 24C,D), no statistically significant difference was observed between groups, although it is possible to identify a trend of HF groups towards higher levels of blood sugar after insulin administration. When analyzing the area under the curves (two-way ANOVA performed separately for each genotype), no significant differences were detected between the wild-type groups, and only a main effect of diet ($F(1,21) = 6.32, p < 0.05$) was found for Cbg ko mice, with HF fed animals presenting higher glucose levels than STD fed mice, regardless of the UCMS. In both the glucose tolerance and the insulin resistance tests, no difference was seen between the basal blood sugar levels of all groups. Altogether, these results indicate that HF diet, especially when combined with UCMS, induces changes in glucose metabolism, reflected as lower glucose tolerance. This lower tolerance to glucose is not likely to be due to an insulin resistance, since all groups had similar results in the insulin resistance test, and is probably a result of lower insulin secretion.

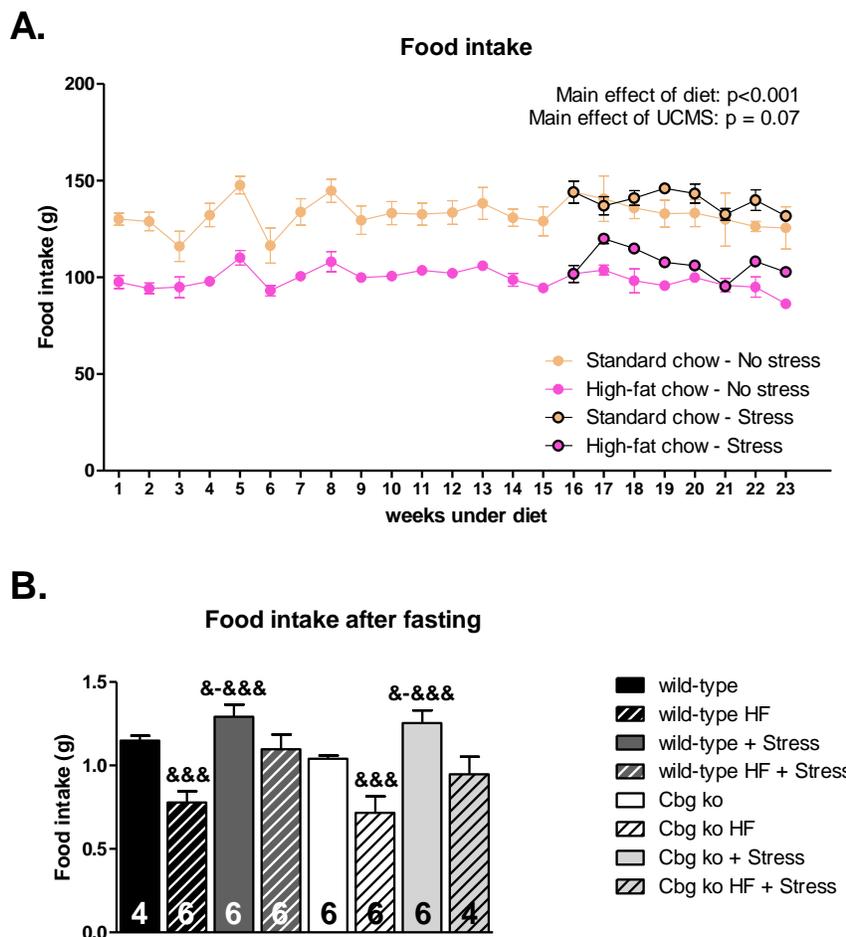


Figure 23 – Food intake throughout the 24 weeks of experiment (A). Yellow symbols and lines represent the standard diet (STD) while pink symbols and lines represent the high-fat diet (HF). The food intake of the groups submitted to chronic stress is outlined in black. Analyses were performed with repeated measures ANOVA followed by Duncan’s posthoc test ($n=3$ cages/diet/stress). Food intake by wild-type (black) and Cbg ko (white) mice during 2 hours after 15 hours fasting (B). Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice, respectively. (&) and (&&&) represent the posthoc analysis of an interaction between diet and UCMS ($p < 0.05$ and $p < 0.001$, respectively). Analyses were performed with two-way ANOVA followed by Duncan’s posthoc test.

Tests evaluating depressive-like behaviors (behavioral despair and anhedonia) were also performed (Figure 25), namely the forced swim test, the splash test and the sucrose preference test. For the forced swim test (Figure 25A), only a main genotype effect ($F(1,39) = 4.98, p < 0.05$) was detected, with Cbg ko mice displaying higher immobility scores regardless of the diet or UCMS. Although this effect corroborates the differences in despair-like behavior our previous work has detected between wild-type and Cbg ko mice ((Richard et al., 2010, Minni et al., 2014), and page 78 of this thesis), a difference in the opposite sense was expected for animals submitted to both HF diet and UCMS. Furthermore, when looking at control groups only, no significant difference is detected between wild-type and Cbg ko mice ($t(0.1) = 0.91$). Although a difference between these groups was expected, it was not the first time we came across similar performances in the last years. We are currently working to identify the variables causing this loss of this previously observed phenotype (higher immobility score of Cbg ko mice).

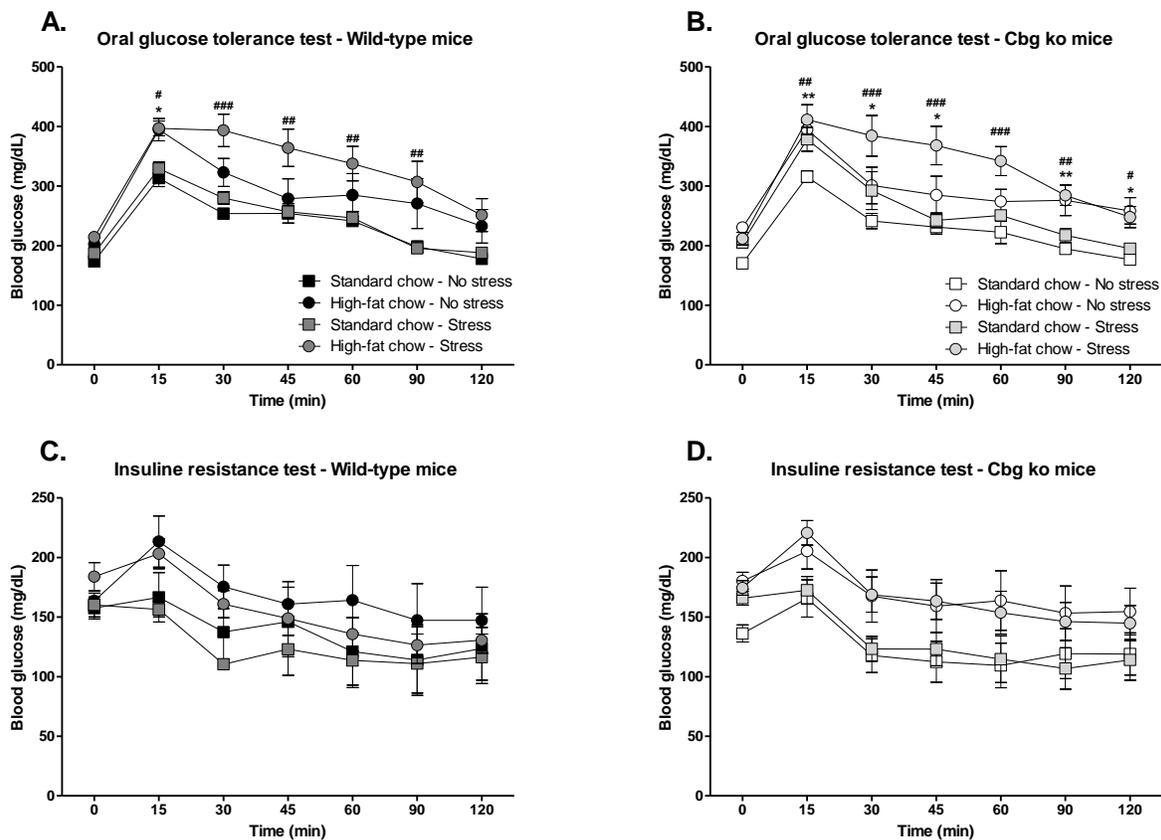


Figure 24 – Oral glucose tolerance (A,B) and insulin resistance (C,D) tests performed in the wild-type (A,C, black) and Cbg ko (B,D, white) groups after 22 weeks of high-fat diet and one week after the end of the chronic stress. Circles represent animals under high-fat diet (HF), while squares represent animals under standard diet (STD). Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice respectively. (*) and (**) represent significant ($p < 0.05$ and $p < 0.01$, respectively) difference between unstressed STD and HF groups, while (#), (##) and (###) represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) difference between STD and the stressed HF groups. Analyses were performed with repeated measures ANOVA followed by Bonferroni post-test ($n = 5-7$ /genotype/diet/stress).

In the splash test (Figure 25B), main effects of diet ($F(1,36) = 8.88, p < 0.01$) and UCMS ($F(1,36) = 25.00, p < 0.001$) were detected. Moreover, a marginally significant interaction between these two factors was observed ($F(1,36) = 3.85, p = 0.06$). The posthoc analysis has revealed that animals under HF diet only spent significantly less time grooming than all the other groups ($p < 0.01$ when compared to controls and $p < 0.001$ when compared to the other groups). Decreased time grooming in this test is related with depressive-like phenotypes, and thus only unstressed HF fed animals would be displaying this profile. These results might be interpreted in the light that HF fed mice display increased adiposity, which promotes inflammation. This chronic low-grade inflammation has been consistently linked to a series of psychopathologies, notably increasing depression-related symptoms (Capuron and Castanon, 2016).

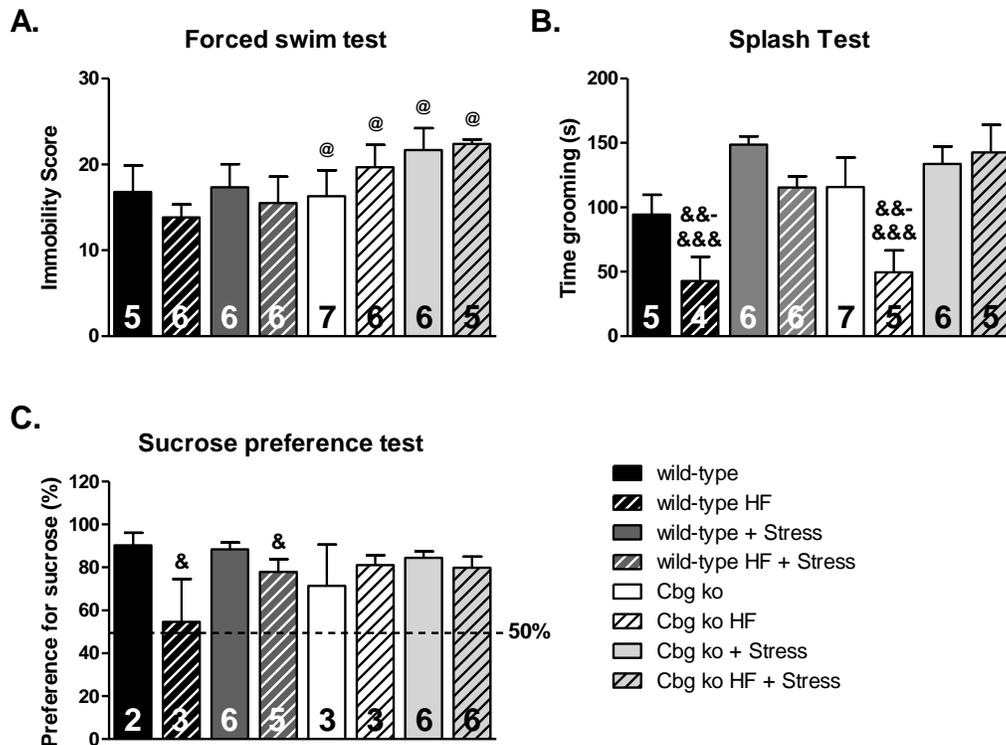


Figure 25 – Performance of wild-type (black) and Cbg ko (white) mice in three depression-related behavioral tests: the forced swim test (A) the splash test (B) and the sucrose preference test (C). Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice respectively. (@) represents a main effect of diet ($p < 0.05$), while (&), (&&) and (&&&) represent the posthoc analysis of interactions between diet and UCMS (B) or genotype and diet (C) ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). Analyses were performed with two-way ANOVA followed by Duncan’s posthoc test.

For the sucrose preference test (Figure 25C), only an interaction between genotype and diet was detected ($F(1,26) = 4.54, p < 0.05$). The posthoc analysis revealed a significant effect of the HF diet lowering the sucrose consumption of wild-type mice only ($p < 0.05$), regardless of the UCMS status. Similarly to what was previously observed in the splash test, this increase in depressive-like behavior presented by the wild-type groups under HF diet might be explained by increased adiposity, leading to a higher inflammatory status which would favor the manifestation of depressive-like symptoms. Nevertheless, the reason why this effect was only observed in wild-type mice is not clear. An unexpected result was the absence of effect of UCMS. A possible bias

on the results of the sucrose preference test is the fact that we have analyzed the preference of the stressed animals individually, since they were already individually housed for the chronic stress procedure, while non-stressed groups were housed collectively, and for practical reasons were only split per genotype (2 animals per cage) during this test. This means that the sample size of the non-stressed groups is half of that from the stressed groups (since the first considers the preference as a result of the mean consumption of the two animals housed together), and this smaller effective might have lowered the statistical power of the analysis.

Finally, as already expected based on previous results from our group on anxiety-related behaviors, no difference between groups was found in the open field test (Figure 26).

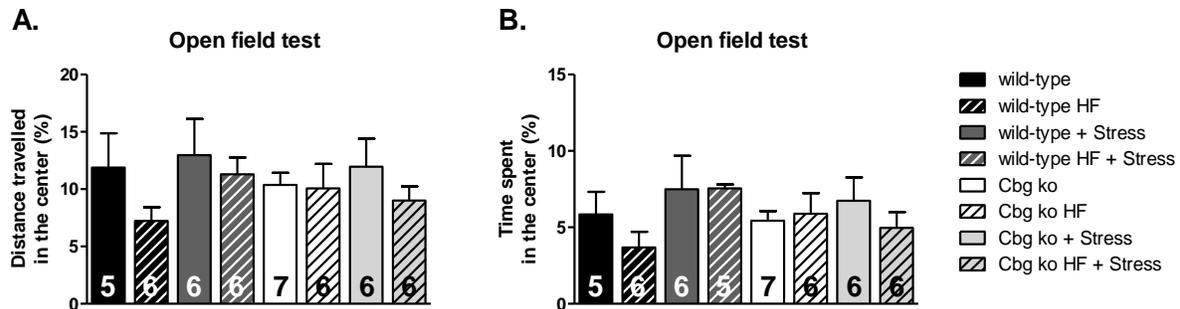


Figure 26 – Percentage of distance travelled (A) and of time spent (B) in the center of the open field test by wild-type (black) and Cbg ko (white) mice. Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice respectively. No effect of genotype, diet or stress was detected for the variables analyzed. Analyses were performed with a two-way ANOVA

Overall, although we were able to see some differences between groups linked to the diet, stress or the combination of both (as for the percentage of fat and lean mass, the percentage of subcutaneous and visceral fat, and the glucose tolerance test, for example), but no clear and consistent difference between genotypes was observed in any of the variables measured. Based on the previous experiments performed by our team, we expected animals from both genotypes to differ at least on the weight gain (with wild-type mice gaining and Cbg ko mice losing weight after submitted to HF and UCMS combined) and on the despair-like behavior (with Cbg ko mice displaying a poor performance in the forced swim test under basal conditions, and improving after submitted to the combination of HF and UCMS, and wild-type animals showing the opposite profile). For this reason, tissues and blood samples collected in this experiment weren't further analyzed.

Puzzled by this difference between the original western lifestyle experiment and this replicate, we have exhaustively searched for sources of variation among both experiments, and have found a few possible explanations for our results (I) the different animal facility in which the latter experiment was carried out, that might implicate in differential temperature and humidity regulation, isolation from sound and other stressors, animal facility workers, bedding, and even a different microbiota (II) a change in the HF chow composition or stability, although the manufacturer has assured us that the formula has not changed in the last years (III) genetic drift or substrain divergence in our mice that, in spite of not affecting the expression of CBG or corticosterone levels, might be influencing another metabolic phenotype that could affect weight gain.

We have designed two complementary experiments to shed a light on this issue, the first exploring the effect of different types of high fat diet and of different types of bedding on the metabolic consequences of the HF diet consumption, and the second one replicating once again the western lifestyle experiment, but this time using very high-fat (VHF) chow instead of the regular HF chow.

The influence of different types of high-fat chow and bedding on metabolic parameters of C57BL/6 mice

Recent studies have shown that different bedding composition, more specifically corncob grit, can interfere on behavioral aspects and even on feed conversion efficiency of animals submitted to HF diets (Melhorn et al., 2010, Villalon Landeros et al., 2012). This experiment was designed to better understand the influence of different diets and types of bedding on the weight gain and other metabolic parameters in mice. For that, we have submitted C57BL/6 mice (background strain of your knock-out mice) to either STD chow, HF chow or VHF chow, and their food intake, weight gain, body composition, corticosterone levels and other metabolic parameters, such as glucose, insulin and leptin levels, were evaluated. The effect of two different types of bedding: spruce sawdust and corncob grit, was also assessed on animals submitted to HF diet only.

Methods

Eight-month-old C57BL/6J mice (background strain of your knock-out mice) were housed into collective cages (4 mice/cage) and submitted to either STD chow (A04, from SAFE - Scientific Animal Food & Engineering, 2 cages), HF chow (D12451, from Research Diets, 4 cages) or VHF chow (D12492, from Research Diets, 2 cages) for 21 weeks. The composition and the caloric content of each diet are illustrated in Figure 27. All cages had spruce sawdust bedding, except for two cages of the HF group which had corncob grit bedding.

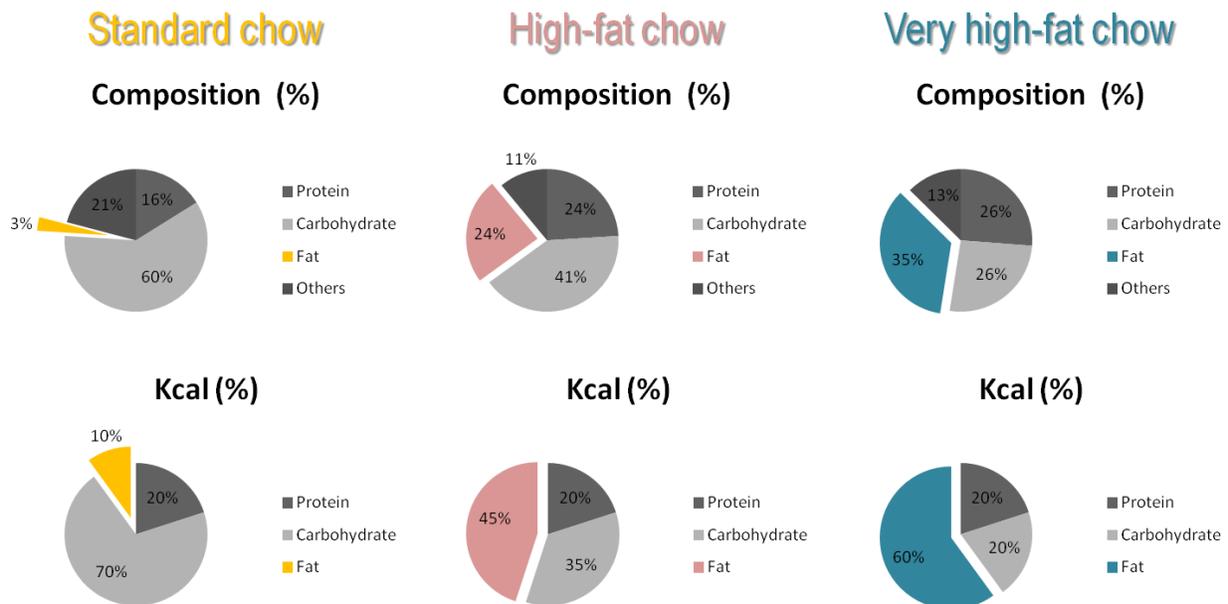


Figure 27 – Composition and caloric content of standard chow (A04, from SAFE - Scientific Animal Food & Engineering), high-fat chow (D12451, from Research Diets) and very high-fat chow (D12492, from Research Diets).

Blood sampling

Blood samples were taken every 4 weeks from unstressed animals in the morning (0900 h) and in the evening (1900 h). The time between taking each cage from

the animal facility to the experimental room and collecting the blood from all animals in that cage did not exceed 3 minutes. Blood was collected from the facial vein or by cardiac puncture (during sacrifice) into tubes containing 10% EDTA. Samples were centrifuged at 10000 rpm for 10 min, and the supernatant (plasma) was stored at -80°C.

Corticosterone dosing

Total corticosterone was measured at every 4 weeks throughout the 21 weeks of experiment, as previously described on page 160.

Blood glucose measurement

At the beginning of the diet and every 4 weeks, after an overnight food deprivation, as well as during the glucose tolerance and the insulin resistance tests, blood glucose concentration was measured as previously described on page 126.

Nuclear magnetic resonance

Body composition was evaluated at the beginning of the diet and every 4 weeks as previously described on page 160.

Glucose tolerance and the insulin resistance tests

The glucose tolerance and the insulin resistance tests were performed on weeks 20 and 21, respectively, with a 3-day interval. Tests were performed as previously described on page 161.

Insulin and leptin levels

The levels of leptin and insulin throughout the different groups were assessed through an enzyme-linked immunosorbent assay (ELISA), following the manufacturers' protocol (Millipore rat/mouse ELISA insulin kit (EZRMI-13K) and Millipore mouse ELISA leptin kit (EZML-82K)). Briefly, plasmatic insulin/leptin will bind an antiserum and the resulting complexes will be immobilized in the wells of a microtiter plate. After washing, a purified biotinylated detection antibody is added and allowed to bind to the immobilized insulin/leptin. After a second wash, horseradish peroxidase is added and binds the biotinylated detection antibody. Then after the wash away of free enzyme conjugates, the quantification of immobilized antibody-enzyme conjugates is made by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically. Since the increase in absorbance is directly proportional to the amount of captured insulin/leptin in the sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of mouse insulin/leptin.

Sacrifice and tissue collection

Animals were sacrificed after 21 weeks of diet. Food was restricted for about 3-4 hours before sacrifice. Mice were anesthetized with isoflurane (Aerrane, Baxter SA,

Maurepas, France) and had about 1mL of blood taken by cardiac puncture. Then, animals were sacrificed by decapitation. Adipose tissue (representative samples of brown (interscapular), visceral (perirenal) and subcutaneous (flank)), muscle, liver, adrenal glands and (whole) brain were collected and immediately frozen into dry ice and kept at -80°C.

Statistical analyses

Analyses involving repeated measures (weight, percentage of weight gain, food intake, leptin levels, percentage of body fat, blood glucose levels, insulin levels, and total corticosterone levels (morning and evening) throughout the experiment, as well as the oral glucose tolerance and the insulin resistance tests) were performed with a repeated measures ANOVA followed by Bonferroni post-test. Analyses of the percentage of brown, visceral and subcutaneous adipose tissue were performed with a one-way ANOVA followed by Dunnett's multiple comparison tests, comparing all groups with the STD chow fed one. Finally, Pearson's correlation analyses were performed between leptin levels and percentage of body fat. All analyzes were performed with the software Prism 5 for Windows (GraphPad Software, Inc.).

Results and discussion

When analyzing the body weight on the animals under different conditions, the two-way ANOVA has detected main effects of diet ($F(3,440) = 7.20$, $p < 0.01$) and time ($F(20,440) = 190.00$, $p < 0.001$), besides an interaction between these two factors ($F(60,440) = 5.50$, $p < 0.001$). The posthoc test results are illustrated on Figure 28A, but briefly no body weight difference was detected between both HF groups, which were significantly lighter than the VHF group on most weeks from the 10th week of diet on, and HF (corncob) mice were significantly heavier than STD fed mice only on the last couple of weeks. Furthermore, VHF fed mice were significantly heavier than STD mice from the 7th week of test on (Figure 28A). One VHF fed mouse did not put on weight and was therefore excluded from all the analyses.

As for the percentage of weight gained, a similar profile is observed. Main effects of diet ($F(3,440) = 8.15$, $p < 0.001$) and time ($F(20,440) = 186.10$, $p < 0.001$) and an interaction between these two factors ($F(60,440) = 5.58$, $p < 0.001$) were detected. The posthoc test results are illustrated on Figure 28B and reproduce the profile found for the body weight.

Although not statistically significant, all animals from this experiment had a greater percentage of weight gain when compared to those from the western lifestyle experiment replicate. This might be explained by the lower weight of the animals from this experiment in the beginning of the diet (Table 1).

Regarding the food intake (Figure 28C), main effects of diet ($F(3,68) = 13.00$, $p < 0.05$) and time ($F(17,68) = 8.00$, $p < 0.001$), besides an interaction between these two factors ($F(51,68) = 2.3$, $p < 0.001$) were detected. The posthoc test revealed that all HF and VHF groups displayed similar food intake through all weeks of test, and differed from STD group in almost all weeks (effects illustrated on Figure 28C). This calorie intake regulation has already been described in the literature (Woods et al., 2004, Melhorn et al., 2010) and this same pattern has already been observed in our previous experiments involving HF diet.

As for the percentage of the weight represented by BAT (Figure 29A), the one-way ANOVA detected group effect ($F(3,22) = 4.09$, $p < 0.05$). Dunnett's posthoc test revealed that HF (corncob) and VHF groups presented significantly higher percentage of their weights represented by BAT than the control group ($p < 0.05$ for both). Similarly, group effects were detected for the percentage of the weight represented by VAT ($F(3,22) = 10.87$, $p < 0.001$) and for ScAT ($F(3,22) = 7.80$, $p < 0.001$) (Figure 29B,C). Posthoc analyses showed that both HF groups ($p < 0.01$ for VAT and $p < 0.05$ for Scat) and VHF group ($p < 0.001$ for both VAT and ScAT) differed significantly from controls regarding the percentage of the weight represented by VAT and Scat.

	Mean body weight (g)	Mean weight gain (%)
Western lifestyle experiment		
Initial weight = ~27 g		
Standard diet (n=12)	32.1	20.1
High-fat diet (n=12)	39.5	32.1
High fat vs bedding experiment		
Initial weight = ~24 g		
Standard diet (n=7)	32.5	37.7
High-fat diet (sawdust) (n=7)	37.3	59.8
High-fat diet (corncob) (n=6)	38.9 *	63.3 *
Very high-fat diet (n=6)	43.9 ***	88.3 ***

Table 6- Mean body weight and mean percentage of weight gain after 21 weeks of diet of C57BL/6J mice from the western lifestyle experiment replicate and from this experiment. (*) and (*) represent significant ($p < 0.05$ and $p < 0.001$, respectively) difference from control groups (standard chow fed animals from the same experiment) on week 21. Analyses were performed with repeated measures ANOVA followed by Bonferroni post-test.**

Interestingly, the effect of HF diet (regardless of the bedding composition) on BAT and ScAT depots is present both in this and in the former experiment (western lifestyle 2014). Furthermore, in this experiment we also observe an effect on the VAT depot, which was only previously observed when UCMS was associated to the HF. Despite the percentage of body weight that each fat sample represented in STD fed animals being very similar between both experiments (0.47% vs. 0.49% for BAT; 0.21% vs. 0.30% for VAT; 0.41 vs. 0.51% for ScAT in the 2014 and in the present experiments, respectively), we observe that the depots of HF mice housed under corn cob bedding are greater in the present experiment (0.69% vs. 0.78% for BAT; 0.31% vs. 0.94% for VAT; 0.73 vs. 1.39% for ScAT in the 2014 and in the present experiments, respectively). The type of bedding associated to the HF diet does not seem to change this profile.

The repeated measures ANOVA has detected main effects of diet ($F(3,88) = 18.00$, $p < 0.001$) and time ($F(4,88) = 40.00$, $p < 0.001$), as well as an interaction ($F(12,88) = 4.70$, $p < 0.001$) between these two factors, for plasma leptin levels (Figure 30A). The posthoc revealed that leptin levels start increasing on the 8th week of diet for the VHF group and on the 12th week of diet for the HF groups when compared to controls. Similarly, the ANOVA has detected main effects of diet ($F(3,88) = 14.34$, $p < 0.001$) and time ($F(4,88) = 67.17$, $p < 0.001$), and an interaction ($F(12,88) = 6.46$, $p < 0.001$) between diet and time for the percentage of body fat (Figure 30B). As expected (Frederich et al., 1995, Considine et al., 1996), plasma leptin levels were highly correlated to body fat, as illustrated in Figure 30C.

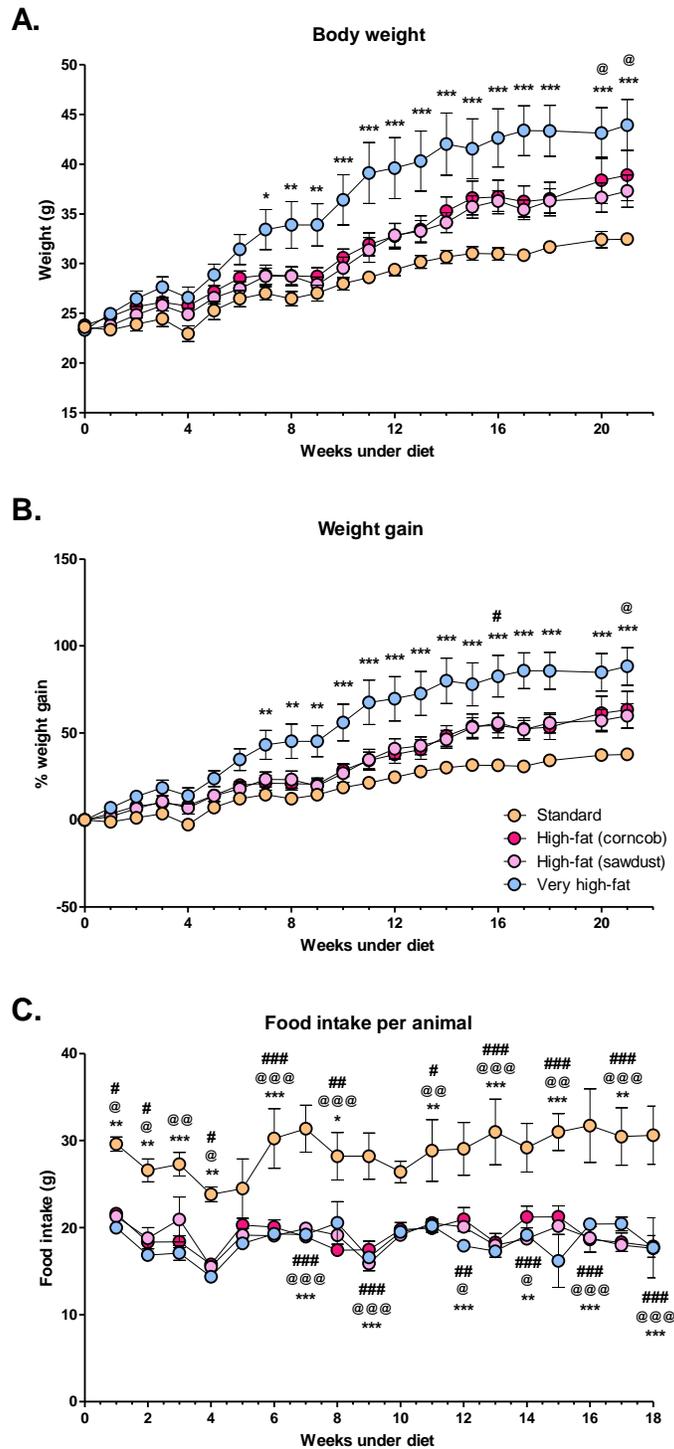


Figure 28 – Weight (A), percentage of weight gained (B) and food intake (C) of C57BL6/J mice submitted to either standard (stud, yellow), high-fat (HF, pink) or very high-fat (VHF, blue) diet, and housed over either spruce sawdust (light pink) or corncob grit (dark pink) bedding. (#), (##) and (###) represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) difference between HF (sawdust) and STD animals. (@), (@@) and (@@@) represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) difference between HF (corn cob) and STD animals. (*), (**) and (***) represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) difference between VHF and STD animals. Analyses were performed with repeated measures ANOVA followed by Bonferroni post-test ($n = 6-7/\text{diet}$).

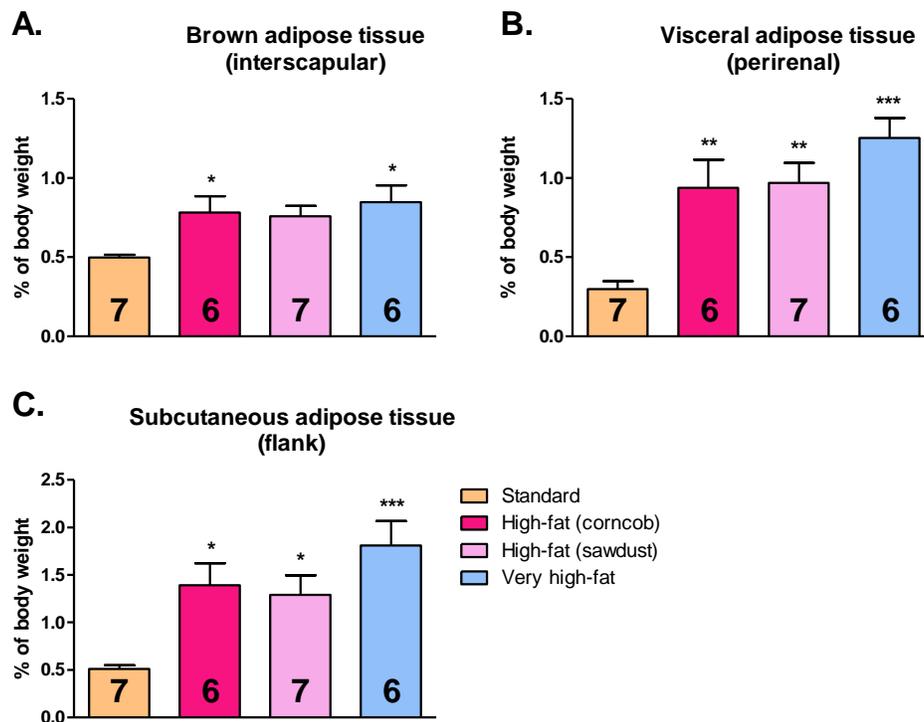


Figure 29 – Percentage of body brown (A), visceral (B) and subcutaneous (C) adipose tissue in C57BL6/J mice submitted to either standard (STD, yellow), high-fat (HF, pink) or very high-fat (VHF, blue) diet, and housed over either spruce sawdust (light pink) or corncob grit (dark pink) bedding. (*), (), and (***) represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) difference from STD animals. Analyses were performed with one-way ANOVA followed by Dunnett’s multiple comparison tests, using STD animals as controls.**

For fasting blood glucose levels, the repeated measures ANOVA has detected only overall effects of diet ($F(3,88) = 7.20$, $p < 0.01$) and time ($F(4,88) = 43.00$, $p < 0.001$). Despite no significant interaction was detected, a posthoc revealed that VHF mice display higher blood glucose than controls from week 12 until the end of the experiment, and HF (corn cob) also display higher levels than controls on the last week of experiment (Figure 31A). As for plasma insulin levels, an effect of diet ($F(3,22) = 11.00$, $p < 0.001$) and an interaction between diet and time ($F(3,22) = 4.10$, $p < 0.01$) were detected (Figure 31B). The posthoc test showed that VHF mice display higher plasma insulin levels than controls from week 8 until the end of the experiment, and both HF groups also display higher levels than controls on the last week of experiment. Glucose metabolism was found altered in all HF and VHF groups, which displayed lower glucose tolerance and higher insulin resistance (Figure 31C,D). The repeated measures ANOVA detected main effects of diet ($F(3,132) = 4.00$, $p < 0.05$) and time ($F(6,132) = 100.00$, $p < 0.001$) on the oral glucose tolerance test. Even though no interaction between factors was detected, a posthoc test was performed and revealed that all groups differ on their response to oral glucose administration when compared to controls (detailed effects on Figure 31C). We have also analyzed the area under the curve (AUC) for all groups, and a one-way ANOVA revealed significantly greater areas ($F(3,22) = 4.45$, $p < 0.05$) for all groups when compared to that from animals under STD diet ($p < 0.05$ for HF groups and $p < 0.01$ for VHF groups). Regarding the insulin resistance test, effects of diet ($F(3,132) = 5.50$, $p < 0.01$), time ($F(6,132) = 11.00$, $p < 0.001$) and interaction between both factors ($F(18,132) = 2.70$, $p < 0.001$) were detected. The

posthoc analysis revealed that, similarly to what was observed in the glucose tolerance test, all groups differ on their glucose response to insulin administration when compared to controls (detailed effects on Figure 31D). We have also analyzed the AUC for the insulin test and found significantly greater areas ($F(3,22) = 5.59$, $p < 0.01$) for HF sawdust ($p < 0.05$) and VHF ($p < 0.01$) groups when compared to that from animals under STD diet.

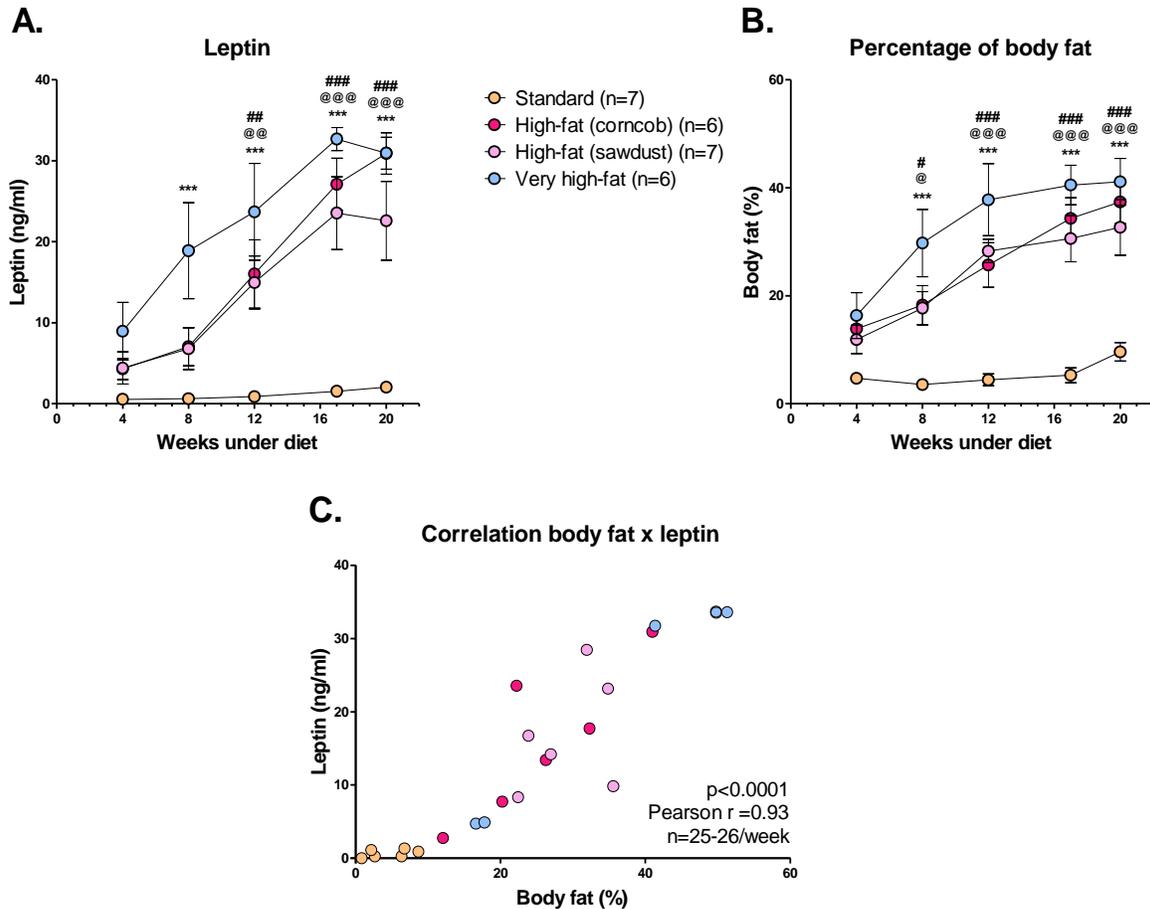


Figure 30 – Plasma leptin levels (A), percentage of body fat (B) and the correlation between both factors (C) in C57BL6/J mice submitted to either standard (STD, yellow), high-fat (HF, pink) or very high-fat (VHF, blue) diet, and housed over either spruce sawdust (light pink) or corncob grit (dark pink) bedding. (#), (##) and (###) represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) difference between HF (sawdust) and STD animals. (@), (@@) and (@@@) represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) difference between HF (corncob) and STD animals. (*) represents significant ($p < 0.001$) difference between VHF and STD animals. Analyses were performed with repeated measures ANOVA followed by Bonferroni post-test (A,B) or Pearson’s correlation (C).**

Finally, for both morning and evening corticosterone levels only main effects of time were detected ($F(4,56) = 5.9$, $p < 0.001$ for morning and $F(4,56) = 7.5$, $p < 0.001$) (Figure 32A,B). A posthoc test only revealed significantly higher morning levels of corticosterone for VHF and HF (sawdust) groups on the last week of the experiment when compared to STD mice. Although a great intragroup variability is observed and significant effects are not detected, both HF groups seem to present higher corticosterone levels in the evening than the control group, while the VHF group keeps

its levels very close to the controls. This is an interesting observation, since the VHF group – which seems to have its metabolism more affected by the diet – does not reflect these alterations on its corticosterone status. A possible factor influencing this trend is the sugar content of these diets: the HF diet contains 20.1 % of sucrose in its composition (corresponding to 17.0% of its calories), while the VHF diet presents only 8.9% of sucrose (corresponding to 6.8% of its calories). It is thus possible that the combination of HF and high sugar concentrations is more prone to alter the HPA axis functioning than fat only. Further studies are needed to confirm and better understand this observation.

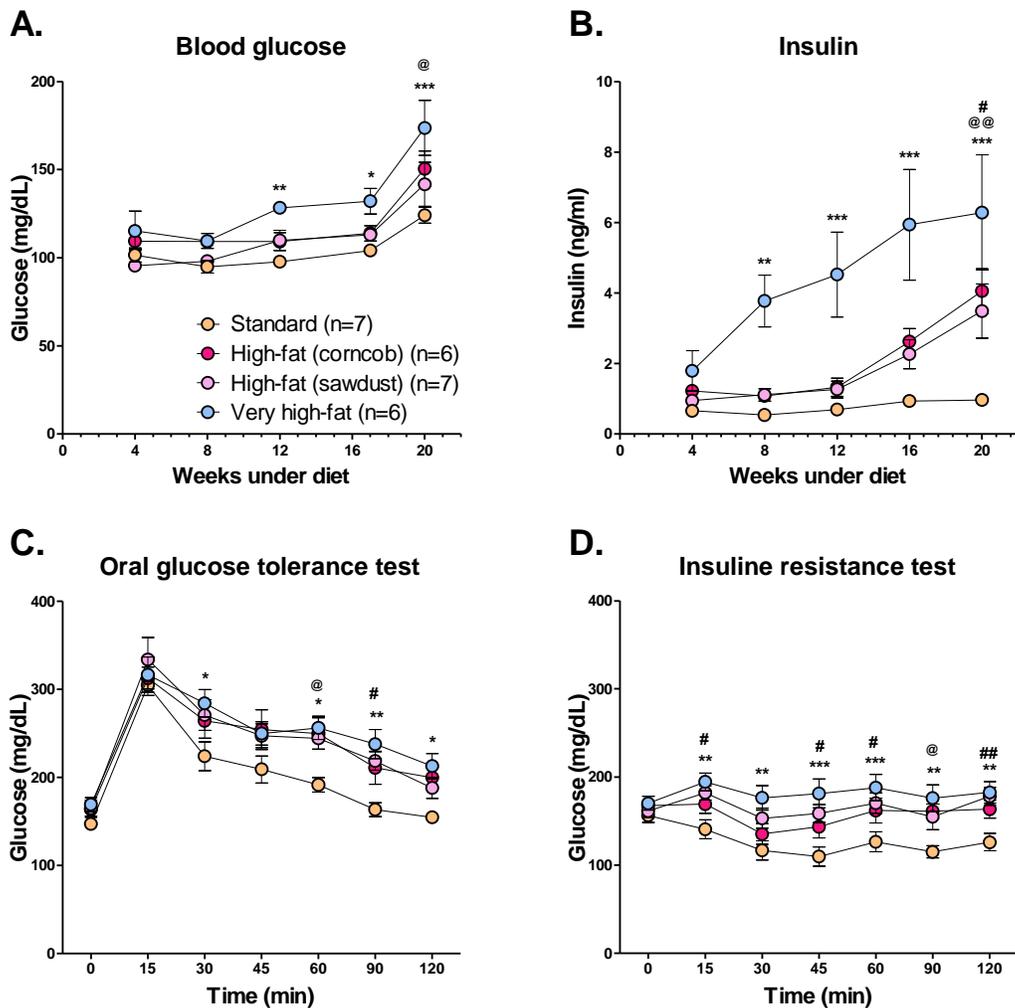


Figure 31 – Blood glucose after overnight fasting (A), plasma insulin levels (B), and blood glucose levels during the oral glucose tolerance (C) and the insulin resistance (D) tests in C57BL6/J mice submitted to either standard (STD, yellow), high-fat (HF, pink) or very high-fat (VHF, blue) diet, and housed over either spruce sawdust (light pink) or corncob grit (dark pink) bedding. (#) and (##) represent significant ($p < 0.05$ and $p < 0.01$, respectively) difference between HF (sawdust) and STD animals. (@) represents significant ($p < 0.05$) difference between HF (corncob) and STD animals. (*), (), and (***) represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) difference between VHF and STD animals. Analyses were performed with repeated measures ANOVA followed by Bonferroni post-test.**

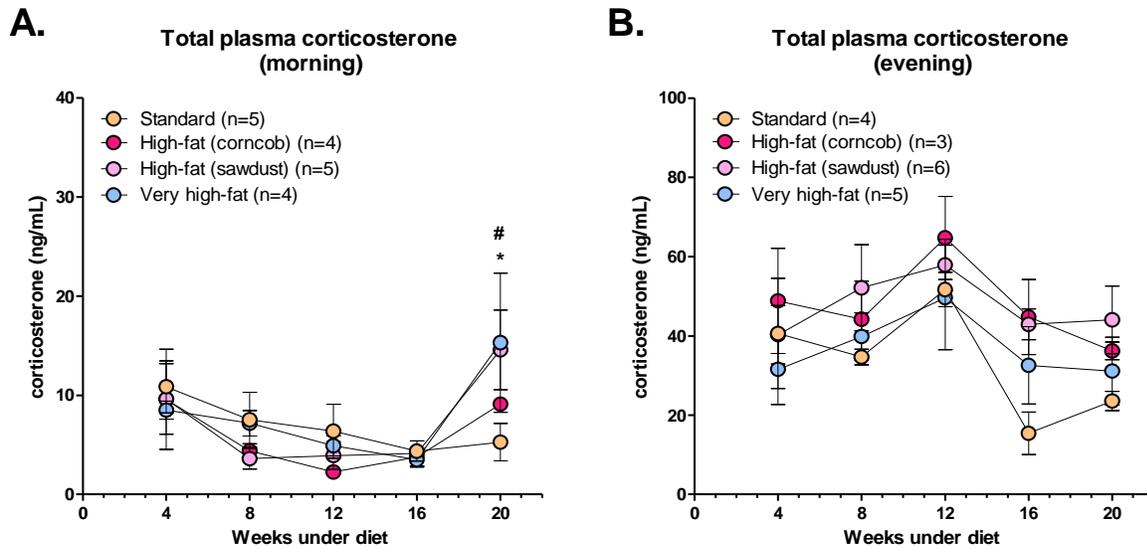


Figure 32 – Total corticosterone levels in the morning (A) and in the evening (B) of C57BL6/J mice submitted to either standard (STD, yellow), high-fat (HF, pink) or very high-fat (VHF, blue) diet, and housed over either spruce sawdust (light pink) or corncob grit (dark pink) bedding. (#) represents significant ($p < 0.05$) difference between HF (sawdust) and STD animals, while (*) represents significant ($p < 0.05$) difference between VHF and STD animals. Analyses were performed with repeated measures ANOVA followed by Bonferroni post-test.

The data obtained in this experiment have helped us screening thoroughly some of the factors we had hypothesized that could have influenced the unexpected results obtained in the replicate of the western lifestyle experiment. We have seen no clear influence of the different bedding materials on the several phenotypes analyzed, and therefore the hypothesis that the difference between the results from the different western lifestyle experiments stems from an influence of the corncob grit bedding on the effects of HF diets is unlikely. On the other hand, this experiment has revealed that – as expected – the deleterious effects of a hyperlipidic diet are more pronounced in animals exposed to the VHF diet. This is particularly true for the body weight and the percentage of weight gain, as VHF diet fed animals are already heavier than the STD controls from the second month of diet, while the HF groups only differ from the STD controls by the end of the experiment. The percentage of body fat – notably VAT and ScAT – also seems to be more impacted by VHF diet. The deleterious effect of VHF diet is also clear when looking at their high insulin levels.

Another hypothesis for our unexpected results in the replicate of the western lifestyle experiment was that the discrete weight gain of the HF groups of the replicate experiment was not sufficient to interact with the chronic stress to generate the difference between genotypes. Based on that, we have decided to perform a new replicate of the western lifestyle experiment, but this time using the VHF diet as a hyperlipidic diet.

Behavioral and metabolic consequences of CBG deficiency on male mice submitted to very high fat diet combined to chronic mild stress

This experiment shares the same background of the western lifestyle experiment replicate. It was designed to verify if the unexpected results from that experiment, i.e. the absence of weight loss of Cbg ko mice when submitted to both HF diet and to the UCMS procedure, was related to the abnormal and discrete weight gain presented by the animals under HF diet. After testing the metabolic effects of STD, HF and VHF diets on C57BL/6J male mice, we have found that the VHF diet produces the most robust effects on weight gain and metabolic parameters when compared to STD chow. We have then decided to perform a new western lifestyle experiment replicate using the VHF diet as a hyperlipidic diet. In this new experiment we have decided to evaluate despair-like behaviors through the TST instead of the FST, since the first is performed with an automated system and thus allows a more precise behavioral evaluation. Furthermore, we included a fear memory evaluation through the contextual fear conditioning, once our studies indicate that Cbg ko mice display an impairment in some memory tasks (see page 111) and we wondered if these impairments could be affected by HF diet, UCMS or the combination of both.

Methods

Cbg ko and wild-type mice (MGI ID: 4833641, *Serpina6tm1.1Mmp*, produced in our laboratory in Bordeaux - France) used in this experiments were littermates, obtained by breeding males and females heterozygous for the CBG mutation, and genotyped from a tail biopsy. Our mice have a C57BL/6J genetic background above 95%. Animals were maintained, from birth and throughout the whole experiment, in an animal facility with constant temperature (23 °C), under a 12 h light/dark cycle (lights on at 0700 h) and with ad libitum access to food and water.

40 male mice – 20 Cbg ko and 20 wild-types – of about 15 weeks of age were split into 10 collective cages (4 mice/cage, mixed genotypes). Before being submitted to the VHF diet, blood samples were collected from all mice (afternoon, after 5h fasting) as control samples (as described on page 159). Blood glucose after overnight fasting and body composition were also evaluated (as described on pages 160). Then, mice were then put under either VHF chow (D12492, from Research Diets) or STD chow (A04, from SAFE - Scientific Animal Food & Engineering) (2 cages of standard and 8 cages of very HF diet) and had their weight and food intake evaluated once a week until the end of the experiment. After 14 weeks under diet, blood samples were again collected, and blood glucose after overnight fasting and body composition were measured. Half of the mice from the VHF group (4 cages, 8 animals from each genotype) were placed into individual cages and an UCMS protocol was imposed, identically to that from the original and replicate of the western lifestyle experiment and as described in Figure 33. The remaining mice were maintained in collective cages and left undisturbed. On the 18th week, after the end of the UCMS, new blood samples from all animals were collected, blood glucose was evaluated after overnight fasting and mice had their body composition measured through magnetic resonance imaging. On the 19th week mice were submitted to the glucose tolerance test (as described on page 161). Still on the 19th week, they were submitted to the TST (as described on page 80). Finally, on the 20th week, memory was evaluated in the contextual fear conditioned (as

described on page 113). Sacrifice was performed in the end of the 20th week, identically to that from mice submitted to the western lifestyle experiment replicate.

Analyses involving repeated measures (weight, percentage of weight gain, food intake, percentage of body fat/lean mass and the oral glucose tolerance test) were performed with a repeated measures ANOVA followed by Bonferroni post-test. Analyses of the percentage of brown, visceral and subcutaneous adipose tissue were performed with a one-way ANOVA followed by Dunnett's multiple comparison tests, comparing all groups with the STD chow fed one. All analyzes were performed with the software Prism 5 for Windows (GraphPad Software, Inc.).

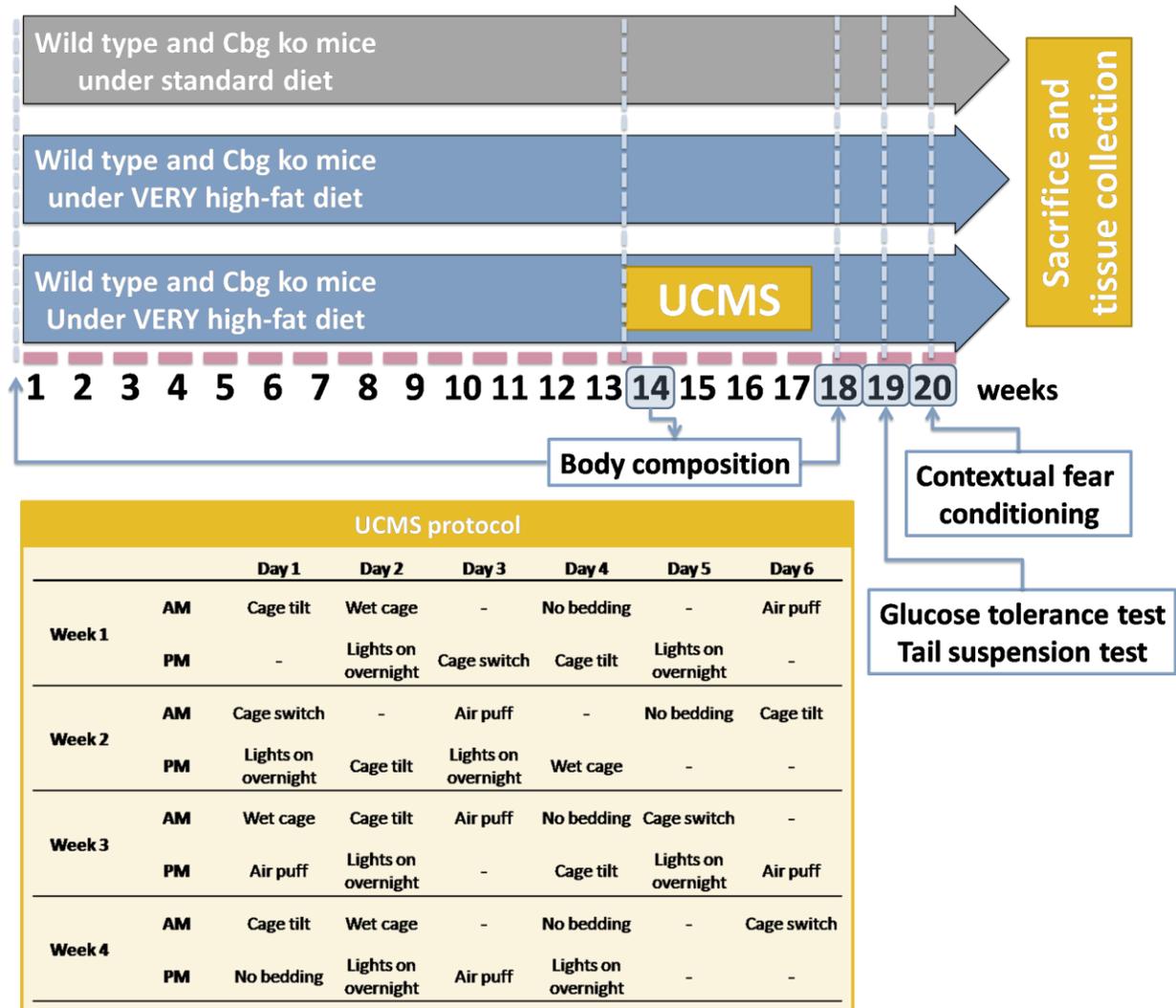


Figure 33 – The unpredictable chronic mild stress protocol.

Results and discussion

The repeated measures ANOVA for weight from weeks 0 to 14 revealed main effects of group ($F(3,434) = 5.5, p < 0.01$), time ($F(14,434) = 140.00, p < 0.001$) and an interaction between time and group ($F(42,434) = 23.00, p < 0.001$). The post hoc test revealed that both wild-type and Cbg ko mice from VHF groups were significantly heavier than mice from STD groups either from week 8 (Cbg ko) or week 9 (wild-type) (Figure 34A,B). As for the weight gain in this same period, the ANOVA also revealed

effects of group ($F(3,390) = 30.00$, $p < 0.001$), time ($F(13,390) = 140.00$, $p < 0.001$) and interaction between time and group ($F(39,390) = 24.00$, $p < 0.001$). The post hoc revealed the same difference observed for the weight gain, but in an even more pronounced way, and from week 4 (Cbg ko) and 5 (wild-type) on (Figure 34C,D).

When the UCMS is associated to the VHF diet (from week 15 on), we still observe effects of group ($F(5,112) = 13.57$, $p < 0.001$), time ($F(4,112) = 7.00$, $p < 0.001$) and interaction between time and group ($F(20,112) = 2.61$, $p < 0.001$). The posthoc reveals that, regardless of the UCMS, VHF groups from both genotypes remain heavier than their controls under STD diet until the end of the experiment. Nevertheless, the UCMS procedure only significantly impacted the weight of wild-type mice, and only from the 17th week (third week of UCMS) on. Still, a trend can be observed towards a weight loss in Cbg ko mice too. When looking at the percentage of weight gain after the

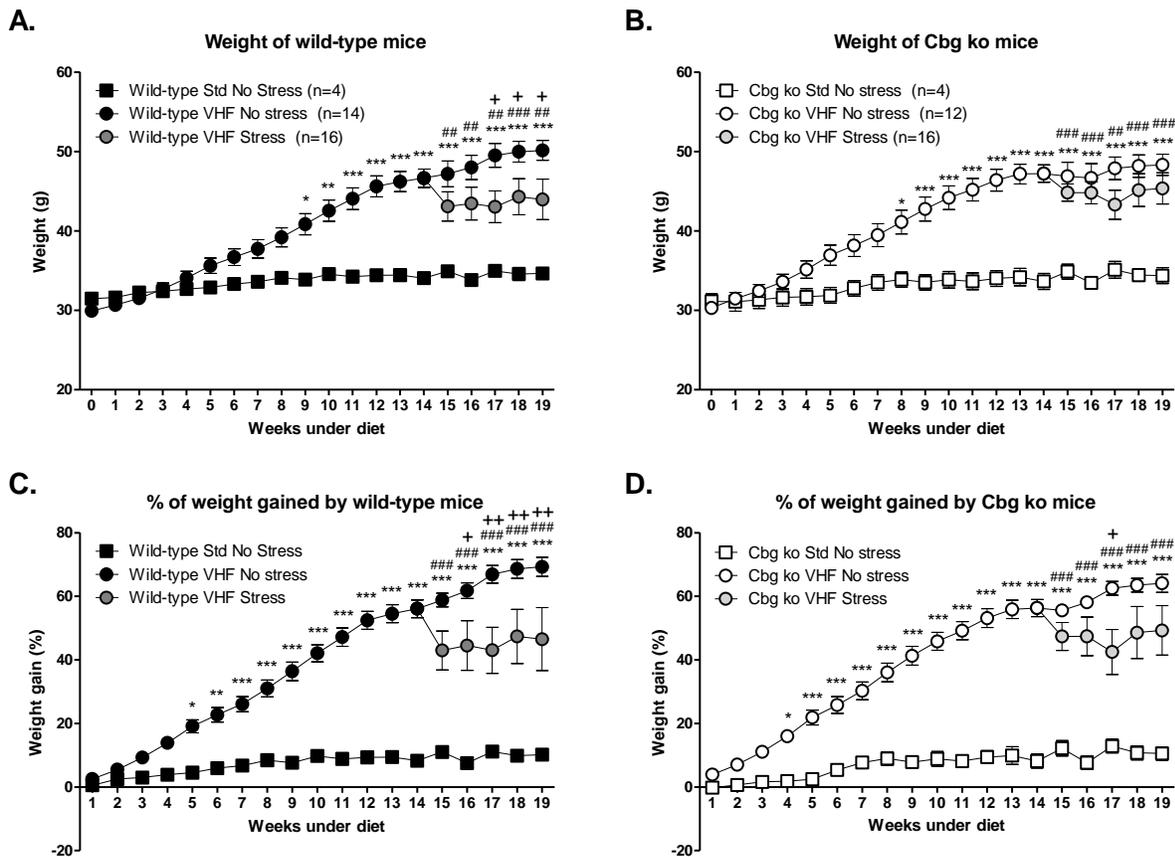


Figure 34 – Weight (A,B) and percentage of weight gained (C,D) by wild-type (A,C) and Cbg ko (B,D). Circles represent animals under very high-fat diet (VHF), while squares represent animals under standard diet (STD). Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice respectively. (*), () and (***) represent significant differences ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) between VHF fed and STD fed groups, while (##) and (###) represent significant differences between VHF stressed and STD groups ($p < 0.01$ and $p < 0.001$, respectively), and (+) and (++) represent significant differences between VHF stressed and VHF unstressed groups ($p < 0.05$ and $p < 0.01$, respectively). Analyses were performed with repeated measures ANOVA followed by Bonferroni post-test. Absolute weight data, as well as percentage of weight gain data, for animals from both genotypes were analyzed in a same ANOVA, but are displayed in separate graphs.**

beginning of the UCMS, effects of group ($F(5,112) = 22.07, p < 0.001$), time ($F(4,112) = 8.01, p < 0.001$) and interaction between time and group ($F(20,112) = 2.79, p < 0.001$) are also observed. Once again, the posthoc reveals that, regardless of the UCMS, VHF groups from both genotypes maintain an increased weight gain when compared to their controls under STD diet until the end of the experiment. Similarly, wild-type mice under VHF were more impacted by the UCMS and display significant weight loss when compared to unstressed VHF animals from the second week of UCMS until the end of the experiment. On the other hand, Cbg ko mice under VHF and UCMS only present a significant weight loss when compared to Cbg ko VHF on week 17.

Once again, the results regarding weight and weight gain do not confirm those from the original western lifestyle experiment. The hypothesis that the divergences between these experiments' results stemmed from the different bedding used was already ruled out in the previous experiment (presented on page 172). Now, we also found that the discrete weight gain of the HF group from the western lifestyle experiment replicate was not responsible for those divergences, once mice from this experiment gained an important amount of weight when submitted to VHF diet and still the expected effects of the combination of VHF and UCMS on body weight were not found. Five animals (two wild-type and two Cbg ko) under HF diet were excluded from all analyses for displaying a lower weight gain.

Similarly to what has been described in the literature and for the previous experiments, VHF fed mice tend to consume less chow than STD fed mice (Figure 35A). The ANOVA detected main effects of diet ($F(1,76) = 280.00, p < 0.001$) and time ($F(19,76) = 8.90, p < 0.001$), besides an interaction between both factors ($F(19,76) = 2.30, p < 0.001$). The post hoc test revealed higher consumption for groups under STD diet throughout the whole experiment. Curiously, the effect of UCMS on food intake (increase in the consumption by stressed groups) observed in the western lifestyle experiment replicate was not observed in this experiment (Figure 35B).

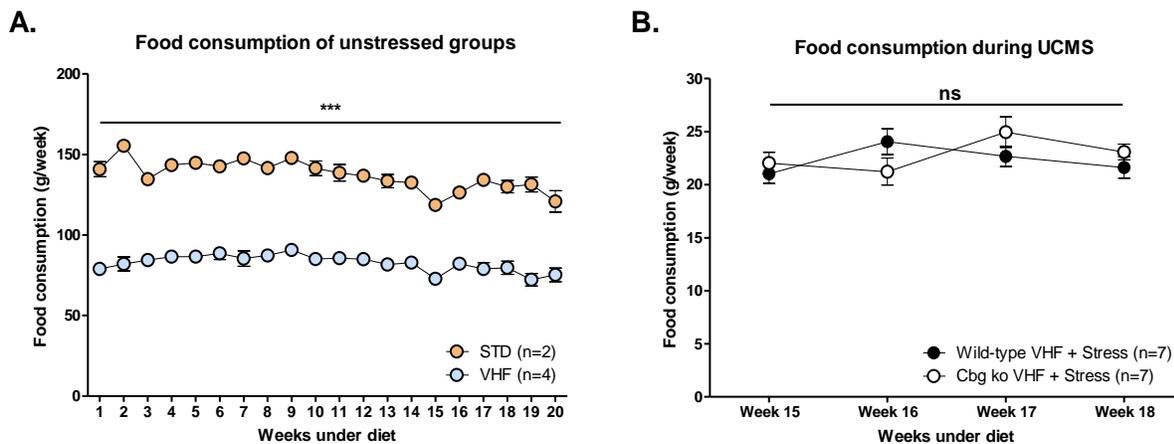


Figure 35 – Food intake throughout the 20 weeks of experiment for the unstressed groups (A) and throughout the four weeks of UCMS for the stressed groups (B). In (A), brown symbols represent the standard diet (STD) while blue symbols represent the very high-fat diet (VHF). In (B), black symbols represent wild-type mice, while white symbols represent Cbg ko mice. (*) represents significant ($p < 0.001$) difference between the STD group and the VHF group for every week analyzed. Analyses were performed with repeated measures ANOVA followed by Bonferroni post-test.**

Blood glucose was not different between wild-type and Cbg ko mice before the beginning of the experiment (Figure 36A). Nevertheless, the values obtained were considered high for animals that were fasted overnight. We hypothesize that as blood glucose values were obtained the day after mice were housed into new cage configurations and transferred to a new experimental room, the stress caused by those changes might have induced this rise in blood glucose levels. This elevation in blood glucose values in response to physical or psychological stress has already been described in human and rodents (McCowen et al., 2001, Kang et al., 2015). Values obtained for control mice on week 14 (Figure 36B), on the other hand, were similar to baseline values of unstressed mice. No difference between wild-type and Cbg ko mice was found on that week, but VHF fed animals presented higher levels of blood glucose than STD chow fed ones, regardless of the genotype (main effect of diet, $F(1,35) = 25.00$, $p < 0.001$). This effect of HF or VHF diet on blood sugar levels has already been described in the literature (Carpene et al., 2014), and was also found in the experiment described on page 141. Finally, blood glucose levels of unstressed wild-type and Cbg ko mice under STD chow did not differ on week 18, after the end of the UCMS procedure (Figure 36C). Interestingly, their values were found higher than on week 14, but still lower than on week 0. We hypothesize that stress might be once again responsible for this increase in blood glucose levels. Despite not being directly submitted to the UCMS procedure, unstressed groups were housed in the same experimental room as stressed groups, and might therefore have been indirectly impacted by the UCMS. No difference between experimental groups was found on week 18.

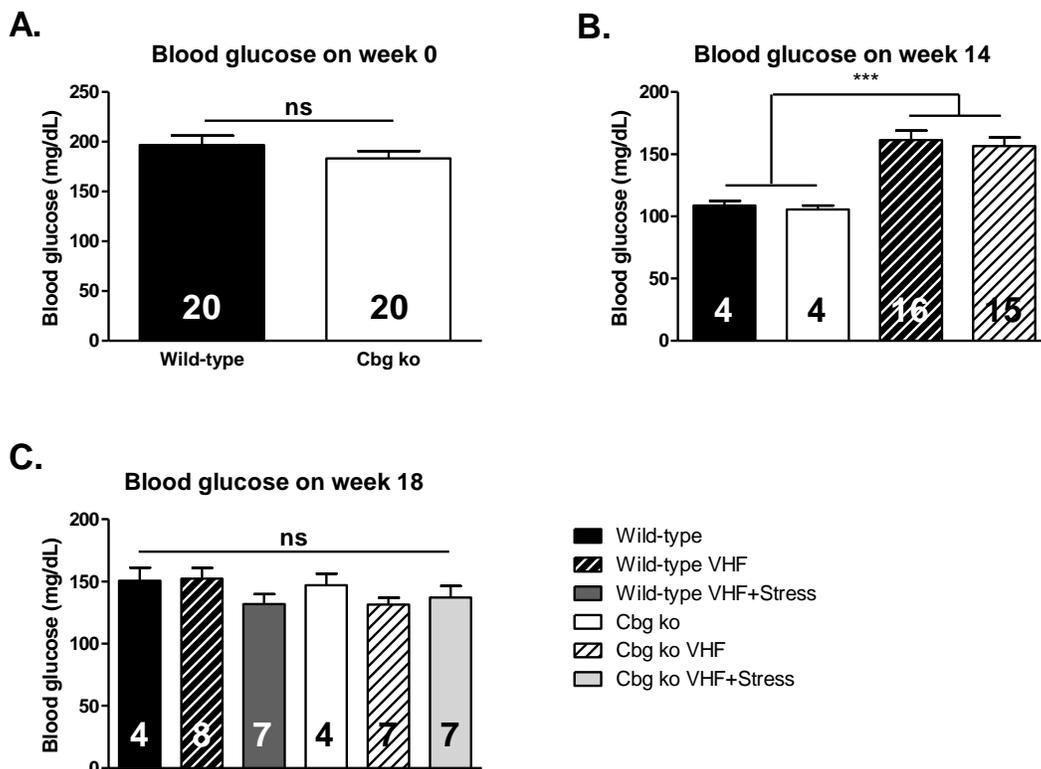


Figure 36 – Blood glucose after overnight fasting before the beginning of the experiment (A), after 14 weeks of very high-fat diet (B) and after 4 weeks of unpredictable chronic mild stress (C). (*) represents significant ($p < 0.001$) effect of genotype. Analyses were performed through a t-test (A), two-way ANOVA followed by Bonferroni post-test (B), and one-way ANOVA followed by Dunnett’s multiple comparison test, using STD animals as controls (C).**

As expected, VHF fed groups had a higher percentage of body fat (main effects of group ($F(5,56) = 53.55$, $p < 0.001$) and time ($F(2,56) = 421.6$, $p < 0.001$), and interaction between both factors ($F(10,56) = 21.53$, $p < 0.001$), with the post hoc results represented in Figure 37A,B) and, complementarily, lower percentage of lean mass (main effects of group ($F(5,56) = 53.18$, $p < 0.001$) and time ($F(2,56) = 415.8$, $p < 0.001$), and interaction between both factors ($F(10,56) = 21.45$, $p < 0.001$), with the post hoc results represented in Figure 37C,D) on weeks 14 and 18. This effect was not influenced by the UCMS procedure or the genotype of the mice. Curiously, the percentage of lean mass on Cbg ko groups was lower on week 0, that is, before the onset of the VHF diet and UCMS. This was probably a random effect artificially produced when selecting the animals for the experiment, since weight was not a parameter used in their selection.

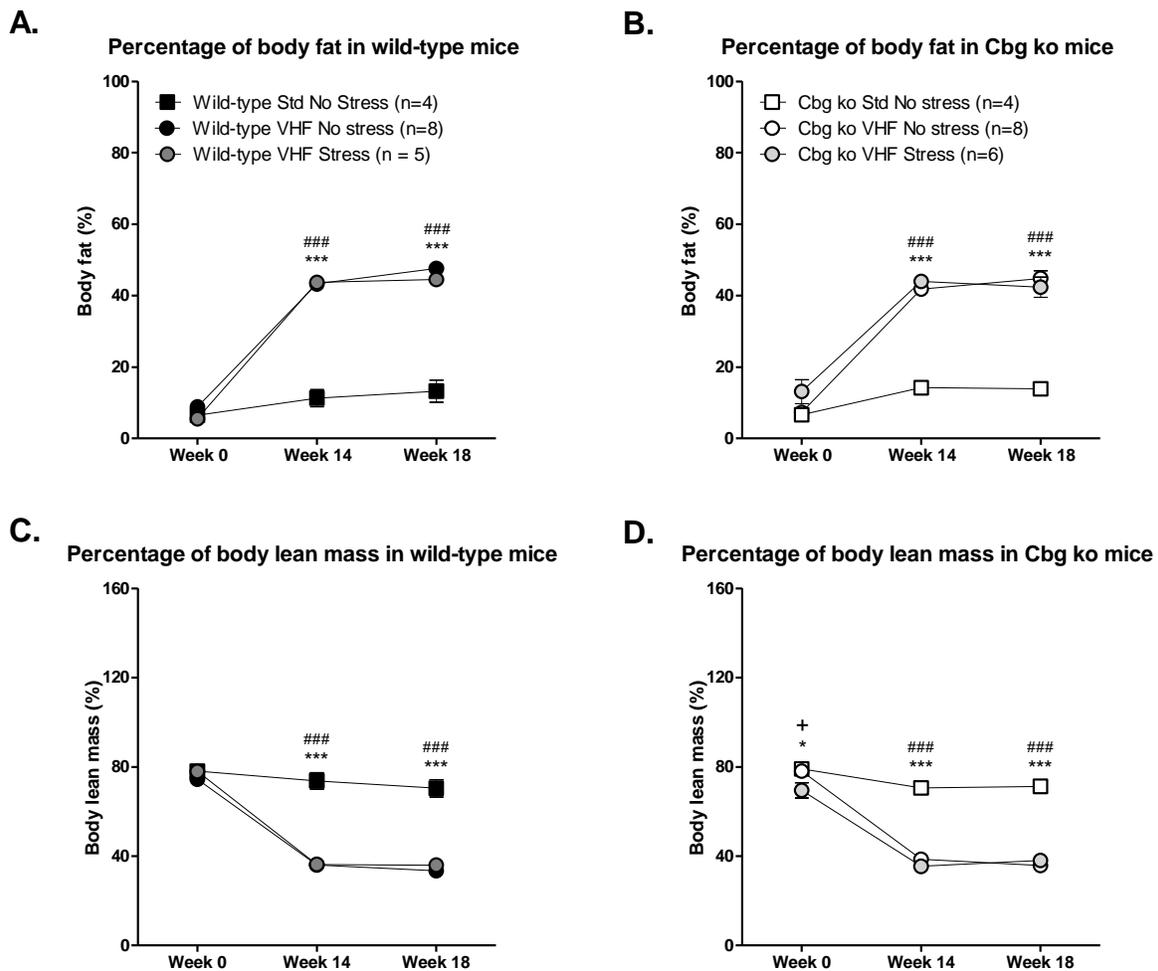


Figure 37 – Percentage of fat (A,B) and lean (C,D) mass of wild-type (black) and Cbg ko (white) mice on weeks 0, 14 and 18. Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice respectively. (*) and (*) represent significant differences ($p < 0.05$ and $p < 0.001$, respectively) between very high-fat (VHF) fed and standard chow (STD) fed groups, while (####) represents significant differences between VHF stressed and STD groups ($p < 0.001$), and (+) represents significant difference between VHF stressed and VHF unstressed groups ($p < 0.05$). Analyses were performed with repeated measures ANOVA followed by Bonferroni post-test. Data for animals from both genotypes were analyzed in a same ANOVA, but are displayed in separate graphs.**

For the oral glucose tolerance test, the ANOVA has detected main effects of group ($F(5,180) = 4.00$, $p < 0.01$) and time ($F(6,180) = 110.00$, $p < 0.001$), but no interaction between factors. Nevertheless, a post hoc test was performed and the results are illustrated on Figure 38A,B. Similarly to what was observed in the western lifestyle experiment replicate, VHF fed mice are less tolerant to glucose, what is reflected in higher blood concentrations of glucose after an oral administration of a glucose solution. This effect seems to be more pronounced in wild-type mice, although no significant difference was detected between wild-type and Cbg ko groups. When analyzing the AUC through a two-way ANOVA, we observe a main effect of treatment ($F(2,30) = 8.23$, $p < 0.01$). A post hoc test revealed that STD groups displayed lower blood glucose levels than VHF groups, regardless of the genotype ($p < 0.05$ for both). Nevertheless, in wild-type mice only, VHF groups also presented higher glucose levels than VHF animal under UCMS, while no difference regarding the glucose levels of these groups was detected in Cbg ko mice.

Interestingly, stress seems to improve tolerance to glucose, lowering blood levels of VHF fed animals towards the values of STD fed mice as already described on the literature (Packard et al., 2014). The effect of stress on glucose tolerance is more pronounced in wild-type mice, where blood glucose levels of mice under VHF diet reach those from STD fed mice when stress is associated.

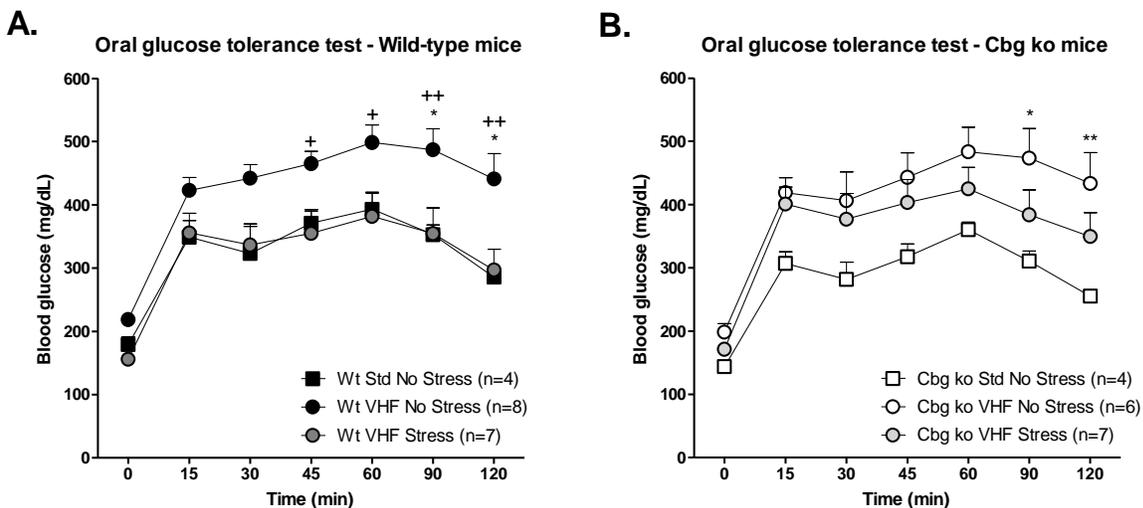


Figure 38 – Oral glucose tolerance test performed in wild-type (A, black) and Cbg ko (B, white) groups after 18 weeks of very high-fat diet and one week after the end of the chronic stress. Circles represent animals under high-fat diet (HF), while squares represent animals under standard diet (STD). Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice respectively. (*) and () represent significant ($p < 0.05$ and $p < 0.01$, respectively) difference between STD and unstressed VHF groups, while (+) and (++) represent significant ($p < 0.05$ and $p < 0.01$, respectively) difference between stressed VHF and unstressed VHF groups. Analyses were performed with repeated measures ANOVA followed by Bonferroni post-test. Data from animals from both genotypes were analyzed in a same ANOVA, but are displayed in separate graphs.**

For the TST (Figure 39A), the one-way ANOVA detected group effects for wild-type ($F(2,15) = 10.90$, $p < 0.01$) and Cbg ko mice ($F(2,13) = 7.93$, $p < 0.01$). The post hoc tests revealed reduced immobility for wild-type mice fed with VHF, regardless of the association with UCMS, and for Cbg ko fed with VHF diet only when associated with

UCMS. Mice under different kinds of hypercaloric diets were already reported to display lower levels of despair-like behavior (Leffa et al., 2015, Del Rio et al., 2016), and this effect might be related to the comfort food theory, as described on page 33. Interestingly, opposite results – with hypercaloric diet increasing despair-like behavior and the association with chronic stress increasing it even more (Kurhe et al., 2014). The origin of these differences is not clear, but the composition of the diet might be a potential confounding factor, once it tends to vary greatly between studies.

We did not find the expected difference in the immobility scores of wild-type and Cbg ko control mice in the TST ($t(1.06) = 0.33$). This difference in despair-like behavior was also not found in the FST performed in the western lifestyle experiment replicate, and that raises the question whether it is just a matter of a small sample size ($n=4$ /genotype for the TST in this experiment and $n=5-7$ /genotype for the FST in the western lifestyle experiment replicate) or if somehow the previously found effect of CBG deficiency on despair-like behaviors (that has been consistently found and described in the articles from our group) was lost during the breeding of our mice. Still, a discrete trend towards higher immobility is found in control and VHF Cbg ko mice, but interestingly identical scores are found for wild-type and Cbg ko mice under both VHF diet and UCMS.

Finally, no difference was detected between wild-type and Cbg ko mice regarding fear learning in the contextual fear conditioning (Figure 39B). This finding contradicts that described in this thesis (Page 118), where we describe that CBG deficient mice display impairment in this task when compared to wild-type mice. Again, this absence of effect can be explained by the small sample size of the control groups, what is corroborated by a non-significant trend of Cbg ko mice towards a lower percentage of time freezing when compared to wild-type mice. VHF diet seems to impair aversive memory and the association between VHF and UCMS results in marginally significant memory impairment ($p = 0.06$). This profile is similar between wild-type and Cbg ko animals.

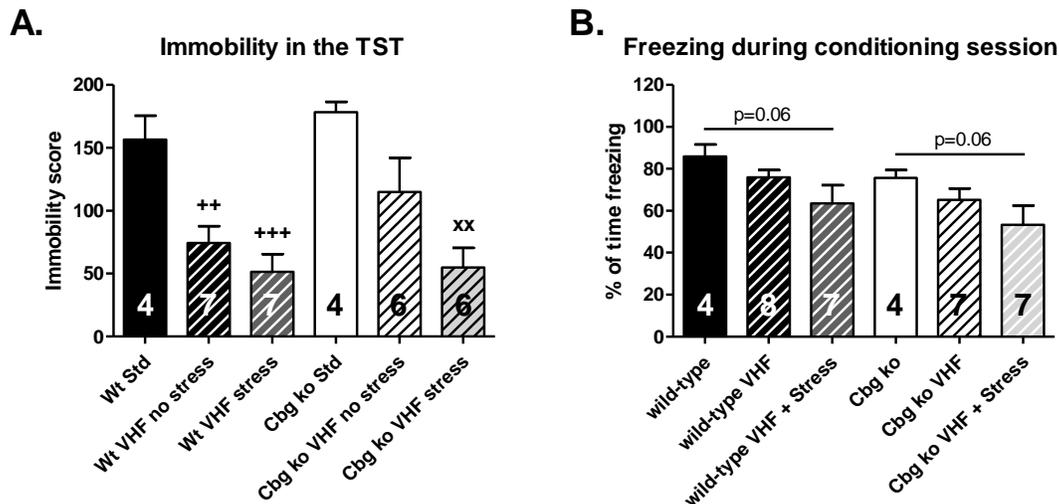


Figure 39 – Performance of wild-type (black) and Cbg ko (white) mice in the tail suspension test (TST) and in the contextual fear conditioning (B). Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice respectively. (##) and (###) represent significant ($p<0.01$ and $p<0.001$) difference and (0.06) represents a non-significant trend towards a difference from the respective Standard chow fed group. Analyses were performed with a one-way ANOVA followed by Dunnett’s multiple comparison tests, using STD animals as controls.

When analyzing the percentage of body weight represented by BAT and VAT (Figure 40A,B), the one-way ANOVA only revealed group effects for wild-type mice ($F(2,15) = 4.07$, $p < 0.05$ for BAT and $F(2,15) = 8.91$, $p < 0.01$ for VAT). The post hoc analyses revealed wild-type mice increase their BAT and VAT composition when under VHF diet only, but not when VHF diet is associated with UCMS. Indeed, BAT does not seem to be affected by diet or stress in the Cbg ko mice, while VAT displays a similar profile to that from wild-type mice, but blunted. Scat (Figure 40C) was highly increased by VHF diet in both wild-type and Cbg ko groups ($F(2,15) = 18.02$, $p < 0.001$, and $F(2,14) = 5.11$, $p < 0.05$, respectively). No significant differences were observed between wild-type and Cbg ko control groups.

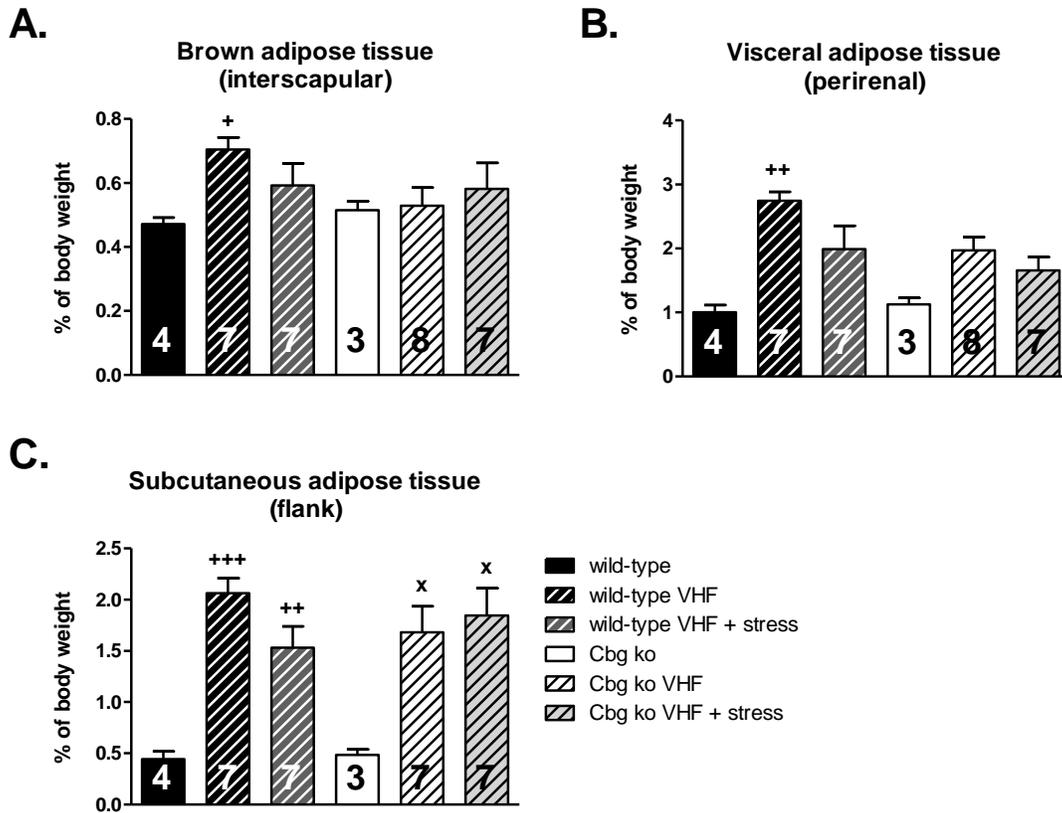


Figure 40 – Percentage of body weight represented by brown adipose tissue (A, BAT), visceral adipose tissue (B, VAT) and subcutaneous adipose tissue (C, ScAT) in wild-type (black) and Cbg ko (white) mice. Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice respectively. (+), (++) and (+++) represent significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) difference from the wild-type control group, while (x) represents significant ($p < 0.05$) difference from the Cbg ko control group. Analyses were performed with a one-way ANOVA separately for each genotype followed by Dunnett’s multiple comparison tests, using STD animals as controls.

For total corticosterone values (Figure 41), the one-way ANOVA did not reveal any group effect in the morning or in the evening. Post hoc analyses revealed only a decrease in morning corticosterone levels in Cbg ko mice submitted to both VHF diet and UCMS when compared to their controls ($p < 0.05$). As expected, a t test detected significant differences between Cbg ko and wild-type levels in the evening, with Cbg ko mice displaying lower levels of corticosterone ($t(3.16) = 0.02$).

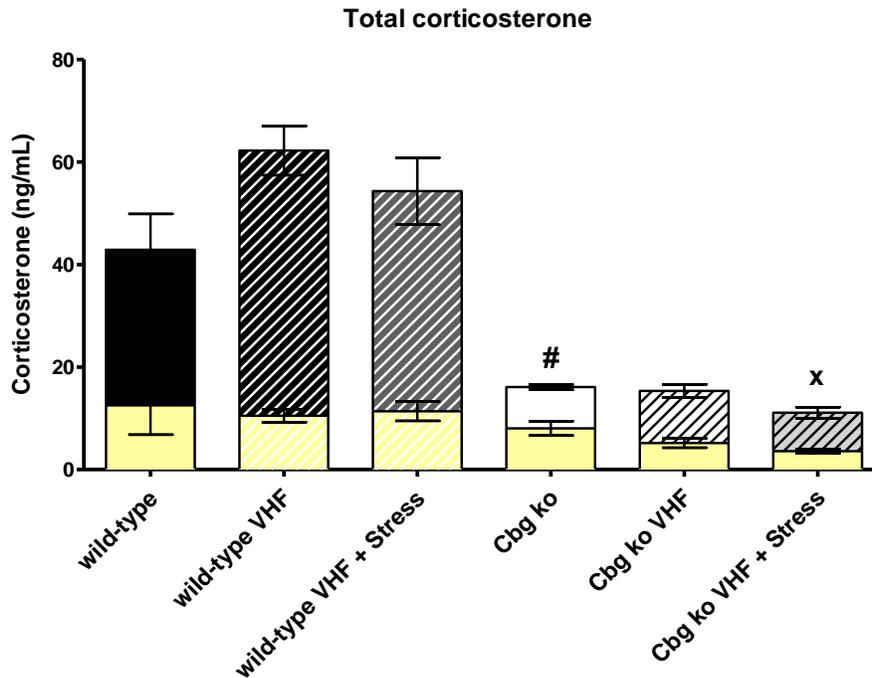


Figure 41 – Total corticosterone in the morning and evening on wild-type (black) and Cbg ko (white) mice. Groups submitted to chronic stress are represented in dark or light gray, for wild-type and Cbg ko mice respectively. Morning values are represented as yellow bars inside the bars of each group. (x) represents significant ($p < 0.05$) difference from the Cbg ko control group, while (#) represents significant difference between wild-type and Cbg ko controls ($p < 0.05$). Analyses were performed with a one-way ANOVA separately for each genotype followed by Dunnett's multiple comparison test, using STD animals as controls, except for the comparison between control groups, which was performed through a t test.

The results from this experiment using VHF diet as a hyperlipidic diet have confirmed some of the previously obtained data from the experiment involving different types of HF diet, notably that animals submitted to VHF have pronounced deleterious effects on body weight and percentage of body fat, glucose metabolism and on emotional and cognitive behaviors. Nevertheless, CBG deficiency did not show a clear influence on the effects of VHF or UCMS on the phenotypes studied. Since the expected effects of CBG deficiency were not reproduced in this experiment, tissues and blood samples collected in this experiment weren't further analyzed.

Overall, we observed an interesting pattern regarding weight gain in the original western lifestyle experiment: while both wild-type and Cbg ko mice gain weight under HF diet, once UCMS is associated Cbg ko mice lose weight, while wild-type maintain their weight gain. This result was not confirmed in the western lifestyle replicate carried out in 2014, where Cbg ko mice maintained their body weight when UCMS was combined to the HF diet, while wild-type mice lost weight. A more careful analysis has revealed that mice from this experiment did not gain as much weight as mice from the original experiment when under HF diet. Furthermore, an unexpected bias was found: wild-type mice from the unstressed groups were heavier than all the other groups from the beginning of the experiment. A subsequent experiment has allowed us to rule out the possible influence of the type of bedding on the weight gain of mice under HF, and also revealed that VHF diet leads to a more important weight gain than HF, which was similar to that observed in the original experiment for mice under HF. A last experiment was performed to evaluate the impact of CBG deficiency on the physiological and

behavioral effects of the western lifestyle, but this time using VHF diet instead of HF diet. In this experiment, mice from both genotypes gained weight when under VHF, but both lost weight when UCMS was associated to the VHF. Furthermore, the weight loss was more pronounced in wild-type mice. Thus, we were not able to replicate the effect of CBG deficiency on the effects of HF diet and UCMS combined on weight gain. In fact, the weight data obtained from the western lifestyle replicate and from the VHF experiment are in accordance with some literature reports that show that mice submitted to both hypercaloric diets and chronic stress display decreased weight gain or even weight loss (Michel et al., 2005, Finger et al., 2011, Paternain et al., 2011, Zeeni et al., 2013, Balsevich et al., 2014). Kuo and colleagues have reported that the weight gain of mice submitted to stress depends greatly on the background strain, type of diet, and stress protocol. They propose that mild stressors (to which mice can habituate to) do not promote changes in body weight, while stronger stimuli may increase or decrease body weight, depending on the type of diet mice are submitted to. When combined with a hypercaloric “comfort food” diet, strong stressors would stimulate sympathetic nerves to upregulate NPY expression. Furthermore, they would increase glucocorticoid levels, primarily in the visceral fat, which in turn would further upregulate the expression of NPY and its receptors in fat tissues, leading to increased fat mass (Kuo et al., 2008). Finally, Cbg ko mice were reported heavier (8.8%) than wild-type mice on day 0 of the original western lifestyle experiment, but this difference was not further observed when mice from both genotypes were split by diet. A study by Gulfo and colleagues has found that, under standard feeding conditions, CBG deficient mice display a slight but highly significant increase in body weight when compared to wild-type mice, associated with higher food intake in the first. This difference disappears when mice are submitted to a hypercaloric diet (Gulfo et al., 2016). Controversially, Petersen and colleagues found no effect of CBG deficiency or hypercaloric diet on the weight of wild-type and CBG deficient mice after six weeks of diet (Petersen et al., 2006). In our experiments, wild-type mice under VHF diet displayed increased weight when compared to those under STD diet after 7 weeks of diet, while those under HF diet only presented a significantly increased body weight after 20 weeks of diet. This suggests that the absence of a diet effect (and eventually of a genotype effect) on Petersen’s experiment might be due to a short diet period.

A consistent finding throughout all these experiments was the reduced food intake presented by HF fed animals, in an attempt to balance energy intake. Interestingly, UCMS had no effect on food intake in any of the experiments. The percentage of body fat was not measured in the original western lifestyle experiment, but the subsequent experiments found no impact of CBG deficiency on this parameter. All experiments found a consistent increase in the percentage of body fat in animals under HF diet, regardless of the stress status. Furthermore, VHF induced an even higher increase in body fat, which was highly correlated with plasma leptin levels. ScAT was invariably increased by both HF and VHF diets, regardless of the association to UCMS. BAT and VAT displayed a more variable and inconsistent profile throughout the experiments, but also generally increasing with HF/VHF diets. Paternain and colleagues have reported that different white adipose tissue depots were increased in HF fed mice, and decreased when a chronic stress protocol was associated (Paternain et al., 2011). We do observe a similar trend in wild-type mice from the VHF experiment, but not in those from the western lifestyle replicate or in Cbg ko mice from any of the experiments. A recent study has evaluated how CBG deficiency affects adipose tissue in obesity, and found that CBG deficient mice display a shift from subcutaneous to visceral fat tissue

accumulation, but those findings were not observed in any of our experiments. These authors also found that adipocytes from both depots of CBG deficient mice fed with control diet tended to be smaller than those of wild-type mice. However, when under hypercaloric diet, visceral adipocytes from CBG deficient mice exhibited a higher increase in area, while those from the subcutaneous depot showed a minor increase when compared to wild-type mice (Gulfo et al., 2016).

Blood glucose showed an interesting pattern in both the original western lifestyle experiment and in the VHF one: HF and VHF fed mice displayed higher blood glucose levels before the beginning of UCMS, but by the end of the UCMS protocol no difference was observed between groups. This phenomenon does not seem to be influenced by the time under diet, since the experiment with different types of diet showed that blood glucose of animals under HF or VHF diet only tend to increase throughout time. We hypothesize that this might be due to a confounding effect of the UCMS protocol on unstressed mice, since all animals were housed in the same experimental room. In all experiments, HF and VHF diet decreased glucose tolerance, but the effects of UCMS were not consistent throughout the different experiments. In the western lifestyle replicate, animals under both HF diet and UCMS had the most impaired tolerance to glucose, followed by those under either HF diet or UCMS only and finally the controls. This response was similar between both genotypes. On the VHF experiment we do not observe this potentiating effect of the combination between VHF diet and UCMS, and CBG deficiency seems to influence the animals' tolerance to glucose. In wild-type mice, animals under VHF diet only seem to have the most impaired tolerance to glucose, and when UCMS is combined to VHF their tolerance returns to baseline. On Cbg ko mice, the same profile is observed for mice under VHF diet, but the association of UCMS has little impact on their tolerance to glucose, with the blood sugar levels of this group remaining similar to that from the VHF diet only group. As for the insulin resistance test, we also do observe higher resistance in HF and VHF animals when compared to controls (despite no statistical significance being detected on the western lifestyle replicate). Like in the glucose tolerance test, a different pattern is observed for the UCMS influence on the insulin resistance among the two genotypes. While wild-type mice under either STD or HF diet seem to improve their response to insulin when submitted to UCMS, Cbg ko mice do not seem to have their response affected by UCMS. Insulin levels were shown to increase with HF diets, mainly with VHF diet, but the levels of this hormone weren't measured in the experiments involving HF/VHF and CBG deficient mice. In accordance with our results, the literature shows that hypercaloric diets lead to impaired glucose tolerance (Collier et al., 1985, Gnudi et al., 1995, Huang et al., 2004, Thompson et al., 2015) and insulin resistance (Storlien et al., 1986, Pedersen et al., 1991, Paternain et al., 2011, Antunes et al., 2016). Chronic stress or corticosterone administration, on the other hand, have been associated with enhanced vulnerability to diet-related metabolic consequences in both animal models and humans (Auvinen et al., 2013, Aschbacher et al., 2014), but some studies suggest that chronic stress may have relatively modest metabolic effects which occur primarily during initial stress exposure (Thompson et al., 2015)

Although variable throughout time and sometimes not reaching statistical significance, it was also observed that HF or VHF diet increases total corticosterone levels in animals from both genotypes, and the increases are more visible in wild-type mice. The literature also brings variable results regarding how corticosterone levels are affected by hypercaloric diets, with some studies reporting increases (Fu et al., 2009, Swierczynska et al., 2015, Gulfo et al., 2016) and other decreases (Paternain et al.,

2011, Auvinen et al., 2012). In our chronic stress conditions, only a slight decrease in plasma corticosterone levels was observed. The literature also presents controversial findings regarding corticosterone levels after chronic stress, with some studies finding increased levels (Fu et al., 2009, Monteiro et al., 2015) and others decreased levels after chronic stress protocols (Paternain et al., 2011, de Medeiros et al., 2016). The association between hypercaloric diets and chronic stress, on the other hand, seems to consistently increase corticosterone levels (Fu et al., 2009, Balsevich et al., 2014). We did not measure free corticosterone in the plasma of these animals, but it remains as an interesting parameter to assess the free and active fraction of this hormone throughout the different conditions. Moreover, none of the experiments we performed has evaluated the corticosterone increase after stress. We have shown in previous experiments (Richard et al., 2010) that the differences between corticosterone levels between wild-type and Cbg ko are greater after stressful conditions, and this measure could provide some interesting data on the influence of diet and UCMS on corticosterone reactivity. CBG binding capacity in wild-type mice was found to be higher in HF fed animals, similarly to what was found by (Gulfo et al., 2016) and to be unaffected by UCMS.

Finally, despite no behavioral difference being detected in the sucrose preference test performed during the original western lifestyle experiment, an interesting profile was found in the forced swim test. Cbg ko mice under standard conditions displayed increased despair-like behavior when compared to wild-type mice in the same conditions. The combination of HF diet and UCMS increased the despair-like behaviors of wild-type mice, but normalized those of Cbg ko animals. We did not confirm these results in the following experiments. In the western lifestyle replicate, we observed no difference between wild-type and Cbg ko mice under standard conditions. Interestingly, Cbg ko mice seemed to increase their despair-like behaviors in all conditions (HF, UCMS and HF + UCMS), while wild-type maintained identical levels, but these trends were not significant. In this same experiment, some other effects were observed in the splash and sucrose preference tests. In the splash test, HF diet (but not HF + UCMS) increased the depressive-like phenotype of both wild-type and Cbg ko mice, while in the sucrose preference test HF increased the depressive-like phenotype of wild-type only. Despite also assessing depressive-related traits, the effects observed in the splash and sucrose preference tests do not seem to relate to those found in the FST performed during the original western lifestyle experiment. Finally, on the experiment involving the VHF diet, we observed a trend towards an increased despair like behavior of Cbg ko mice when compared to wild-type mice under standard conditions, resembling the findings from the original western lifestyle experiment. Nevertheless, this trend was not statistically significant, possibly due to the low sample size of these groups (n=4 for both). Moreover, despite an unexpected decrease in the despair-like behavior of wild-type mice was observed when VHF and UCMS were associated, wild-type and Cbg ko mice do not differ within these two conditions combined. Similarly to what we have found for wild-type mice in the splash and in the sucrose preference tests – but not in the forced swim test – hypercaloric diets have been usually associated to increased depressive like behavior (Sharma and Fulton, 2013, Zemdegs et al., 2016). However, the unpredictable chronic mild stress – one of the most extensively investigated animal models of depression (Willner et al., 1987, Willner, 2005) – only induced depressive-like behaviors in wild-type mice from the original western lifestyle experiment.

It can be tricky to compare the results from our studies with the literature, since hypercaloric diets vary greatly in composition (from industrialized pellets containing different caloric contents from different sources to actual cafeteria diet) and chronic

stress protocols are not homogeneous (from different durations to different types of stressors and different intensities). Nevertheless, most of our findings regarding the effects of HF diet, chronic stress wild-type and the combination of both in wild-type mice were also observed elsewhere.

In a nutshell, our original western lifestyle experiment had brought up an interesting and differential effect of HF diet and UCMS on the weight gain and despair-like behaviors presented by wild-type mice and Cbg ko. These differences seemed to be linked to increased lipolysis in Cbg ko mice submitted to both HF diet and UCMS. Further experiments failed to confirm these differences, but pointed out new metabolic differences between wild-type and Cbg ko mice regarding glucose tolerance and insulin resistance, which deserve further investigation. We remain with two main hypotheses to explain the divergences between our experiments: (I) a genetic drift or a substrain divergence; or (II) other environmental differences existent between the animal facilities in which the experiments were performed. It is possible that not CBG (since Cbg ko mice still do not express this protein), but instead the product of one or more genes located near the excised *locus* were in fact responsible for the observed phenotypes. Throughout several generations of breeding in our laboratory, these genes might have had their expression affected through genetic drift or a substrain divergence, explaining the current absence of the expected phenotypes. Alternatively, despite some main differences already being ruled out, it is still possible that some apparently minor factors – such as the animal facility workers or the frequency of cage cleaning – might play a role in the metabolism and behavior of these mice.

GENERAL DISCUSSION

CBG has long been considered as a mere transporter of glucocorticoid hormones within the bloodstream. As presented in the introduction of this thesis, studies are increasingly showing that CBG transcends this modest role and its central importance on other processes starts to be unraveled. Given the important, yet frequently neglected, role of CBG in modulating glucocorticoid levels and action, our team has developed a mouse model of CBG deficiency to further explore its actions. This model presents blunted glucocorticoid circadian rhythmicity and glucocorticoid hyporesponse to stress (Richard et al., 2010). It has also been shown to display increased despair-like behaviors and is protected from the deleterious effects of stress on memory retrieval (Richard et al., 2010, Minni et al., 2012). This thesis' objectives were to further explore the physiological and behavioral alterations presented by the CBG-deficient mice in order to better understand the role of CBG in modulating the effects of glucocorticoids.

Study I – Role of corticosteroid binding globulin in emotional reactivity sex differences in mice

Sex differences exist for stress reactivity as well as for the prevalence of depression, which is more frequent in women than in men, and is often precipitated by stressful events. This differential effect of stress on males' and females' emotional behavior has been well documented. Sexual dimorphism regarding CBG levels have also already been described in the literature, and the first study presented in this thesis aimed at investigating if there was a sexual dimorphism in the already described modulation of glucocorticoids by CBG. Since CBG is also known to be regulated by estrogens, we also aimed at evidencing if there was an influence of estrogen in CBG's modulation of glucocorticoids.

Our main findings are that, as Cbg ko males, Cbg ko females also presented markedly reduced corticosterone levels across the circadian cycle and after stress. Plasma free corticosterone levels in Cbg ko mice, measured immediately after stress, were blunted in both sexes when compared to wild-type mice. Furthermore, we also showed that Cbg ko mice, regardless of the sex, have a faster corticosterone clearance from plasma than wild-type mice. This seems to be the main source of differences in plasma corticosterone levels between Cbg ko and wild-type mice, since neither adrenal sensitivity to ACTH nor CRH levels were found to differ among these mice. Regarding their behavioral profile, we detected the expected genotype differences in male mice – with Cbg ko mice displaying increased despair-like behaviors – but not in female mice. In fact, both wild-type and Cbg ko female mice display elevated despair-like behavior, similar to the levels observed in Cbg ko male mice. Then, female mice were ovariectomized, and on this group we observe a difference similar to that observed in male mice, notably by the decrease of the despair-like behaviors presented by wild-type females. When ovariectomized females were replaced with E2, their behavior on despair-like behavior tests resembled again that from naïve females, indicating that endogenous E2 in naive females explains the absence of behavioral difference between wild-type and Cbg ko female mice. Thus, we have demonstrated that the increase in despair-like behaviors triggered by CBG deficiency is masked in cycling females by circulating estrogens.

This first study has contributed to a better understanding of the sex differences on the consequences of CBG deficiency, more specifically regarding emotionality-

related phenotypes. The complementary studies that followed on this subject present a limited interpretation, since the expected and well documented differences in despair-like behavior between wild-type and Cbg ko male mice, and between wild-type males and females were not observed. Nevertheless, animals from both sexes and genotypes seem to respond to the treatment with the antidepressant imipramine, decreasing the display of despair-like behavior indexes. Finally, we have also observed that the treatment with this antidepressant increases the levels of free corticosterone in the plasma of male mice from both genotypes after stress, and a trend in the same direction is observed in wild-type female mice. This puts the increase in free corticosterone levels as a possible mechanism behind the effects of this drug. Future work on this topic require investigating the absence of the expected differences in despair-like behaviors between wild-type and Cbg ko mice in the anti-depressant experiment. Once we are able to recover these expected phenotypes, a more precise evaluation of the effects of a tricyclic antidepressant – and eventually other classes of antidepressants – could be performed to confirm our results.

Study II – Chronic stress does not further exacerbate the abnormal psychoneuroendocrine phenotype of CBG-deficient male mice

Chronic stress is known to firstly increase glucocorticoid levels and subsequently dysregulate the HPA axis due to the prolonged activation of the stress response. The dysregulation of the HPA axis (leading to either hyper- or hypo-activity) is linked to the deleterious effects of chronic stress. We have hence hypothesized that, due to their known glucocorticoid hyporesponse to stress, our Cbg ko mouse model could be protected from the deleterious effects of chronic stress. To address this question, in the second study we submitted Cbg ko and wild-type male mice to two different chronic stress paradigms – the UCMS and the social defeat. The impact of these two paradigms on neuroendocrine function – assessed through corticosterone and CBG measurement – and on behavioral responses – evaluated via anxiety and despair-like behavioral tests – was investigated.

Our main findings were that individuals with CBG deficiency do not have their abnormal psychoneuroendocrine phenotype further affected by chronic stress. We showed that both chronic stress paradigms increased the display of despair-like behavior in wild-type mice, while that from Cbg ko mice – which was already high – was not aggravated. We have also found that control and stressed Cbg ko mice show no difference in social interaction, while wild-type mice reduce their interaction time when stressed. These changes in behavioral response were accompanied by a similar pattern for corticosterone levels: while both chronic stress paradigms lowered the corticosterone levels of WILD-TYPE mice, those from Cbg ko mice remained low and unaltered. Thus, through Cbg ko mice, we demonstrated that CBG is crucial in modulating the effects of stress on plasma corticosterone levels and consequently on behavior. Additionally, we have shown that free – and therefore active – plasma corticosterone fraction remains higher in Cbg ko mice and does not vary within genotypes with chronic stress. These apparently conflicting results might stem from the fact that these measures were performed in plasma obtained in the morning and under resting conditions. We believe this profile may change in situations where the HPA axis is more active such as during the active phase (night) or after an acute stressor. We have also investigated the expression of genes involved in the HPA axis regulation or behavioral modulation. Some of these genes showed different expression profiles among wild-type and Cbg ko mice,

or within unstressed and stressed conditions. Nevertheless, most of these differences were only observed as trends and did not reach statistical significance, suggesting that new studies should be carried out to eventually confirm these effects.

This work highlighted the importance of CBG in modulating the alterations imposed by chronic stress on the HPA axis. Even though no chronic stress-induced variation in plasma levels of CBG were observed in wild-type mice, its presence seems to be of great importance to allow variations in the levels of plasma corticosterone. These variations in corticosterone levels, on their turn, seem to underlie the behavioral changes observed in wild-type mice submitted to chronic stress.

The main results of this work consist on behavioral observations. To further broaden the contribution of this study to the understanding of the role of CBG on chronic stress, a larger screening of the mechanism involved in these effects should be performed. For that, we should ideally submit a new group of wild-type and Cbg ko mice to one of these chronic stressors and evaluate several parameters, not only in resting conditions, but also after the stimulation of the HPA axis by an acute stressor. Among the parameters that could be analyzed are: (i) the expression of genes involved in the HPA axis regulation, regulated by glucocorticoids or modulated by chronic stress (similarly to what was performed in this study); (ii) the plasma levels of free and total corticosterone at trough at peak of secretion, and after stress – as well as CBG binding capacity; and (iii) the neuronal activation, especially after an acute stressor (through c-fos or Egr1 immunohistochemistry).

Study III – CBG deficiency specifically impairs hippocampus-dependent memory consolidation in male mice

Brain glucocorticoid concentration is a crucial factor influencing memory processes. Since we know that CBG is as an important regulator of glucocorticoid availability, we wondered whether CBG deficiency could impact memory processes. Studies from our team had already shown that CBG deficiency prevents the deleterious effect of stress on memory retrieval. In the third study of this thesis we wanted to investigate if CBG also had a role regulating memory formation. Our hypothesis was that the glucocorticoid hyporesponse to stress observed in the brain of CBG ko mice would lead to a lower activation of its receptors in the brain, compromising their memory formation and impairing the subsequent memory performance.

We showed that CBG affects memory consolidation. Cbg ko mice have a deficit in long-term, but not in short-term memory. Moreover, this deficit seems to be specific of tasks that depend on the hippocampus, since the performance of Cbg ko mice on amygdala- and perirhinal-cortex-dependent tasks was not affected by CBG deficiency. Variations in the protocols of the hippocampus-dependent tasks that influence the level of stress of the mice after the training/conditioning sessions reflect in different performances, following the Yerkes-Dodson law. These findings corroborate the numerous studies showing that suboptimal levels of glucocorticoids can lead to memory impairments, and supports the view that memory consolidation processes depend on post-training activation of glucocorticoid-sensitive pathways. We are now carrying out immunohistochemistry analyses of these mice's brains to investigate if there are any differences between wild-type and Cbg ko mice regarding neuronal activation after acquisition, after retrieval or in baseline conditions. We hypothesize that the deficit in hippocampus-dependent tasks observed in Cbg ko mice is accompanied by decreased neuronal activation after the training/conditioning sessions, but not in baseline

conditions. We are also processing these same brains through mass spectrometry imaging to assess the spatial distribution of corticosterone in their brains throughout these different situations. Similarly, and based on what has been previously shown by our team through microdialysis after an acute stress (Minni et al., 2012), we expect to find a weaker staining in the brains of Cbg ko mice than of wild-type, reflecting lower glucocorticoid access. If we confirm our hypotheses, future work could involve *in vivo* microdialysis to investigate the dynamics of corticosterone increase in the brain of these mice throughout different tasks and their modifications.

Study IV - Behavioral and metabolic consequences of CBG deficiency on male mice submitted to high fat diet combined to chronic mild stress

The HPA axis is the major neuroendocrine system involved in the stress response. Dysregulation of this axis activity has been increasingly associated to obesity and to mood disorders, which are in turn reported as highly comorbid. As already mentioned, our CBG deficient mouse model displays a dysregulation of the HPA axis, notably a blunted glucocorticoid response to the circadian rhythm and to stress, associated with increased despair-like behaviors. Furthermore, we had evidence from a pilot study showing that Cbg ko mice display different metabolic and behavioral responses to the combination of HF diet and UCMS – mimicking a Western lifestyle, and that this difference seems to be linked to increased lipolysis in Cbg ko mice. In the fourth study presented in this thesis we aimed at further characterizing these metabolic and behavioral alterations resulting from the combination of HF diet and UCMS.

After one replicate of the original Western lifestyle experiment and two other studies manipulating different aspects of the original protocol (different types of bedding and different types of HF diet), we were not able to confirm the findings from the original experiment. The impact of CBG deficiency on the effects of HF diet and UCMS combined was not observed in these animals' weight or in their behavior, and the origins of these discrepancies are still unknown. Nevertheless, an interesting and not previously described influence of CBG on glucose metabolism was observed. In wild-type mice, animals under VHF diet only seem to have the most impaired tolerance to glucose, and when UCMS is combined to VHF their tolerance returns to baseline. On Cbg ko mice, the same profile is observed for mice under VHF diet, but the association of UCMS has little impact on their tolerance to glucose, with the blood sugar levels of this group remaining similar to that from the VHF diet only group. Likewise, a different pattern is observed for the UCMS influence on the insulin resistance among the two genotypes. While wild-type mice under HF diet improve their response to insulin when submitted to UCMS, Cbg ko mice do not have their response affected by UCMS. These findings suggest that CBG deficiency prevents the amelioration by UCMS of the glucose intolerant and insulin resistant phenotypes induced by hyperlipidic diets. These effects do not seem to relate to the total corticosterone measured in these same studies, but measuring the free and active fraction of this hormone could give us a more precise idea about this relationship.

This new finding of a difference in glucose metabolism between wild-type and Cbg ko mice submitted to both HF diet and UCMS opens a whole new range of phenotypes that could be investigated in these animals. Besides replicating and confirming these findings, further analyses could involve the expression of genes involved in glucose metabolism (in glycolysis, in gluconeogenesis, in the regulation of those processes...) in liver and in muscle, as well as a more complete phenotyping of

their glucose metabolism, possibly including hyperglycemic and hyperinsulinemic clamps. The relationship between these glucose metabolism-related phenotypes and HPA axis alterations also needs to be elucidated. A starting point would be measuring the free corticosterone levels in the peak and trough of the circadian cycle, and eventually after being stimulated with glucose or insulin administration.

Limitations and future perspectives

As some obvious future perspectives for these studies are the investigation of the effects of chronic stress, HF diet, as well as the memory performance in *Cbg* ko female mice as well. We have highlighted the importance of studying females in the first study presented in this thesis, and showed that some effects of CBG deficiency are sex-specific due to an influence of estrogens on these effects. Yet, it is not clear if this crosstalk between CBG and estrogens specifically influences despair-like behaviors or if it can also have an impact on other phenotypes we have shown to be affected by CBG – such as memory formation or glucose metabolism under HF diet and UCMS conditions combined.

As with any other model, the work performed with the *Cbg* ko mouse model presents some limitations. A relevant issue is the possibility of a compensatory effect on *Cbg* ko mice, which could be masking other physiological effects of total CBG deficiency. To further explore this matter, it would be interesting to develop an inducible *Cbg* ko model, in which *Cbg* expression could be specifically inactivated at a given time point. For that, mice carrying the *Cbg* gene *locus* floxed by LoxP sites could be crossed with transgenic mice that express Cre recombinase under the regulation of a promoter that can be activated by a transcription factor not found in mammalian cells. This way, the excision of the *Cbg* gene would be conditioned to the administration of this exogenous molecule. Through this approach, we could bypass some critical embryonic and early development phases in which these compensatory effects may take place. Another limitation is that, for having a full ko model, we cannot confirm the origin of the CBG which is involved in the phenotypes we observe. The main source of systemic CBG is the liver. It is known that this protein does not cross the blood brain barrier, and as CBG mRNA and protein expression have been detected in brain cells (despite in very low amounts), we cannot exclude the possibility of a local action of brain CBG modulating especially the behavioral traits observed in this study. To address this matter, our team has developed a brain specific *Cbg* ko (brain *Cbg* ko) by crossing mice carrying the *Cbg* gene *locus* floxed by LoxP sites with transgenic mice expressing Cre recombinase under the regulation of calcium/calmodulin-dependent kinase II (CaMKII), an enzyme mainly expressed in the neurons of the forebrain. Preliminary studies have evaluated despair-like behavior and memory function in these mice, besides total and free corticosterone measures, and to this date no difference between the brain *Cbg* ko and our wild-type mice were observed. This indicates that the behavioral effects of CBG deficiency are likely to stem from the reduced access of free glucocorticoids in the brain caused by the absence of liver-produced CBG.

An interesting perspective for these studies would be rescuing the “normal” phenotype in the *Cbg* ko mice. A few strategies could be used for that purpose. A first one would be the purification of CBG (Berdusco et al., 1993) from mice blood and further administration of this protein in *Cbg* ko mice. Some difficulties in this approach are obtaining the protein – once a great amount of blood is needed and the blood volume in each mouse is of only 2.0 ml in average (Riches et al., 1973). Furthermore,

the delivery routine should consider not only the desired plasma concentration of this protein, but also its half-life (which is known to be of 14.5 hours in rats, to be determined in mice). Finally, for an intravenous infusion, mice should either be immobilized for a venous puncture at least once a day or remain cannulated throughout the duration of the experiments, which could configure a source of stress and possibly compromise the behavioral analyses. Another approach could involve the liver-directed transfer of the Cbg gene to the Cbg ko mice through a viral gene delivery system, such as the adeno-associated viral (AAV) vectors. AAV vectors are highly efficient for liver-targeted gene delivery in murine models, which is very convenient for our model once the liver is the main expression site of CBG. Depending on the dose and on the type of AAV employed, this technique can transduce up to 90-95% of hepatocytes in the mouse liver following a single intraportal vein injection (Sands, 2011, Dane et al., 2013). This approach could restore the phenotype of Cbg ko mice in a more endogenous way, since all steps from transcription to glycosylation would be performed by the mouse itself. Moreover, it would reduce the delivery-associated stress, once after the infection the expression is maintained for several months. Finding out whether the glucocorticoid hyporesponse of Cbg ko mice could be rescued by these approaches could pave the way for more ambitious studies aiming at restoring CBG function in patients with CBG deficiency or carrying mutations in the Cbg gene.

Conclusions

The deleterious consequences of HPA axis hyperactivity and glucocorticoid excess have been exhaustively described in the literature, while much less attention has been given to HPA axis hypoactivity and glucocorticoid deficiency. This thesis has characterized an interesting model of glucocorticoid hyporesponse due to CBG deficiency, the Cbg ko mice. Besides representing the main feature in Addison's disease, hypocortisolism in humans has also been reported in a series of disorders, such as irritable bowel syndrome, burnout syndrome, fibromyalgia, chronic fatigue syndrome, post-traumatic stress disorder, and atypical depression (Fries, 2008). Many of these conditions are becoming increasingly frequent, and often present with alterations in emotional and cognitive functions.

We showed in this thesis that the lower corticosterone levels observed in CBG deficient mice is caused by increased clearance of corticosterone from the plasma of these mice. We have also shown that Cbg ko mice present increased despair-like behavior, which is masked in female mice by their endogenous estrogens. Furthermore, we evidenced that CBG deficiency leads to a lower sensitivity to chronic stress, reflected in no changes in behavioral or corticosterone levels after chronic stress. Long-term memory impairment was also observed in Cbg ko mice, notably in tasks that depend on the hippocampus. Finally, we have evidenced altered glucose metabolism in CBG deficient animals when under extreme physiological situations (hyperlipidic diet associated to chronic stress).

These findings reinforce the important role of CBG influencing the broad range of actions of glucocorticoids by modulating their levels and availability. Together with previous data from our team and from others, our results highlight the importance of also evaluating CBG levels (and, complementarily, free glucocorticoid levels) when investigating alterations in glucocorticoid-influenced traits.

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