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Molecular mechanisms and individual vulnerability in an animal model (*Mus musculus*) of obesity induced by chronic social stress

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to Zofia Zukowska

my American mother, who thought me to dream big

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Part I

Chapter 1

Introduction

In today's society, the complex biological machinery presiding over energy balance is facing multiple challenges. Amongst the elements that may contribute to positive energy balance and to the development of metabolic diseases western diet, sedentary lifestyle, and environmental stress can be included. The link between chronic stress and energy homeostasis has been convincingly demonstrated in various preclinical models, clinical population and epidemiological studies. For example the Finnish twin study has identified a group of monozygotic twins showing divergent BMI and obesity around puberty [Pietilainen et al., 2004,Pietilainen et al., 2008]. A follow-up study determined that the obese co-twin showed increased neuroendocrine markers of stress (increased urinary cortisol and catecholamines) as well as increased emotional reactivity [Marniemi et al., 2002]. Overall the obese co-twin seems to be "more stressed" than the lean co-twin. In line with this finding, psychosocial and socio-economic challenges such as low income, low education, unemployment and divorce have been associated with neuroendocrine-autonomic dysregulation followed by visceral obesity and associated risk factors for disease [Rosmond and Bjorntorp, 2000a]. Chronic work stress, for example, correlates with obesity in humans and can double the risk of metabolic syndrome [Branth et al., 2007]. Recent meta-analyses have revealed that a plethora of emotional stressors can be found in association with increased risk of obesity and type 2 diabetes (T2D) [Mooy et al., 2000, Raikkonen et al., 2007]. The Whitehall II study clearly established a link between lifetime stress exposure and the development of the Metabolic Syndrome (MetS) and insulin resistance [Chandola et al., 2006]. Other important studies such as the National Health and Nutrition Examination Survey and the MacArthur Studies of Successful Aging, also clearly established a connection between individual socioeconomic status health and mortality. Interestingly, the effect of (objective or subjective) socioeconomic status is particularly relevant for obesity, type-2-diabetes (T2D) and MetS [McEwen and Mirsky, 2002, Mackenbach et al., 1997]. Moreover, stress has been associated to overeating in both men and women [Van Strien et al., 1986, Greeno and Wing, 1994]; notably, stress-driven eaters typically prefer food rich in fats and high in palatability [Laitinen et al., 2002, Dallman et al., 2005].

The impact of stress on metabolic function is well documented in primate models (see [Shively et al., 2009] for review). For example, both in captivity and the wild Cynomolgus monkeys (*Macaca fascicularis*) are organized into linear social hierarchies [Shively et al., 2009,Shively and Willard, 2012]. Subordinate monkeys have higher basal cortisol levels and have higher heart rates in response to challenge than dominants. Moreover a relationship between social subordination, fat distribution, and associated metabolic characteristics has been demonstrated [Shively et al., 2009]. Hyperglycemia and associated cen-

tral fat redistribution in subordinate monkeys is reminiscent of MetS in obese humans, linking social subordination to an overall positive energy balance outcome [Shively et al., 2009]. Subordinate females also present evidences of glucocorticoid receptor downregulation in central areas which modulates the Hypothalamus-Pituitary-Adrenocortical (HPA)-axis response to peripheral negative feedback and promotes visceral obesity [Shively et al., 2009]. In turn the metabolic characteristics of visceral obesity (e.g. hyperinsulinemia, leptin, free fatty acids, proinflammatory cytokines, etc.) may activate the HPA-axis response by enhancing sympathetic drive. It is quite likely that these responses to social stress are shared by many primate species [Kaufman et al., 2005, Kavanagh et al., 2007, Wilson et al., 2008, Tardif et al., 2009]. Similar stress-induced behavioral, physiological, and neurochemical alterations have been reported in subordinates tree shrews (*Tupaia belangeri*), a solitary species regarded as intermediate between insectivores and primates [Fuchs, 2005]. However, chronic stress in subordinate tree shrews results in weight loss that has been attributed to stress-induced enhancement of metabolic activity and to a lesser extent to reduced food intake [Fuchs and Flugge, 2002]. This dichotomy is not likely a species-related effect. In fact, despite the majority of human studies point to a positive effect of stress on energy balance (see above), it is also well established that stress affects eating, metabolism and energy balance in a bidirectional way; in humans some subjects decrease while others increase food intake and body weight [Stone and Brownell, 1994, Epel et al., 2004, Lo Sauro et al., 2008].

As it will be detailed in the rest of the review the same dicotomic effect of stress on rodent metabolism and energy balance is well documented. How is possible to reconcile these opposite outcomes of stress? And which are the underlying molecular mechanisms?

A first major challenge is due to the varying conceptualization of stress and how it's differently applied between studies (see below). Additionally, given the procedural differences used to model the consequences of stress, it's not surprising that the direction and the magnitude of the metabolic alteration are far from being unambiguous. Factors such as intensity of the stressor, duration of the exposure/recovery, potential for habituation to the stressor itself, diet, animal species, strain, and sex, are amongst the most crucial contributors to the final metabolic outcome.

1.1 Stress and the Physiological response to Stress

A major difficulty in the study of stress and stress response is the varying definition which has been proposed and used operationally by scientists [McEwen, 1998, Sapolsky et al., 2000, Pacak and Palkovits, 2001, Koolhaas et al., 2011]. The problem of having so many different scientific and popular definitions of stress has recently been discussed and the revised definition of stress that has been proposed will be used in the present review [Koolhaas et al., 2011].

> We suggest that the term *"stress"* should be restricted to conditions where an environmental demand exceeds the natural regulatory capacity of an organism, such as situations that include unpredictability and uncontrollability.

In this view, the physiological reactions that are a prerequisite of any behavior (challenge to homeostasis) should not be called "stress" nor should arousal or adaptation to changing environmental conditions within the regulatory range of a species (i.e., housing at 'cold' and 'hot' temperature that are within the regulatory range) be used as synonymous of "stress".

Several metabolically active hormones stimulate or are involved in the physiological stress response [Sapolsky et al., 2000, Ulrich-Lai and Herman, 2009, Dallman et al., 2003].
The two major components of the stress response are the sympatho-adrenal system (SAS) and the hypothalamic-pituitary-adrenal (HPA) axis.

The central components of the HPA axis are located in the hypothalamus and the brain stem neurons of the hypothalamic paraventricular nucleus (PVN) and in the locus coeruleus-norerpinephrine system (LC-NE) for the sympathetic nervous system (SNS) [Herman et al., 2003]. Under stress the SAS is activated to create quick compensatory changes in homeostasis promoting the "fight or flight" response [Cannon, 1929, Jansen et al., 1995] allowing for a rapid regulation of vital function. Sympathetic nerve terminals originating from the preganglionic fibers innervate peripheral organs where they release norepinephrine (NE), while the hormonal response of the SNS is given by the release of mostly epinephrine (EPI) and some NE, from the cromaffin cells of the adrenal medulla (Figure 1.1).

The HPA axis plays a fundamental role following homeostatic challenge and is ultimately responsible for controlling virtually all stress hormones (Figure 1.2). Specifically, neurons within the parvoventricular nucleus of

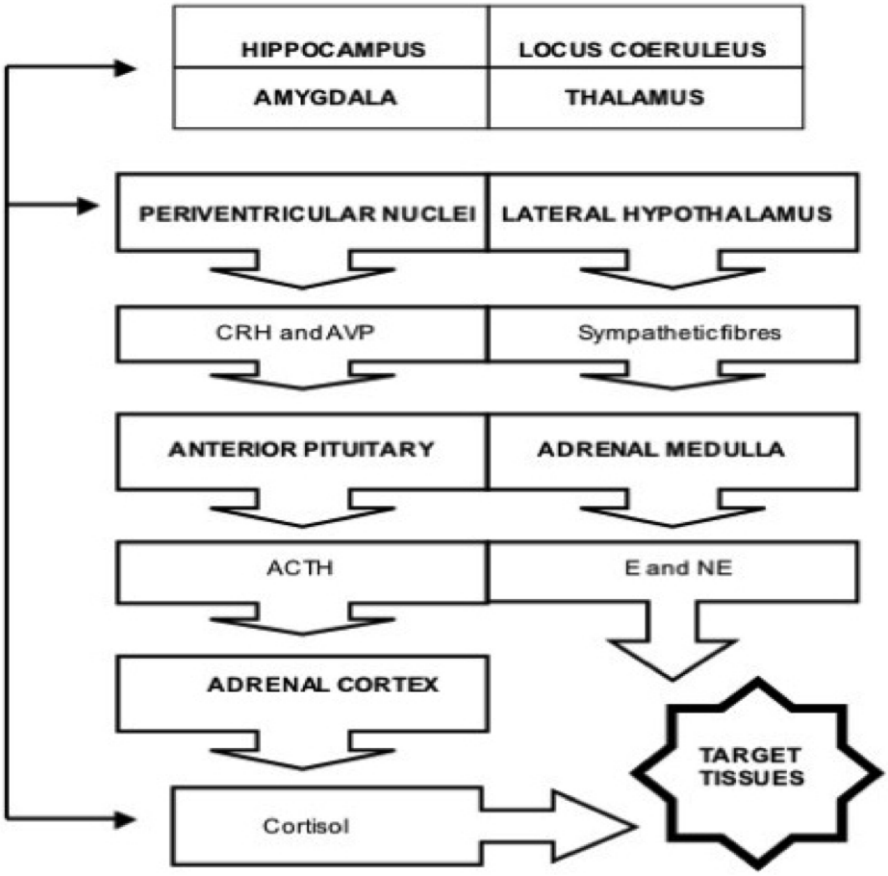


Figure 1.1: Schematic representation of the stress system and response

the hypothalamus (PVN) synthetize and release parvocellular corticotropin-releasing hormone (CHR) and arginine-vasopressin (AVP) hormone into the hypophyseal portal system in a precise circadian rhythm. These hormones reach the anterior pituitary gland and stimulate the secretion of adrenocorticotrophic hormone (ACTH) which in turn ACTH stimulates the adrenal cortex to secrete glucocorticoids, cortisol in primates and corticosterone in rodents, the main effectors of the HPA axis [Herman et al., 1996].
Glucocorticoids control the basal activity of the HPA axis by operating a direct negative feedback mechanism by binding to its own receptors, glucocorticoids receptors (GRs), distributed on the hypothalamus and pituitary. Through genomic and non-genomic mechanism glucocorticoids are able to modify key regulator proteins suppressing the release of CRH, limiting the release of ACTH and further glucocorticoids secretion [Keller-Wood and Dallman, 1984, Dallman et al., 1987] (Figure 1.3).Moreover glucocorticoids appears to either directly or indirectly regulate genes involved in vital metabolic

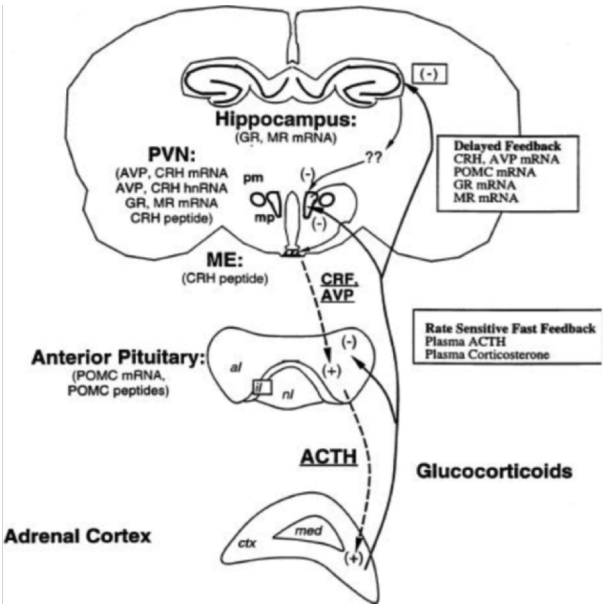


Figure 1.2: Activation of the HPA following stress

pathways, such as the up-regulation of lipoprotein receptors, enzymes involved in glucose, lipid and amino acid metabolism and suppression of CRH, POMC, adiponectin, TNF-α and interleukin-6 and 8 [Bamberger et al., 1996, Dostert and Heinzel, 2004]. Overall while glucocorticoid might exert a pro-adipogenic role (particularly in visceral fat), catecholamines are largely responsible for a pro-lipolytic role via activation of βAR (adrenergic receptors). Therefore one of major unresolved issues in stress physiology is to identify why under certain conditions adipogenic-promoting or lipolytic-promoting mechanisms will prevail.

1.2 Response to Acute and Chronic Stress

During the "fight or flight" response that occurs following acute stress, glucocorticoids act on carbohydrates and proteins metabolisms exerting primarily catabolic effects. Within the liver glucocorticoids promotes gluconeogenesis by promoting conversion of stored hepatic glycogen into glucose that is poured into the blood stream increasing significantly plasma concentration of glucose [Cherrington, 1999]. Protein degradation in muscle and bone are also promoted so that amino acids are available as substrates for oxidative pathways. Through these mechanisms glucocorticoids are able to provide quickly endogenous metabolic fuel necessary for the body to respond to stress.

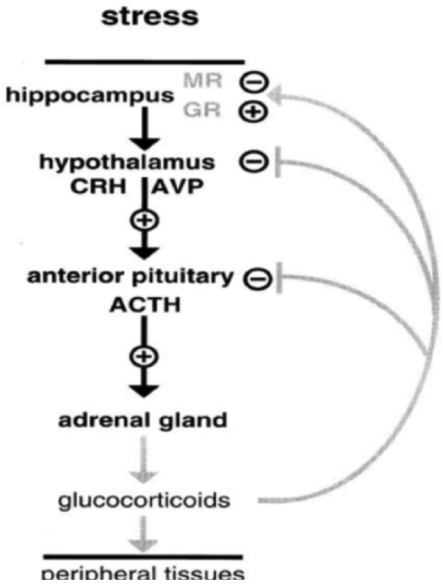


Figure 1.3: Glucocorticoids negative feedback mechanism

The energy stores are then replenished by exogenous energy such as food and nutrients [Cherrington, 1999]. On the other hand the SNS is able to redirect blood flow to threatened areas; indeed it increases perfusion to the aroused brain increasing vigilance, to muscle tissue increasing muscle tone, to the lungs increasing pulmonary ventilation, and to the heart increasing number and strength of heart beats [Sapolsky et al., 2000]. During the stress response energy is diverted from housekeeping activates such as reproduction, growth, food ingestion and digestion, mainly to the brain and muscle tissue to preserve life [Peters et al., 2011]. Together these systems provide us with the required energy to respond to the stress stimulus. This neuroendocrine system plays a vital role in preparing our body to the best conditions to either fight or flight and help our organism to adapt to the homeostatic challenge [Mravec et al., 2008]. Acute stress response is correlated to the increase in plasma cortisol/corticosterone, EPI, glucose concentration, increased adrenal gland weight and proinflammatory cytokine activity; these are used as stress indicators in many preclinical stress models. This response is transient allowing the individual better survival, indeed following a stressful stimulus these adaptive systems are able to efficiently turn off brining the organism to its original equilibrium state [Jankord and Herman, 2008].

In circumstances where the stimulus is prolonged and poorly regulated the system is loaded or overloaded and consequently the stress response is deregulated becoming maladaptive with potential harmful consequences for

our organism. During chronic stress HPA axis is hyperactivated and the feedback mechanism of glucocorticoids is impaired resulting in constant high levels of glucocorticoids. Chronic or prolonged stress does not allow the body to fully recover causing a deregulated stress response that results in difficult maintenance of biochemical balance and as a consequence detrimental effects and pathophysiology arise [Sapolsky, 2005, Selye, 1984].

1.3 Energy balance and metabolic disorders

The physiologic regulation of the body metabolic function is a complex phenomenon that relies on the communication between brain, gut and adipose tissue. To maintain energy homeostasis, the brain tightly monitors the peripheral energy state on the basis of two major groups of metabolic inputs: short term signals produced by the gut system and the autonomic nervous system and long term signals produced by gut adipose tissue, liver, muscle, etc. After central integration of these inputs, the brain generates neuronal and hormonal outputs to balance energy intake with expenditure. Miscommunication between gut, brain, and adipose tissue, or the degradation of input signals once inside the brain may lead to increased energy intake and production, eventually causing metabolic disorders. The efficient maintenance of the delicate homeostatic balance of energy, glucose, and lipid metabolism largely depends on systemic metabolic processes that are centrally regulated. The hypothalamus is a central integrator of metabolic information, with nuclei such as ventral medial hypothalamus (VMH) and arcuate nucleus (Arc) expressing high levels of receptors for adipokines (i. e., cell signaling molecules secreted by adipose tissue), gut and pancreatic hormones [Schwartz et al., 2000, Yi and Tschop, 2012]. In the hindbrain, the nucleus tractus solitarius (NTS) is the detector of metabolic feedback, especially from the gastrointestinal system via vagal afferents or the circulation [Berthoud et al., 2006]. The gut is in charge of energy intake: nutrient digestion and absorption trigger the secretion of gut satiety signals such as colecystokinin, peptide Y (PYY), and glucagon like peptide-1 (GLP1) for stimulation of vagal sensory nerves to provide feedback to the brain [Berthoud, 2008, Yi and Tschop, 2012]. Vagal sensory terminals also express receptors for other gut hormones and metabolically active peptides. Additionally, gut hormones and nutrients can have central effects acting on cirumventricular organs [Cottrell and Ferguson, 2004]. These brain areas then relay the information to other key regulatory areas, such as the PVN (to maintain systemic metabolic homeostasis by modulating neuroendocrine and autonomic outputs) and the suprachiasmatic nucleus (SCN) (to synchronize the behavior and physiology of the autonomic

outflow). Malfunctioning at the central level can result in abnormal elevation of sympathetic outflow. On the other hand, defective or weakened signal feedback by the brain in response to satiety and nutrients from the gut can cause overfeeding and disinhibition of liver glucose production, and thereby promote metabolic disease [Berthoud, 2002]. The fact that stress can lead to under- and over- eating exemplifies the "stress-eating paradox" [O'Connor et al., 2008, Serlachius et al., 2007, Coccurello et al., 2009]. Highly palatable food has properties that promote dependence; palatable food can in fact activate the brain reward system, comprising opioid, dopamine, and endocannabinoid acting via both fast sensory inputs as well as slower post-ingestive processes, such as increased blood glucose, adiposity, and possibly gut signals [Coccurello et al., 2009, Dallman et al., 2003]. Repeated stimulation of the central reward pathways through highly palatable food may lead to neurobiological adaptations that eventually increase the compulsive nature of overeating characterized by frequent drive to initiate eating [Coccurello et al., 2009].
It must be noted that the activation of the HPA axis elicits — among other neurotransmitter systems — the release of endogenous opioids. Opioids decrease activity of the HPA axis on different levels. Opioid release increases palatable food intake and palatable food sustains opioid release (see [Cota et al., 2006] for a review on food and reward). Consumption of palatable foods clearly reduces CRH mRNA expression in the PVN, the central drive of the HPA axis [Pecoraro et al., 2004]. Similarly daily limited access to sucrose or saccharin solutions also results in reduced PVN CRH mRNA expression [Ulrich-Lai et al., 2007].

Obesity can be defined as excessive body weight and adiposity to such an extent to damage health increasing the likelihood of life threatening conditions such as hypertension, stroke, T2D, cardiovascular disease, and cancer [Danaei et al., 2009]. Adipose depot size is a result of the balance between actions which promote lipid accumulation vs. those that promote lipolysis [Thompson et al., 2010, Cinti, 2012]. Energy balance is also dependent upon signals to the brain from the white adipose tissue via sensory nerves [Bartness et al., 2010a, Murphy et al., 2013]. Adipose tissue produces adipokines that inform the brain about whole-body long-term energy storage status, and drives the brain's control of energy balance and the long-term regulation of body weight. To date, several adipokines have been identified. Leptin, is sensed by the central nervous system (CNS) and sensory neurons in the white adipose tissuefor feeding and energy expenditure regulation [Halaas et al., 1995, Maffei et al., 1995, Murphy et al., 2013]. Central leptin resistance is caused by defective leptin sensing in these brain regions [Gautron and Elmquist,

2011]. Generally, leptin resistance can cause the brain to misunderstand long term energy stores and, as a result, initiate extra energy production, i.e. overfeeding (hyperphagia) and glucose production (hyperglycemia), both typical symptoms of metabolic disorders. The dominant autonomic-white adipose tissue connections are neuronal sympathetic outputs that initiate lipid mobilization in white adipose tissue [Foster et al., 2010]. Loss of autonomic control from the PVN can increase the accumulation of lipids in fat depots without influencing lipid mobilization, suggesting that obesity could be caused by excess energy deposition owing to malfunction of the sympathetic autonomic nervous system. Uncoupling protein 1 (UCP1) is a mitochondrial protein mainly expressed in brown adipose tissue with the primary function of dissipating chemical energy to form heat through the process of non-shivering thermogenesis [Cannon and Nedergaard, 2004]. The activation of UCP1 by NE and subsequent signaling cAMP-PKA-p38 pathway has been well established and leads to increases of energy expenditure that is crucial to the regulation of whole body energy and metabolism [Cannon and Nedergaard, 2004, Collins et al., 2004]. The UCP1 gene is regulated by the sympathetic nervous system and its transcription is stimulated by NE released from sympathetic nerves innervating brown adipose tissue; this induces the increase in heat production and therefore increases energy expenditure as seen in response to cold exposure [Cassard-Doulcier et al., 1993, Lowell and Bachman, 2003].

1.3.1 Pro-adipogenic stress-mediators:

Prolonged stress causes the onset of a pathological condition due to the dysregulation of the HPA and SAM, respectively, with excessive secretion of glucocorticoids and catecholamines. Cortisol, secreted with a specific circadian rhythm, peaks during the night in rodents while early morning in humans [Dallman et al., 1987]. Under constant stimulation of the HPA aixs the circadian rhythmicity of cortisol secretion is lost causing constant high levels. The long-term effects of glucocorticoids on adipocyte metabolic processes are thought to promote visceral obesity [Bjorntorp, 2001, Black, 2006].

Hypercortisolemia is highly influenced on the sensitivity of the peripheral tissue to glucocorticoids, which depend on the concentration, and activity of glucocorticoid receptors (GRs) and local expression of the reductase 11-β-hydroxysteriod dehydrogenese type I the enzyme responsible of the conversion of inactive cortisone to cortisol (11β-HSD1). Hypercortisolemia induces down-regulation and less sensitive of GRs [Bjorntorp, 2001, McEwen, 1998]. Intrabdominal or visceral fat have the highest density of GRs com-

pared to other fat depots, consequently less sensitive GRs result in excessive visceral fat accumulation [Bjorntorp, 1996]. Moreover, cortisol increases the expression and activity of lipoprotein lipase (LPL), the main enzyme responsible for fatty acid uptake and storage of triglycerides promoting lipid accumulation and storage. Cortisol enhances the activity of 11β-HSD1 that is highly present in adipocytes and muscle cells contributing to the development of central obesity and metabolic syndrome in stressed subjects [Lundgren et al., 2008, Ottosson et al., 1994]. The main peripheral metabolic effects of hypercortisolemia are insulin resistance, insulin-dependent glucose uptake and enhanced gluconeogenesis in liver. These effects of cortisol are due to direct or indirect effects on the insulin receptor substrates (IRS) 1 and 2, and glucose transporter, mainly GLUT4 expressed in skeletal muscle cells and adipocytes. High glucocorticoids levels do reduce the transportation of GLUT 4 to the cell surface [Andrews and Walker, 1999]. In addition to the effects of hypercortisolemia on insulin sensitivity, glucocorticoids also inhibit insulin secretion from pancreatic β-cells [Delaunay et al., 1997]. High glucocorticoids levels do reduce the transportation of GLUT 4 to the cell surface. As a result of cortisol induced insulin stimulated glucose uptake, glucose metabolic clearance rate is reduced. Cortisol also stimulates the breakdown of stored triglycerides in the adipose tissue and as a result plasma free fatty acid levels are increased which inhibits the release of insulin from the pancreatic cells worsening insulin resistance and glucose intolerance.

Furthermore, it has been demonstrated that in presence of the anti-lipolytic hormone insulin, glucocorticoids increase craving for food-reach meals and that this may lead to a metabolic derangement leading to increased abdominal fat [Rebuffe-Scrive et al., 1992, Pecoraro et al., 2004, Dallman et al., 2003, Dallman et al., 2004, Dallman et al., 2005, Dallman, 2010, la Fleur et al., 2004]. Beside glucocorticoids and insulin other stress-mediators have a pro-adipogenic role. Obesity, particularly visceral obesity, is now recognized as a systemic low-grade inflammatory state [Hotamisligil et al., 1993, Chawla et al., 2011]. These fat borne proinflammatory cytokines sustain a hypersecretory HPA axis, which in turn promotes visceral fat accumulation. Thus the relationship between stress and visceral obesity may be bidirectional and self-sustaining [Black, 2006, Kyrou and Tsigos, 2007, Beasley et al., 2009]. Finally, sympathetic-derived neuropeptide Y (NPY), induces a pro-adipogenic and pro-angiogenic programming under specific stress conditions and in presence of high fat diet by binding Y2R in adipocytes membranes (results will be discussed in detail below) [Kuo et al., 2008, Kuo et al., 2007].

1.3.2 Pro-lipolytic effect of stress-mediators:

Catecholamines are the major pro-lipolytic and thermogenic factors in mammals via activation of βadrenergic receptors βARs) [Lowell and Spiegelman, 2000, Bachman et al., 2002]. The genetic ablation of the three known βARs in the so called β-less mice led to morphological abnormalities in the brown adipose tissue, defective adaptive thermogenesis, obesity and glucose intolerance [Bachman et al., 2002, Asensio et al., 2005]. In addition other mediators released under certain stress conditions such as TSH and T3/T4, nutrietic peptides, granins and many others have been shown to increase lipolysis (e.g. [Liu and Brent, 2010, Bordicchia et al., 2012]. Overall, increased lipolysis and increased circulating free fatty acids can in turn promote glucose intolerance, hyperinsulinemia, and dyslipidemia by influencing hepatic function [Arner, 1997].

1.4 How does stress affect energy balance?

Simply stated, energy homeostasis reflects the balance between energy input to the system (nutrients) and the energy output (energy expenditure divided into obligatory and adaptive thermogenesis) [Lowell and Spiegelman, 2000]. A negative energy balance, defined as loss of body weight or fat mass, can occur in presence of decreased food intake and/or increased energy expenditure. Viceversa a positive energy balance can occur in presence of increase food intake and unchanged or reduced energy expenditure. Because of the physiological systems evolved to maintain a strict control over energy balance, a sustained weight loss or weight gain usually leads to compensatory responses in the opposite direction. Alternatively in an allostatic (non-homeostatic) perspective [McEwen, 1998], the system might adapt to a different set-point which maintains stability through changes.

In an attempt to shed light on the mechanisms of stress-induced metabolic disorders we will discuss animal models of stress-induced metabolic disorders using an energy balance perspective and a two tiers classification. *The first tier* is the nature of stress: social or non-social (physical, psychological) stress models. *The second tier* is the net positive/negative energy balance (as measured from pre-stress condition and, in rare instances, compared to controls): positive balance defined as increased body weight or fat mass; negative balance defined as loss of weight or fat mass. For each model we will describe first changes in food intake and energy expenditure and then we will summarize changes in neuroendocrine (e.g. HPA-axis produced hormones) or biochemical (e.g. insulin, leptin, glucose, etc.) markers. The metabolic effect

of "recovery" after stress and its molecular mechanisms will be discussed in separate sections.

As far as the nature of the stress, laboratory stressors can be broadly defined as social or physical/psychological (non-social). Many experimental stressors have an acute psychological stress aspect but are primarily physical in nature. Since the likelihood for animals (and humans) to encounter stressors such as restraint, immobilization, or foot shock in nature is very low, these models cannot be considered ethologically relevant. They may trigger different coping responses such as behavioral, physiological, or neurochemical, with limited validity to natural and ethologically relevant conditions [Bartolomucci, 2007, Koolhaas et al., 2011]. Nevertheless, the studies based on non-social stressors have offered insights into the biology of the stress responses and can be considered valid experimental tools to address human coping responses with traumatic events such as traumatic events or natural disasters [Tamashiro et al., 2004].

A shared feature of all models reviewed in this chapter is that the stressors, whatever their nature, are applied over time (i.e., chronically, repeatedly, intermittently). Acute stress-models will not be discussed because of uncertain effect on long term energy balance.

1.5 Animal models of chronic stress and their impact on energy balance

1.5.1 Physical and psychological (non-social) chronic stress models

1.5.1.1 Mild chronic pain models - mild tail pinch, foot shock

Following Antelman and colleagues observations that mild tail pinch could reliably induce a syndrome of gnawing and eating behavior in rats, mild pain has been proposed as a tool to produce hyperphagia [Antelman et al., 1975, Rowland and Antelman, 1976]. Most studies have addressed the short term mechanisms responsible for the acute hyperphagic response [Rowland and Antelman, 1976, Robbins et al., 1977, Nemeroff et al., 1978, Morley and Levine, 1980]. The only study describing a long term effect of chronic application of this stressor showed an overall negative energy balance, a decrease in body weight gain with unaffected food intake [Levine and Morley, 1981].

The mild chronic pain model based on electric foot shock is often associ-

ated with caloric restriction (although only results obtained in ad *libitum* fed animals are included in this review) [Artiga et al., 2007,Fu et al., 2010,Ortolani et al., 2011]. This model has validity for human eating behavior disorders, primarily binge eating. Similarly to tail pinch, mild chronic pain induces a body weight loss without any change in food intake. Nevertheless, in combination with high-fat and high-sucrose diet, it can produce a synergistic effect aggravating insulin resistance associated with the elevation of serum NEFAs and the activation of the HPA axis. Other consequences of this procedure are increased TNF-α in the serum and adipose tissue, decreased density of high-affinity receptors and expression of PPARα mRNA in hepatocites, as well as nonalcoholic fatty liver disease, considered to belong to the hepatic manifestation of the MetS [Fu et al., 2010, Fu et al., 2009].

1.5.1.2 Thermal models - cold and heat stress

Housing mice at temperatures lower than the standard housing in the laboratory induces increased food intake and thermogenesis, decrease in weight and fat mass (e.g. [Lowell and Spiegelman, 2000, Cannon and Nedergaard, 2004, Ochi et al., 2008, Ukropec et al., 2008, Zhao et al., 2010]. Mice perceive commonly housing temperatures ($\sim 21^\circ C$) as cold, given that the mouse thermoneutral zone is at $27^\circ \sim 30^\circ C$. Indeed cold stimulates the expression of thermoregulatory genes in both brown and white adipose tissue [Trayhurn et al., 1995, Cinti et al., 2002]. After chronic cold exposure the brown adipose tissue is hypertrophic and presents increased UCP1 mRNA and protein levels [Cinti et al., 2002]. Intuitively cold increases caloric intake to match the increased energetic demand targeting a sustainable homeothermic state; at the same time the cold challenge increases corticosterone levels, and in rats it has been associated to an overall negative energy balance and to a thermogenic switch [Akana et al., 1999]. However based on our definition of stress (see above) exposure to a different ambient temperature within the regulatory range of a species should be considered an adaptive response to a challenge to homeostasis and not a stressor. A more subtle and intermittent cold stress model was developed by Kuo et al by exposing mice daily to ice cold water for several weeks [Kuo et al., 2007]. Under these conditions, mice developed increased visceral fat mass without any change in body weight and food intake while energy expenditure was not assessed [Kuo et al., 2007]. Other studies demonstrated increased inflammation, hyperlipidemia, increased levels of ACTH, hepatic steatosis, and atherosclerosis [Kuo et al., 2007, Li et al., 2011, Han et al., 2012, Najafi et al., 2013]. Most of these metabolic alterations are considered to be linked to a stress-induced facilitated release of NPY and expression of Y2 receptors (Y2Rs) in visceral fat [Kuo et al., 2008]. As it will

be discussed later on, NPY is an adrenergic cotransmitter and a major stress mediator, preferentially released from the sympathetic nerves by intense and prolonged stressors [Zukowska-Grojec, 1995].

At the opposite end of the thermal spectrum, chronic heat treatment is a well-known physical stressor [Bhusari et al., 2008]. Experimental models of chronic heat stress (mice house above thermoneutrality, i.e. $\sim 35^{\circ} C$) are associated to negative energy balance due to decreased body weight and food consumption, and increased water intake and rectal temperature [Morera et al., 2012]. At the hormonal level heat treatment significantly increases both leptin and adiponectin secretion, as well as their receptors, and up-regulates insulin receptor substrate-1 and glucose transporter mRNAs [Morera et al., 2012]. Nevertheless the molecular mechanisms by which heat stress regulates the expression and secretion of adipokines remain largely unknown, although these changes could be considered an acclimatization of homeothermic animals to heat, i.e. a way to increase avenues of heat loss and reduce heat production in an attempt to remain euthermic.

1.5.1.3 Chronic mild stress models: Chronic mild stress, chronic variable stress, etc.

Chronic mild stress was developed to decrease responsiveness to rewards through a variety of behavioral paradigms with the ultimate goal of modeling crucial symptoms of human depression. As stated in a review by Willner [Willner, 1997], the designation of the procedure as chronic mild stress indicates

(i) that the behavioral changes induced may be observed over a period of several weeks of continuous stress administration

(ii) that habituation either does not occur, or occurs to only a limited extent

(iii) that the individual stressors used do not include any of the severely stressful elements such as intense foot shock, prolonged food/water deprivation, etc.

Based on this concept, several procedures based on the chronic application of different mild intensity stressors have being developed and referred quite interchangeably as chronic mild or variable stress. Notwithstanding the lack of standardization in the application of these procedures, they will be treated altogether for the purposes of the present discussion and will be referred to as chronic mild stress. In a typical experiment, rats or mice are exposed

sequentially to a variety of mild stressors (e.g. overnight illumination; periods of food and/or water deprivation; cage tilt; change of cage mate), which change every few hours over a period of weeks or months [Willner, 1997, Willner et al., 1992, Monleon et al., 1995]. Chronic mild stress induces lasting effects on HPA axis regulation and future response to stress [Jankord and Herman, 2008, Flak et al., 2009, Ostrander et al., 2009]. From a metabolic standpoint, chronic mild stress establishes a negative energy balance, mainly including reduced food intake, body weight gain, and adiposity [Levin et al., 2000, Li et al., 2010, Solomon et al., 2010, Flak et al., 2011, Paternain et al., 2011]. The only exception is a study where rats were exposed to restraint and cage rotations on alternate days. In this case, without any effect on fat mass and body weight (food intake and energy expenditure were not assessed) there was an increase in mesenteric fat pad weight associated with high corticosterone [Rebuffe-Scrive et al., 1992]. Studies associating chronic mild stress with high fat diet have shown profound metabolic alterations both in rats and in mice suggesting that prior stress exposure has long term consequences for metabolic regulation [Lin et al., 2005, Teegarden and Bale, 2008, Li et al., 2010, Liu et al., 2012, Castaneda et al., 2011, Manting et al., 2011]. When obesity-prone (C57BL6) and obesity-resistant (AJ) mice are subjected to chronic mild stress and high fat diet, the chronic stress is more catabolic than anabolic even when genes and environment are propitious to obesity [Michel et al., 2005]. Similar effects are induced by this stressor in males and females [Fachin et al., 2008, Solomon et al., 2011]. Chronic mild stress is associated to high plasmatic corticosterone and low leptin levels. In mice fed high fat diet chronic mild stress induces lower plasma adiponectin, free fatty acids, and glycerol paralleled by a lower glucose tolerance and decreased white tissue insulin sensitivity, increased lipogenesis, adipogenesis and adipocyte differentiation, and elevations of plasma resistin levels [Castaneda et al., 2011].

1.5.1.4 Restraint or immobilization

Restraint or immobilization stress models have been used extensively to study stress-related biological, biochemical, and physiological responses in animals [Kvetnansky and Mikulaj, 1970, Marti et al., 1997, Kasuga et al., 1999, Bhatia et al., 2011]. Originally developed induce classic signs of stress (i. e., adrenal hypertrophy and thymic involution) [Selye, 1984], nowadays it is the more commonly employed model for the induction of acute stress and for studying stress-induced neurodegeneration and post-traumatic disorders [Southwick et al., 1994, Kumari et al., 2007]. Restraint stress is induced by placing rats and mice in restrainers for different durations and with different schedules (once/twice day) over a number of days (consecutive or

not) [Marin et al., 2007, Kaur et al., 2010, Manchanda et al., 2011]; only multiple exposures to this stressor will be discussed in this chapter. Restraint stress is able to induce an overall negative energy balance, causing weight loss and metabolic alterations that might persist following the restoration of HPA alterations [Harris et al., 2002, Zardooz et al., 2006, Sweis et al., 2013]. The hypermetabolic syndrome caused by restraint stress exposure appears to be triggered by transiently increased energy expenditure and reduced food intake, both of which normalize at different rates depending on the severity of the stress induced neuroendocrine dysregulation and also on species, sex, strain, duration and time of stress application [Depke et al., 2008]. Habituation can occur during chronic restraint protocols, as most of the component of the HPA axis appear to normalize [Harris et al., 2002, Harris et al., 2006, Pecoraro et al., 2004, Zardooz et al., 2006, Depke et al., 2008, Macedo et al., 2012]. The decreased body weight is concurrent with an initial increase in corticosterone and decrease in leptin levels [Zardooz et al., 2006, Macedo et al., 2012]. Corticosterone levels are later normalized with a parallel change of plasma glucose fasting levels; however these changes are not necessarily mimicked by insulin the levels of which can remain decreased [Zardooz et al., 2006]. Restraint stress administered with a calorie-dense diet is associated with a blunted HPA response due to a reduction of hypothalamic mRNA expression and secretion of CRH and ACTH in presence of increased glucose and insulin levels [Pecoraro et al., 2004]. Despite a reduction of total caloric intake due to restraint stress, rats exposed to a choice between chow and palatable diet show increased consumption of the "comfort food". Nevertheless, rats exposed to restraint stress show a negative food efficiency when compared to unrestrained rats that can only partially reversed by comfort food. Finally and importantly the fat mass of control and stressed rats ingesting the comfort food is indistinguishable and higher than the adipose mass of rats fed a standard chow exposed or not to restraint stress [Pecoraro et al., 2004].

1.5.2 Chronic social stress models

Experimental models of social stress offer the best ethological approximation to the social factors that characterize animal natural life, as well as to human stressogenic situations [Sapolsky, 2005, Bartolomucci, 2007, Koolhaas et al., 2011]. Intuitively the relevance of experimentally manipulating social dynamics (social hierarchy formation, social group stability, social group numerosity, etc.) may be more or less adequate depending on the extent to which the animals studied live in social groups or in close proximity to other members of either their or different species. Similarly to non-human primates discussed in the introduction, social factors are also commonly used

as stressful interventions on laboratory rodents.

1.5.2.1 Social isolation, individual housing

The physiological and behavioral syndrome elicited by social isolation has been classically considered a model of stress-related pathologies [Valzelli, 1973]. Social isolation has been widely described as inducing numerous behavioral and neurochemical changes linked to HPA dysregulation, emotionality, and hypertension, although the nature and severity of these effects depend on age at isolation, species and strain, and specific testing conditions [Bartolomucci et al., 2003, Nonogaki et al., 2007]. Traditionally the effects of social isolation upon food intake and body weight have been investigated using two different approaches [Yamada et al., 2000]. In isolation rearing, isolation begins at weaning and body weight of isolated animals is found to be greater than socially reared controls, especially in rats [Morgan and Einon, 1975, Fiala et al., 1977]. The individual housing at a post weaning age generally leads to decreased body weight gain and food intake than group-housed mice fed standard diet [Goodrick, 1974, Meisel et al., 1990, Kabuki et al., 2009, Bartolomucci et al., 2003, Bartolomucci et al., 2009]. Although contrasting findings have also been reported Sakakibara [Sakakibara et al., 2012]. However when individually housed mice are fed a high fat diet they can show a paradoxical positive energy balance with increased body weight, fat mass and food intake [Bartolomucci et al., 2009]. Rats mainly react to social isolation with an overall positive energy balance, increasing their body weight and food intake [Morgan and Einon, 1975, Fiala et al., 1977, Sahakian et al., 1982, Scalera, 1992]. In general individual housing is not associated with marked activation of the HPA axis unless mice are expose to an heterotypical stressor, e.g. open field or restraint, in which case they show increased HPA activation when compared to group housed counterparts (See discussion in [Bartolomucci et al., 2003, Bartolomucci et al., 2009].

1.5.2.2 Unstable social settings

We group here a heterogeneous category of animal models that includes procedures based on the alternate application of social defeat/overcrowding/isolation/ group composition change/etc. Particularly interesting on this perspective are the studies on Syrian hamsters. Syrian hamsters are naturally solitary and highly territorial [Murphy, 1977]. For these species group housing represents a social stressor with an overall anabolic outcome [Borer et al., 1988, Meisel et al., 1990, Fritzsche et al., 2000]. Group-housed female Syrian hamsters increase their food intake, body and lipid mass compared with singly

housed hamsters [Borer et al., 1988, Meisel et al., 1990, Fritzsche et al., 2000]. Exacerbating the group housing stress through social crowding aggravatesthe deposition of excessive fat mass and the diminution of energy expenditure with consequent increase of body weight [Borer et al., 1988, Meisel et al., 1990]. On the contrary in mice the alternation of social defeat and overcrowding leads to a negative energy balance [Reber et al., 2006]. Independently from the diet available, in this procedure mice decrease body weight and food intake, in parallel to decrease of body fat and circulating leptin and increase in lean mass [Finger et al., 2011]. The availability of high fat diet contributes only to the amelioration of the behavioral sequelae of the social defeat/overcrowding procedure, with no impact on any of the metabolic parameters assessed. Furthermore, in the context of unstable social settings a shift in food choice from healthier to calorie-rich foods is observed without a notable effect on energy balance [Adam and Epel, 2007, Macht, 2008].

1.5.2.3 Visible Burrow System

The visible burrow system is a paradigm used to study dominance hierarchies in laboratory settings. Typically rats are housed in mixed-sex groups in a seminatural social environment consisting of tunnels and chambers. A male rat will become dominant, while the others will be subordinate; once formed the hierarchy remains stable, with subordinate rats not habituating to the stress of social subordination [Blanchard et al., 1995, McKittrick et al., 2000, Hardy et al., 2002, Nguyen et al., 2007, Tamashiro et al., 2007b]. The visible burrow system also elicits diverging metabolic phenotypes in dominant and subordinate animals. Subordinate male rats consistently lose $10\% - 15\%$ of their original body weight, whereas dominant males maintain their body weight. The weight loss in subordinate is attributed to loss of adipose and lean tissue. In contrast, dominant rats maintain their weight, but alter their body composition by losing adipose tissue and gaining lean body mass. Both dominant and subordinate rats are hypophagic while the dominance hierarchy is forming. Once the hierarchy is established, food intake of subordinate rats remains low whereas that of dominant rats returns to normal and this pattern persists for the remainder of the visible burrow system housing. The observed altered meal patterns suggests that signals normally controlling ingestive behaviors become impaired or overridden during social stress. In line with this phenotype subordinate rats develop lower leptin and insulin levels in the presence of high levels of corticosterone and low levels of testosterone, while dominant differ from control rats only in their lower leptin levels [Tamashiro et al., 2004, Tamashiro et al., 2007a].

1.5.2.4 Intermittent social defeat (Resident/Intruder procedure)

The resident/intruder procedure was developed manipulating rodent territorial disparity, by allowing brief confrontations between a resident and an intruder subject to study mechanisms that may contribute to affective and neuroendocrine disorders [Miczek and Tornatzky, 1996]. After displaying defeat, the intruder is protected from the potential injury of the resident's attack and returned to his home cage. Few episodes of social defeat can decrease weigh gain, food intake, and leptin levels in intruder animals along with increased corticosterone, ACTH and body temperature [Raab et al., 1986, Meerlo et al., 1996, Koolhaas et al., 1997, Ruis et al., 1999, Bhatnagar et al., 2006]. Increased food intake (although only during the light phase) in presence of decreased body weight has also been reported [Bhatnagar et al., 2006]. In mice the combination of this stressor with high fat diet regimen is able to induce an overall increase in adipose fat mass, but not body weight gain, in absence of any change in food intake. As will be described later this effect seems to be mediated by the NPY/Y2R system in the adipose tissue [Kuo et al., 2007]. In hamsters intermittent chronic social defeat increases weight gain, food intake, and adipose depots as well as plasma levels of leptin in absence of any change in corticosterone, ACTH, or insulin [Foster et al., 2006]. In this species a clear-cut difference between dominant and subordinate liability to weight gain, increased food intake, white adipose masses, and leptin serum levels has been also demonstrated [Solomon et al., 2007].

1.5.2.5 Chronic psychosocial stress, Sensory contact and Chronic defeat stress

The sensory contact (also known as chronic defeat stress) and the chronic psychosocial stress models are very similar and based on male mouse aggressive behavior. Two unfamiliar male mice are paired and allowed to aggressively interact for a short period of time daily; they are left thereafter in sensory contact allowed by a perforated partition in the housing cage. A dominant and a subordinate mouse can be identified by behavioral observations. The vast majority of the study focused so far on the metabolic consequences of the subordinate mice. Accordingly, unless otherwise stated we will refer to the phenotype of subordinate mice only. The sensory contact model was originally developed in C57BL/6J mice by Kudryavsteva and colleagues (review [Kudryavtseva et al., 1991, Kudryavtseva, 2000, Kudriavtseva, 2002, Kudryavtseva et al., 2010]) and later modified and popularized by Nestler's group [Berton et al., 2006, Tsankova et al., 2006, Krishnan et al., 2007, Lutter et al., 2008b], to induce behavioral changes relevant for human

major depression. In this paradigm, mice dyads are composed by an experienced aggressive mouse (CD1) and an unexperienced intruder mouse (usually C57BL6J) allowed to live in sensory contact and to interact on a daily base for several days/weeks. This configuration allows to induce and to maintain subordination through social defeat of the C57BL6J male mouse. The subordinate individual is moved to the cage of a different CD1 mouse on a daily basis. Sometimes susceptible vs. resilient mice are identified on the basis of a social avoidance behavioral test [Berton et al., 2006, Krishnan et al., 2007]. Recently more interest has been dedicated to the metabolic consequences of this model [Lutter et al., 2008a, Lutter et al., 2008b, Chuang et al., 2010a, Chuang et al., 2010b, Chuang et al., 2011, Kumar et al., 2013]. A decrease in body weight is consistently being reported during the stress experience [Chuang et al., 2010b, Krishnan et al., 2007, Rodriguez-Sureda et al., 2007], mostly in correspondence of increased corticosterone and ghrelin levels [Lutter et al., 2008b]. Interestingly only one study demonstrated stress-induced weight gain in the same model in a selection of susceptible individuals without any effect on food intake [Tsuneki et al., 2013]. These mice also showed glucose intolerance in the GTT but normal insulin, leptin and homeostatic model assessment of insulin resistance (HOMA-IR).

In the chronic psychosocial stress model stable dyads of male mice live chronically in sensory exposure and interact physically on a daily basis [Bartolomucci et al., 2001, Bartolomucci et al., 2004, Bartolomucci et al., 2005].

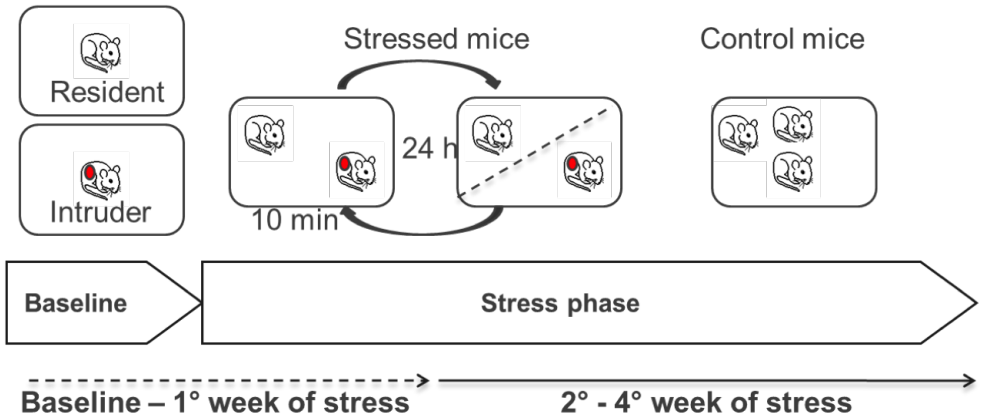


Figure 1.4: Chronic Psychosocial Stress protocol. modified [Bartolomucci et al., 2001]

The model was originally developed with CD1 mice but has been optimized to be used with several inbred and transgenic mice (e.g [Bartolomucci et al.,

2010,Dadomo et al., 2011]). As for the sensory contact model the outbred CD1 is the only reliable dominant. The model has been originally characterized as a model of depression and immune-endocrine changes ([Bartolomucci et al., 2005] for review). Subordinate mice show a clear positive energy balance with increased weight and fat mass [Bartolomucci et al., 2004, Bartolomucci et al., 2009, Sanghez et al., 2013]. What appears to be triggering the cascade of metabolic events leading to positive energy balance is increased food intake [Bartolomucci et al., 2004, Bartolomucci et al., 2009, Bartolomucci et al., 2010, Dadomo et al., 2011]. Furthermore subordinate mice have been shown to prefer kilocalories from fat when offered a choice between pure macronutrients [Moles et al., 2006] or to prefer standard vs. high fat diet [Patterson et al., 2013]. Positive energy balance is also associated with decreased energy output, i.e. low locomotor activity while energy expenditure is only marginally increased [Bartolomucci et al., 2003, Bartolomucci et al., 2009, Bartolomucci et al., 2010, Sanghez et al., 2013]. Subordinate mice show hyperactive HPA axis and when fed a high fat diet show hyperglycemia, hyperinsulinemia, lipid dysfunction, glucose intolerance and insulin resistance [Sanghez et al., 2013, Patterson et al., 2013]. Interestingly dominant mice display an overall negative energy balance (body weight loss and reduced fat mass) despite increased food intake which is due to increased energy expenditure and sympathetic hyperactivity [Moles et al., 2006, Bartolomucci et al., 2010, Bartolomucci et al., 2009]. Remarkably dominant mice are metabolically healthy [Sanghez et al., 2013].

1.5.3 Stress, recovery and maintenance: insights on adaptive and maladaptive effect of stress

Long-term consequences of stress on metabolic function have also, although not extensively, been investigated after the cessation of the stress protocol, i.e. in the recovery phase. In the recovery phase previously stressed animals are usually individually housed and, as it will emerge from our discussion below, the overall effect is constantly a reversal of the catabolic or anabolic effect exerted during the application of the stress procedure. In at least one instance, the recovery (isolation) took place maintainingsome features of a social stress protocol (i.e., avoiding the full interaction/defeat but maintaining the sensory contact between dominant and subordinate mouse in a model of chronic psychosocial stress) with the overall effect of a persistence of the stress-induced metaboliceffect [Moles et al., 2006]. Overall this suggests that at least some of the stress-induced effects are reversible.

When subordinate rats are removed from the visible burrow system environment and allowed to "recover" in individual housing, they immediately become hyperphagic and quickly regain the lost weight primarily as fat, resulting in greater overall and visceral adiposity than dominant and control rats. This effect is further enhanced in rats exposed to a second cycle of visible burrow system stress and recovery. Consistent with increased adiposity, subordinates have elevated plasma leptin and insulin levels [Tamashiro et al., 2007a, Tamashiro, 2011]. Even following two cycles of visible burrow system, subordinate rats still present grater insulin sensitivity compared to dominant and control rats indicating increased glucose uptake and storage in visceral adipose tissue which results in an increase of de novo lipogenesis in adipose tissue casing the gain in fat mass [Tamashiro, 2011]. On a behavioral standpoint, the impact of stress on food intake and body weight in subordinate animals is associated with different feeding strategies during visible burrow system stress and recovery [Tamashiro et al., 2004, Melhorn et al., 2010].

Most of the positive effects on energy balance attributed to the sensory contact or social defeat stress model are indeed observed only after several weeks of recovery in individually housed mice. Mice show a reversal of stress induced weight loss: previously subordinated mice gain weight and fat mass due to hyperphagia due to increased meal size [Chuang et al., 2010a, Chuang et al., 2010b, Kumar et al., 2013]. At the endocrine level, leptin production remains suppressed, and ghrelin secretion is increased to induce a potent feeding response that increases available energy stores [Lutter et al., 2008b, Chuang et al., 2011]. Previously stressed mice also show a decrease in fatty acid synthase in white adipose tissue and increased hypothalamic expression of the orexigenic neuropeptides NPY and AgRP. This activation of NPY/AgRP neurons can then stimulate food intake and body weight after chronic stress and promote the use of carbohydrates as fuel while sparing fat. Interestingly, high fat diet availability doesn't seem to worsen the metabolic phenotype of mice previously subjected to chronic social defeat [Chuang et al., 2010a, Chuang et al., 2010b] and they actually develop less fat mass and associated leptin rise than control mice on high fat diet, in the presence of higher cholesterol levels [Chuang et al., 2010a, Chuang et al., 2010b]. When access to high fat diet is limited to few days throughout recovery [Chuang et al., 2011] previously defeated mice exhibit hyperphagia and increase body weight gain compared to controls. It must be noted however that social isolation exerts metabolic consequences per se (see discussion above) with potential confounding effects on the metabolic sequelae of social stress in rodents [Nonogaki et al., 2007]. For example, it has been demonstrated that chronic social stress has CNS

effects only when isolation follows the social stress but not if animals are group housed [Ruis et al., 1999, Isovich et al., 2001].

Only recently the recovery from chronic psychosocial stress has been investigated. Interestingly subordinate mice isolated following the end of the stress, show an overall drop in body weight while food intake remains elevated, in the presence of increased energy expenditure and adiposity [Patterson et al., 2013]. Furthermore previously stressed mice present increased visceral fat with larger adipocytes, heavier brown adipose tissue, hyperinsulinemia, hyperleptinemia, hyperglycemia, elevated basal corticosterone levels and increase of IL-6 all indicative of increased adiposity and coexisting with increased expression of the hypothalamic orexigenic NPY and AgRP signaling pathways [Patterson et al., 2013]. On the contrary, it's been shown that both the positive energy balance in subordinate mice and the negative energy balance in dominant animals can persist if the stress phase is followed by partial "maintenance" of the stress phase conditions [Moles et al., 2006].

The recovery from unstable social settings stress has also been investigated. Interestingly adult mice that experienced social instability at adolescence show differential fat distribution compared to controls a year after the cessation of the stress [Schmidt et al., 2009]. Nevertheless this phenotype cannot be related to a specific set of biomarkers yet and it must be noted that isolation stress might have played a role as the mice were single-housed for the whole length of the recovery phase.

The recovery from non-social stress models has been investigated as well. At least one study addressed the recovery from chronic mild pain demonstrating persistent negative energy balance after the cessation of the stress as the stress-induced loss of body weight is not recovered in spite of normalized levels of food intake [Levine and Morley, 1981]. The alterations induced by chronic mild stress can normalize following the cessation of the stress regimen [Flak et al., 2011]. Interestingly the recovery occurs with a delay compared to the timing of weight loss/recover observed in animals where the same weight loss seen in restrained animals is induced through food-restriction only. This would indicate that stress attenuates weight gain independently from effects on metabolic parameters. In the recovery from restraint stress a compensatory hyperphagic phase may occur [Depke et al., 2008]. Depending on the severity of the stress induced body weight loss, restrained

rats might fail to return to the body weight of control animals [Kennett et al., 1986, Shimizu et al., 1989, Krahn et al., 1990] or they might show a complete normalization [Babenko et al., 2012]. New evidences are now in support of different timing required by different metabolic parameters to normalize in recovery. For example carbohydrate metabolism might be still altered several weeks after the stress cessation, when hyperglycemia through increased activities of hepatic enzymes is still observed [Nirupama et al., 2013].

1.5.4 Molecular mechanisms of stress-induced negative and positive energy balance

The "neuro-symphony of stress" is emerging [Ulrich-Lai and Herman, 2009, Joels and Baram, 2009] and very elegant pharmacogenomics studies are revealing the neurocircuitry of eating and energy expenditure (e.g. [Balthasar et al., 2005, Kong et al., 2012]). However, to date no study has tested the hypothesis that the same neurocircuitry regulates stress-induced positive or negative energy balance. The most recent mechanistic studies are mostly focused on hypothalamic neuropeptides, adipocytes and gut-derived peptides as well as classical neurotransmitters.

Overall, our understanding of the molecular mechanisms of stress-induced negative and positive energy balance is still in its infancy. This could be due at least in part to the confusion generated by the different, sometime opposite, phenotypes induced by similar models as reviewed above. Furthermore, because the HPA-axis is activated by acute stressor and is up-regulated by chronic stress, it is generally assumed that glucocorticoid-mediated metabolic effect should be observed in mice exposed to stress models. In our opinion this assumption is moslty incorrect because it does not take into account the parallel activation of many other stress-related pathways including the SNS and the SAM-axis which affect metabolism in a direction which is usually opposite to glucocorticoids. For example, the "comfort food" hypothesis, usually discussed as the mechanism linking stress and HPA axis activation to obesity has been originally tested in rats exposed to restraint stress where the restraint-induced negative energy balance was only partially reversed by preference for comfort hypercaloric food [Pecoraro et al., 2004]. On the contrary, in absence of stress and in presence of insulin, glucocorticoids determine a net positive energy balance and visceral fat accumulation [Rebuffe-Scrive et al., 1992, la Fleur et al., 2004, Pecoraro et al., 2004] . Accordingly, the activation of the HPA-axis might be necessary but not sufficient to explain the development of stress-induced positive energy balance.

1.5.4.1 Serotonin (5-hydroxytryptamine, 5HT)

Serotonin is a neurotransmitter synthesized in the serotonergic neurons mainly localized in the caudal and rostral nuclei that projects fibers to a variety of brain regions influencing several body functions, among these mood, behaviour, food intake, energy expenditure and body temperature [Murphy and Lesch, 2008, Gellynck et al., 2013]. Serotonin acts through its specific receptors, 5HTRs 1 to 7, with the exception of 5HT3R being a ligand channel, all the other receptors are Gq coupled protein receptors [Gellynck et al., 2013]. 5-HT_{2C}R is the key mediator of serotonergic suppression of feeding and increased activity, indeed mice and humans with mutations in this receptor are characterized by chronic hyperphagia and locomotor hyperactivity [Nonogaki et al., 2007, Vickers et al., 1999]. 5HT acting on 5-HT_{2C}R would inhibit ghrelin activity by directly activating POMC/CART neurons and inhibiting ghrelin/NPY signaling causing a decrease in food intake and shifting the phenotype towards a negative energy balance [Fujitsuka et al., 2009]. The serotonergic action is terminated by the reuptake of 5HT by the serotonin transporter (5-HTT) encoded by the *SLC6A4* gene that has two alleles, short and long, which respectively are associated with the diminished and increase transcriptional activity of serotonin reuptake transporter [Murphy and Lesch, 2008]. A correlation between depression, anxiety behavior and the short version of the gene has been reported [Lesch et al., 1996, Caspi et al., 2003], as well as disruption of the 5-HTT gene and increased corticosterone levels and ACTH under stress [Holmes et al., 2003, Holmes et al., 2002]. Jarrell et al demonstrated that primates under a psychosocial stress protocol display a negative energy balance but females rehus monkeys presenting one or both short allele of 5-HTT are less aggressive and more submissive confirming that depressive and anxiolytic behavior are influenced by 5-HTT polymorphisms [Jarrell et al., 2008]. Similarly to the pharmacological inhibition of the serotonin transporter, 5-HTT knockout mice display an overall positive energy balance along with lower locomotor activity and basal plasma corticosterone levels; furthermore they present a dramatic stress hypersensitivity to acute and chronic stimulation [Tjurmina et al., 2002, Holmes et al., 2003, Murphy and Lesch, 2008]. More recently Bartolomucci et al., [Bartolomucci et al., 2010] using the chronic psychosocial stress model in heterozygous 5-HTT and wild type mice did show that stress independent from the genotype induced a positive energy balance, but the 5-$HTT^{+}/^{-}$ stressed mice did display a depressed locomotor activity and increased depressive behavior compared to the non stressed and control animals. In summary, 5-HTT heterozygous mice have an increased behavioral vulnerability but normal metabolic vulnerability to chronic psychosocial stress which could be due to a lower serotonin turnover

in different brain nuclei [Bartolomucci et al., 2010, Boyarskikh et al., 2013]. Very few studies have investigated the role of 5-HT in regulating metabolism under stress.

1.5.4.2 Orexin

Orexins also called hypocretin (two existing forms, orexin-A and -B which will be referred to as orexins in this review) are neuropeptides synthesized in the pirifornical, lateral, and posterior hypothalamic area [Peyron et al., 1998, Nambu et al., 1999]. Signaling through OX1, a G-protein coupled receptors, orexin regulates sleep/wakefulness, appetite/metabolism, energy expenditure, stress response, reward/addiction, and analgesia [Berridge et al., 2010,Teske et al., 2010,Kukkonen, 2013]. In rats central orexins administration increases food intake, while the opposite effect is due to the administration of anti-orexin antibodies [Sakurai et al., 1998, Yamada et al., 2000]. Moreover OX1 antagonist decreased food intake, body weight, fat mass and fasting glucose while increased insulin sensitivity and energy expenditure in *ob/ob* mice (i.e. genetically lacking the leptin gene, see below). Cold exposure, and restraint stress have been reported to activate orexin neurons in rats [Berridge et al., 1999, Ida et al., 2000, Zhu et al., 2002]. Orexin-expressing neurons are thought to be involved in the stress response through direct effects on the hypothalamic CHR/AVP neurons that are central in the stress responses, as well as indirect effects through the brainstem [Nishino and Sakurai, 2005]. After chronic sensory contact stress associated with caloric restriction, only wild type (wt) stressed mice display increase social interaction, while Orexin knock out ($^{-}/^{-}$) mice are unaffected, suggesting that orexin plays a role in the stress and caloric restriction induced behavioral changes [Lutter et al., 2008a]. Moreover, wt stressed mice shown to increase hyston demethylation of the orexin promoter leading to downregulation of orexin mRNA expression and orexin neurons in the lateral hypothalamic area, thus suggesting that chronic stress impairs orexin signaling decreasing the ability to cope with chronic stress [Lutter et al., 2008a]. Interestingly stress susceptible (determined with social avoidance) orexin $^{-}/^{-}$ mice show a reversal of chronic sensory contact stress-induced weight gain without any effect on food intake [Tsuneki et al., 2013]. Remarkably this is the only study showing a positive energy balance for this model of stress. Furthermore, Orexin $^{-}/^{-}$ stressed mice but not wt mice exhibit increased HOMA-IR, hepatic glucose production, and hyperinsulinemia. Surprisingly, despite having higher insulin resistance stressed Orexin $^{-}/^{-}$ mice showed almost normalized stress-induced glucose intolerance as observed in wt mice. Thus central actions of orexins appear to be required for preventing the rapid development of hepatic insulin resistance

under chronic stress conditions [Tsuneki et al., 2013]. Indeed hypothalamic orexin can increase plasma corticosterone levels which in turn modulate hepatic glucose production via the sympathetic nervous system thus suggesting orexins as essential neurotransmitters in maintaining altered glucose and insulin metabolism responses to chronic stress [Yi et al., 2009].

1.5.4.3 Neuropeptide Y (NPY)

NPY, one of the key molecules involved in regulation of energy metabolism, is 36 amino acid neuropeptide largely expressed in almost all the brain areas even though the majority of the NPY secreting neurons are located in the arcuate nucleus. NPY plays an important role both in the central and peripheral nervous system [Wettstein et al., 1995, Zukowska-Grojec, 1995]. Its biological effects are mediated by at least six G-protein coupled receptors, Y1 to Y6, which are widely and differently distributed in the CNS and the periphery; the binding to NPY to specific receptor influences different physiological responses [Parker and Herzog, 1999]. Hypothalamic NPY expression is regulated by starvation, leptin and insulin levels [Parker and Herzog, 1999]. Hypothalamic NPY expression is regulated by starvation, leptin and insulin levels [Schwartz et al., 1996, White and Kershaw, 1990]. In the central nervous system the main function of NPY is to promote hyperphagia: NPY is the most potent orexigenic factor and it mediates promote hyperphagia and anxiety by binding to Y1 and Y5 receptors [Lecklin et al., 2002, Bertocchi et al., 2011]. It has been seen that hypercortisolemia and hyperleptinemia given by chronic stress act on the NPY expressing neurons in the PVN increasing the release of the neuropeptide that exerts an inhibitory effect on the CRH neurons inducing hyperphagia [Cavagnini et al., 2000]. In addition to direct effects on eating behavior NPY also influences energy balance by decreasing energy expenditure as thermogenesis in BAT acting through the Y1Rs [Kushi et al., 1998, Kotz et al., 2000] and promoting lipid storage by increasing lipoprotein lipase activity in WAT. However, NPY also co-localizes with NE-secreting sympathetic neurons in several peripheral tissues, such as heart, blood vessels, smooth muscle, gastrointestinal and adipose. Under specific stimuli both NPY and NE are released from the sympathetic nerve terminals [Lundberg et al., 1983].

In adipose tissue NPY is known to have antilipolytic properties [Kuo et al., 2007] reducing the rate of lipolysis and free fatty acid release from lipid droplets and enhancing fat deposition [Valet et al., 1990]. Mice exposed to cold stress or intermittent social defeat stress models and fed high fat diet showed a decrease in catecholamines but a significant increase in fat mass as well as glucocorticoids, NPY, Y2R and dipetidyl peptidase IV (DPPIV)

mRNA, the enzyme responsible to cleaving NPY1-36 to NPY 13-36 making the fragment of NPY more specific for NPY2R, expression in subcutaneous white adipose tissue [Kuo et al., 2007]. Moreover, Y2R agonist treatment determined an obese phenotype and positive energy balance that were reversed by a Y2R antagonist [Kuo et al., 2007]. Accordingly germline Y2R $^{-}/^{-}$ mice exhibit reduced adiposity when exposed to stress [Kuo et al., 2007]. It has been hypothesized that cold stress and to a certain extent repeated social defeat associated to high fat diet could activate the NPY/Y2R pathway that promotes adipogenesis by blunting the NE/βAR signalling in white adipose tissue [Kuo et al., 2007, Kuo et al., 2008].

More recently, studies have shown that also Y1R is involved in the control of energy metabolism, gremline Y1R $^{-}/^{-}$ have a significant increase in lipid oxidation and obese resistant mice also acts through its Y1R to promote fat accretion [Zhang et al., 2010, Shi and Baldock, 2012] as well as proliferation of adipocytes precursor cells in visceral adipose tissue [Yang et al., 2008].

1.5.4.4 Ghrelin and growth hormone secretagogue receptor (GHSR)

Ghrelin is a hormone secreted by gastro-intestinal tract that rises before meals and stimulates hunger and meal initiation [Kojima et al., 1999, Cummings et al., 2005]. Ghrelin circulates in two forms, acyl-ghrelin and des-n-octanoyl ghrelin (des-acyl ghrelin). While acyl-ghrelin activates growth-hormone secretagogue receptor des-acyl ghrelin does not. GHSR are distributed mainly in the catecholaminergic and dopaminergic neurons of the VTA as well as in some peripheral tissue [Gnanapavan et al., 2002, Abizaid et al., 2006]. Centrally, ghrelin promotes orexigenic effects by stimulating the orexigenic NPY/AgRP neurons in the hypothalamic feeding centers; peripherally it facilitates the accumulation of adipose tissue by inhibiting fat utilization in adipose tissue, overall favoring obesogenic phenotype and positive energy balance. Ghrelin also promotes fat mass accumulation and suppresses sympathetic nerve activity (in brown adipose tissue) decreasing energy expenditure [Tschop et al., 2000, Davies et al., 2009, Mano-Otagiri et al., 2009]. However, the functional significance of GHSR or ghrelin genetic ablation is still controversial [Sun et al., 2003, Wortley et al., 2004]. Generally knockout mice for either ghrelin or GHSR show only a mild metabolic phenotype that is mostly composed of decreased body weight and adiposity, in absence of altered feeding behavior or activity [Sun et al., 2003, Sun et al., 2004, Wortley et al., 2004, Longo et al., 2008]. Negative energy balance develops in GHSR knockout mice only following several weeks of high fat diet [Zigman et al., 2005]. GHSR $^{-}/^{-}$ mice are hypophagic, have increased energy expenditure but normal locomotor activity, and overall they are resistant to diet induced obesity compared

to wt mice. Ghrelin plasma levels are elevated after several types of stress in human and rodent models (e.g. [Rouach et al., 2007, Kristenssson et al., 2006, Ochi et al., 2008, Mundinger et al., 2006]). In contrast plasma ghrelin level is lower in obesity and diabetes (e.g. [Tschop et al., 2000, McLaughlin et al., 2004]). Chronic psychosocial stress or sensory contact stress models increase plasma concentration of acyl-ghrelin [Lutter et al., 2008b, Chuang et al., 2011, Patterson et al., 2013]. Because of the discrepancies in the metabolic effects of the two stress models it is difficult to interpret the present findings. Furthermore Patterson and coworkers [Patterson et al., 2013] clearly showed that subordinate mice exposed to chronic psychosocial stress show weight gain and hyperphagia both of which are reversed by genetic deletion of the ghrelin receptor (GHSR $^{-}/^{-}$ mice) and treatment with ghrelin receptor antagonist ([D-Lys3]-GHRP-6). Conversely, Lutter and coworkers [Lutter et al., 2008a] showed that genetic deletion of the GHSR prevents chronic social defeat stress-induced hyperphagia without any effect on body weight. It must be noted however that in Chuang study [Chuang et al., 2011]. defeated mice did not show the weight loss demonstrated by the same group in other studies. Accordingly it is difficult to interpret the present findings. Nevertheless the role of ghrelin and GHSR in mediating the rewording properties of stress on high fat diet in a conditional place preference test has been well documented [Chuang et al., 2011].

1.5.4.5 Glucagon Like Peptide 1 (GLP1)

GLP1 is derived from the transcription product of the proglucagon gene. The major source of GLP-1 in the body are the intestinal L cells [Holst, 2007]. GLP1 primarily influences the absorption process and is a potent stimulator of insulin secretion with significant effects on the regulation of glucose metabolism [Vella and Rizza, 2004]. However GLP-1 is also centrally expressed in the NTS and ventro-lateral medulla that directly innervate the hypothalamic PVN ([Ghosal et al., 2013] for review). GLP1 neurons play a crucial role in regulating HPA axis functions during basal and stress conditions [Kinzig et al., 2003]. For example, in rats under chronic mixed stress, chronic central administration of GLP1 induces HPA hyperactivity, decreases basal glucose levels, and body weight gain. Since food intake is unchanged, GLP1 might be influencing body weight by acting on energy expenditure, although the exact mechanism is still unclear [Tauchi et al., 2008].

1.5.4.6 Leptin

Encoded by the *ob* gene [Zhang et al., 1994], leptin is primarily secreted by white adipocytes in proportion to adipose mass. Leptin acts through Ob-Rs receptors that are widely expressed both centrally and peripherally. Mutations in the *ob* gene or in Ob-Rs receptors result in excessive obesity, hyperinsulinemia, and hypercorticosterolemia indicating a crucial role for leptin in controlling energy homeostasis [Schwartz et al., 2000, Cowley et al., 2001]. Leptin resistance develops in obesity. Mice fed high fat diet and exposed to the sensory contact stress model develop leptin resistance which seems to be associated with lower hypothalamic pSTAT3 signaling [Chuang et al., 2010a].

1.5.4.7 Amylin

Amylin is co-secreted with insulin from the beta pancreatic cells and acts as adiposity signal [Wielinga et al., 2010]. In positive energy balance states, such the one induced during the recovery of the visible burrow system, chronic amylin treatment reduces food intake and body weight gain by limiting deposition of visceral adipose tissue [Smeltzer et al., 2012]. Although the exact molecular mechanisms behind amylin's action are still unclear, amilyn seems to act primarily on the NPY/Agrp hypothalamic neurons, inhibiting the NPY-induced hyperphagia by reducing meal size, duration, and frequency [Morris and Nguyen, 2001, Melhorn et al., 2010].

1.5.4.8 Norepinephrine and β3-adrenergic receptor

Catecholamines increase lipolysis in adipocytes and thermogenesis in brown adipose tissue via activation of the βARs [Nicholls and Locke, 1984, Goldman et al., 1985, Klaus et al., 1991]. In the sensory contact model mice lose weight during the defeat phase and regain weight, due to hyperphagia, in the recovery phase while fat mass and leptin remain lower than control levels. the pharmacological blockade of β3AR in this context abolishes the stress-induced hyperphagia but paradoxically normalized stress-induced decrease in fat mass and leptin level [Chuang et al., 2010b].

1.6 Conclusion

The vast majority of physical-psychological non-social stress models as well as models of chronic mild stress (social or non-social) induce a negative energy balance that results in body weight and/or fat mass loss. High calories,

"comfort food" ingestion may limit, but not reverse, the non-social stressors induced negative energy balance. The only notable exception is daily short exposure to ice-cold water that increased fat mass in presence of high fat diet which might be linked to the proposed functional role of NPY as a hibernating hormone. On the contrary the metabolic effects of chronic social stress models are more heterogeneous, although when administered in the presence of a high fat diet a positive energy balance generally is the outcome. The major discrepancies exist between models that appear very similar such as the sensory contact (or chronic social defeat) and the chronic psychosocial stress. Overall it is remarkable that the two models induce very similar neuro-endocrine and behavioral effects but very divergent metabolic consequences (negative the first and positive the latter) at least during the sensory-contact social defeat phase. We believe that the factor responsible for this stricking difference is the stability (chronic psychosocial stress) vs. the instability (sensory contact) of the housing environment for the subordinate animal, although this hypothesis is yet to be tested. On the contrary, removing the mice from the stress experience by individually housing leads to a reversal of stress-induced effects. Finally, although poorly investigated, it is clear that social status plays a major role in energy homeostasis in condition of chronic stress ([Sanghez et al., 2013] for further discussion).

The mechanistic understanding of how stress affects energy balance is still at at an early stage where hypothesis have been mostly tested with one model of stress and not generalized to other models. On the one hand, serotonin (via $5H_{2C}$ receptors and 5-HTT), orexin, amylin and ghrelin seem to play a major central role, while peripherally NPY/Y2R receptor in the sympathetic nervous system/adipocytes synapse in the adipose fat pads. Remarkably, the role of HPA-axis secreted hormones and catecholamines, which are crucial to both stress-physiology and energy balance, is not been unambiguously determined in the context of stress-induced energy balance disorders.

Chapter 2

Goal and Outline

Stress, primarily prolonged stress has been shown to alter eating behaviors and energy homeostasis [Adam and Epel, 2007] and has been associated to adiposity, in particular abdominal adiposity, that leads to the development of metabolic disturbances such as cardiovascular disease, type II diabetes and obesity [Raikkonen et al., 1996, Chandola et al., 2006, McEwen and Mirsky, 2002, Chrousos and Gold, 1998]. Moreover, chronic stress has also been associated to overeating, mainly high sugar and high fat foods [Greeno and Wing, 1994, Dallman et al., 2005] accelerating and aggravating the onset of the consequences of stress. Aside from the, environmental and social-economical factors [Rosmond and Bjorntorp, 2000b, Sapolsky, 2005], genetic predisposition has likewise been shown to influence and impact eating behaviors and metabolism [Committee on Assessing Interactions Among Social et al., 2006, Grimm and Steinle, 2011].

To understand the relationship between stress induced-metabolic disorders and obesity, animal models have been studied extensively as discussed in detail in the introduction section of this thesis. However, different animal models of stress, mainly rodent models, produce divergent metabolic phenotypes where some animals develop a negative energy balance phenotype and lose body weight while others increase food intake and body mass and become vulnerable to the development of metabolic disturbances [Patterson and Abizaid, 2013, Neubauer and Kulkarni, 2006].

In this thesis we used a model of chronic psychosocial stress [Bartolomucci et al., 2009, Bartolomucci et al., 2005, Bartolomucci et al., 2010] to determine the molecular mechanism of stress-induced obesity and methabolic syndrome. In doing so we specifically investigated the role played by social status (dominant *vs.* subordinate mice) individual variability (genetic strains of mice) and diet (normal and high fat diets). The strains of mice investigated are:

- C57BL/6J, obese and diabetic-prone strain [Chatzigeorgiou et al., 2009, Plum et al., 2005]
- CD1, an outbred strain showing high territorial aggression previously shown to be prone to obesity and glucose intolerant [Mathews et al., 2004]
- 129SvEv strain considered to be obese resistant *but resulted to have metabolic vulnerability to our model of chronic psychosocial stress* [Chatzigeorgiou et al., 2009, Plum et al., 2005]

Genetically modified animals were also included in our studies to gain more knowledge about the role of specific genes and molecules in stress induced obesity studies:

- transgenic 5-HTT$^{+}/^{-}$, a good model to study depression, anxiety and stress behavior [Caspi et al., 2003], but had normal metabolic vulnerability to stress under a standard fed diet [Bartolomucci et al., 2010]
- β-AR knock-out mice that globally lack β1, β2 and β3 adrenergic receptors which are crucial for thermogenesis and body weight homeostasis [Bachman et al., 2002, Lowell and Bachman, 2003]

The main aim of this thesis project was to undercover the underlying molecular mechanisms involved in the pathophysiological of chronic social stress induced obesity and metabolic syndrome. This projects' focus was to determine changes in candidate gene in visceral (epididymal, retroperitoneal, perirenal), subcutaneous (scapular, inguinal) and brown adipose tissue as well as liver and muscle tissues that are insulin sensitive in mice under basal and after being exposed to chronic psychosocial stress conditions. In vivo experiments and physiological assessment were performed in Bartolomucci Lab for many years and the current thesis specifically aims at analyzing the tissue biopsies collected in different published and unpublished experiments. Each chapter starts with a brief introduction of the metabolic phenotype observed for each strain and diet. I have personally been involved in the execution of many of the experiments included in this thesis. To achieve this goal the genes that I investigated were:

- β3-AR, the main adrenergic receptor present on adipocytes membrane promoting catecholamine induced lipolysis [Cannon and Nedergaard, 2004]
- mitochondrial-uncoupling protein 1 (UCP1), key molecule of brown adipocytes that uncouples respiration and dissipates chemical energy as heat, is a key molecule of brown adipocytes [Nicholls and Locke, 1984, Cannon and Nedergaard, 2008] and tyrosine hydroxylase, rate limiting enzyme in the synthesis of catecholamines [Daubner et al., 2011]
- adipogenic and pro-diabetogeneic marker PPARγ [Lehrke and Lazar, 2005] a highly expressed transcription regulator in white adipose tissue that regulates adipocytes' differentiation
- α2-AR one of the main anti-lipolytic genes that inhibits adenylyl cyclase activity blocking the breakdown of triglycerides [Cannon and Nedergaard, 2004]

- the NPY system genes (NPY, Y1R, Y2R, Y5R and DPPIV) that have been shown to be peripherally up-regulted under chronic stress and HFD promoting adiposity [Kuo et al., 2007]
- genes implicated in insulin signaling, the insulin receptor substrate 1 and 2 (IRS1 and IRS2) [Kubota et al., 2008, Kubota et al., 2011]

Specifically the questions asked were:

- Does exposure to chronic stress per se (i.e. in absence of high fat diet) results in fat depot-specific gene expression? (chapter 1)
- Do heterozygous 5-HTT mice, vulnerable to subordination stress and depressed like disorders, alter their molecular machinery following exposure to chronic stress? (chapter 1)
- What are the molecular differences between CD1 dominant and subordinate mice following chronic stress? (chapter 2)
- Which are the key genes that differ between strains under basal conditions and after the stressful challenge?
  - 5-HTT$^{+}/^{-}$ and wild type (chapter 1)
  - CD1, C57BL/6J and 129SvEv (chapter 3)
  - βless and wild type mice (chapter 4)
- What is the molecular signature of the interaction between stess vulnerability and diet? (chapter 1, chapter 2, chapter 3 and chapter 4)

Chapter 3

Materials and Methods

3.1 Animals

Only male mice were used in our experiments.

3.1.1 Chapter One:

Wild-type and heterozygous male 5-HTT knockout littermates were produced by mating wild-type females (C57BL/6J, JAX mice; Charles River Italia, Calco, Italy) and heterozygous 5-HTT mice [B6.129(Cg)-*Slc6a4*^{tm1Kpl}/J] [Bengel et al., 1998]. C57BL/6J male mice, used as controls, were bought from Charles River Italia (Calco, Italy). Swiss CD-1 male mice from an outbred stock originally obtained from Charles River Italia (Lecco, Italy) and maintained at the University of Parma were used through this study. Mice were born and reared at the University of Parma. At weaning, 21 days for the C57BL/6J mice and 28 days for the CD-1 mice, the mice were housed in same-sex groups of siblings (3-6 per cage) in Plexiglas cages ($38 \times 20 \times 18$ cm) with wood shaving bedding changed weekly to avoid excessive manipulation. Animal care was provided according to protocols designed by the European Communities Council Directive of November 24, 1986 (86/EEC) and approved by the ethical committees of the University of Parma and the Italian Institute of Health.

3.1.2 Chapter Two:

Male Swiss CD1 mice where derived from an outbred stock originally obtain by Charles River Italia. At 28 days of age the mice were weaned and groups of siblings males were housed in plexiglass cages ($38 \times 20 \times 18$ cm) and bedding was changed weekly. In vivo experiments were conducted at University of Parma (Italy) and University of Minnesota (USA). All animal experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/EEC) and approve by ethical committees of University of Parma and the Italian Institute of Health.

3.1.3 Chapter Three:

129SvEv were derived from breeders supplied by Takeda Cambridge (U.K.); C57BL/6J were generated from breeders supplied by Charles River Italia (C57BL/6J, JAX mice; Charles River Italia, Calco, Italy) and Swiss CD-1 mice were derived from an outbred stock originally obtained from Charles River Italia (Lecco, Italy). Mice were born and reared at the University of Parma. 129SvEv mice were weaned at postnatal day 25, C57BL/6J mice at

day 21 and CD1 mice at day 28 days. After weaning mice were housed in same-sex groups of siblings (3-6 per cage) in plexiglass cages ($38 \times 20 \times 18$ cm) and bedding was changed weekly. In vivo experiments were conducted at University of Parma (Italy) and University of Minnesota (USA). All animal experiments were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/EEC) and approve by the ethical committees of University of Parma and the Italian Institute of Health.

3.1.4 Chapter Four:

A pair of βless mice on a mixture of 129SvJ, C57Bl6/J, and DBA/2 strain background and their wild type liettremates were kindly donated by Dr. B Lowell (Harvard Medical School, Boston, USA) pmid12161655. A colony of each strain was genrated and maintained in at the University of Minnesota, USA. At weaning, 21 days after birth, the mice were housed in same-sex groups of siblings (3-4 per cage). Swiss CD1 male mice were purchased from Charles River (USA). Animal care and experiments were approved and performed according to Institutional Animal Care and Use Committee, University of Minnesota.

3.2 Housing conditions

In vivo experiments were conducted at University of Parma (Italy) and University of Minnesota (USA). Mice were housed under standard laboratory conditions at a constant temperature of $20 \pm 2^{\circ} C$ in a 12h light / 12h dark cycle, with food and water ad libitum.

3.3 Experimental groups

Seven days before the beginning of the experiment, all animals were single housed in a different experimental room (same conditions as described above). On Day 0, the following experimental groups were formed:

(i) group-housed siblings of 3 animals used as controls (Con)

(ii) animals living under chronic psychosocial stress conditions (Stress)

3.4 Chronic psychosocial stress paradigm

The procedure used in these experiment is a modified version of our standard Chronic Subordination Stress Paradigm [Bartolomucci et al., 2001, Bartolomucci et al., 2005]. This procedure is validated etiological model to investigate the vulnerability to stress induce obesity and metabolic syndrome [Bartolomucci et al., 2009].

Briefly, three-months old heterozygous 5-HTT, C57BL/6J, 129SvEv, CD1, βless and their wild type mice were individually housed in Plexiglas cages ($38 \times 20 \times 18$ cm) for a 7 days baseline period for basal parameter measurements and used as residents.

On day 1 of baseline a cage a wire-mesh partition was introduced in each resident's cage bisecting the cage longitudinally so that only half of it was accessible recalling the conditions of the stress phase (detailed below) and allowing recording of baseline individual locomotor activity. On day 6 of the baseline phase, the wire-mesh partition was removed allowing the animal to have access to the entire cage re-establishing individual territory in the whole cage. On day 7, a three-week stress phase began, each resident mouse received a same age CD-1 intruder mouse in its cage. Based on previous observations, outbred CD-1 males were selected as intruders because of their higher aggressive and competitive behavior over other strains [Bartolomucci et al., 2010, Parmigiani et al., 1999], indeed in our experiment almost all the resident mice undergoing repeated agonist interaction with CD-1 became subordinates.

The two animals were allowed to freely interact for 10 minutes to allow the mice to interact and establish a social status. In order to prevent injuries, whenever social agonism escalated the fighting was interrupted. After the interaction, the two animals were separated by a wire-mesh partition, which allowed the two subjects to be in continuous sensory contact but no physical interaction. The same procedure was repeated for all the 21 days of stress between 8 a.m. and 10 a.m. During the social interaction offensive behaviors of the animals were manually recorded and mice social status was determined as follows: the aggressive chasing and biting animal was defined as 'Dominant' (Dom), while the mouse displaying upright posture flight behavior and squeaking vocalization was the 'Subordinate' (Sub). The number of attack bouts performed by each animal was quantified by direct observation by a trained observer [Bartolomucci et al., 2005]. During baseline period and first week of stress phase all mice were fed a standard diet, while starting from the 2nd week of chronic social stress the diet was switched to a high fat diet (except for the C57BL/6J and heterozygous 5-HTT experimental group that were kept on a standard diet throughout the entire experimental protocol). Food

and water were at all time available ad libitum. Body weight and food intake were monitored on day 0 and 7 of the baseline phase and weekly during the stress phase. Locomotor activity was monitored continuously except during the aggressive interaction. Age-matched heterozegous 5-HTT, C57BL/6J, 129SvEv, CD1, βless and their wild type mice were group-housed siblings of 3 littermates were included as the non stress control group and fed the same diet ad the experimental individuals [Bartolomucci et al., 2001] . Stressed and control males were littermates to reduce the possible confounds of inter-litter variability. The choice of using group-housed siblings is based on previous studies that show no metabolic, immune-endocrine, behavioral evidence of stress activation or anxiety [Bartolomucci et al., 2001, Bartolomucci et al., 2005, Bartolomucci et al., 2009].

3.5 Diets

- Standard chow (6.55% kcal from fat and 3.9 kcal/g; 4RF21, Mucedola, Italy) was used throughout the entire study in chapter one and baseline and phase one for experimental mice in chapter two and three
- High fat diet: (45% kcal from fat and 5.2 kcal/g; modified 4RF21, Mucedola, Italy) was used as high caloric diet in chapter two and three
- Standard chow (18% kcal from fat and 3.1 kcal/g; 2018 Teklad global, Harlan Lab, USA) and high fat diet (45% kcal from fat and 4.73 kcal/g; D12451, Research Diets Inc., USA) were used in the βless and wild type study that was entirely performed at the University of Minnesota

3.6 Tissue collection

After 24 hours of the last agonist interaction, on day 21 of the stress procedure between 10 a.m and 12 a.m all the experimental mice were humanly sacrificed by decapitation following a brief CO_2 exposure. Five different type of white adipose tissue (WAT), three visceral (perigonadal, perirenal and retroperitoneal) and two subcutaneous (scapolar and inguinal) were manually dissected and weighted. Also, liver and skeletal muscle were dissected but not weighted. Tissue samples were immediately frozen in liquid nitrogen or dry ice and stored at $-80^{\circ}C$ until RNA isolation.

3.7 RNA preparation and extraction

RNA was obtained by homogenizing of 50 to 100mg of frozen tissue in 1ml of TRI REAGENT (Molecular Research Center, Inc., Cincinnati, Ohio) on ice following manufacturer's instructions. Total RNA was digested with Dnase I using DNA-free (Ambion, Austin, TX) and tested for the presence of DNA contamination using PCR. Total RNA concentration and purity was then determined using spectophotometry at the UV absorbency of 260 nm on a Spectronic ® Genesys ™ 5 or using the NanoDrop ND-1000 spectophotometer (NanoDrop Technologies, Inc., Wilmington, Delaware USA).

3.8 Reverse Transcription and Quantitative Real-Time PCR

1μg of RNA was converted into cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) and relative quantification of mRNAs was performed with 5μl of cDNA used in each 20μl real-time-RT-PCR reaction using using ICycler iQ Detection System (Bio-Rad Laboratories, Hercules, CA). PCR reactions were carried out using TaqMan Universal PCR Master Mix and pre-designed primers and fluorescein-labeled probes (Applied Biosystems, Foster City, CA) for the NPY system genes and PPARγ. The primers from Applied Biosystems were: (NPY:16403380), (Y1R:16403381), (Y2R:14381272), (DDPIV:16403393) and (PPARγ:Mm01184322). All other PCR reactions were carried out using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and custom-designed primers (Integrated DNA Technolgies). Primers for the target genes were UCP1: forward 5'-GTC CCC TGC CAT TTA CTG TCA G-3' and reverse 5'-TTT ATT CGT GGT CTC CCA GCA TAG-3'; β3-ADR: forward 5'-CCA GCC AGC CCT GTT GA-3' and reverse 5'-GGA CGC GCA CCT TCA TAG C-3'; α2-ADR: forward 5'-TTC TTT TTC ACC TAC ACG CTC A-3' and reverse 5'-TGT AGA TAA CAG GGT TCA GCG A-3'; and the reference gene β-actin: forward 5'-GGC ACC ACA CCT TCT ACA ATG-3' and reverse 5'-GGG GTG TTG AAG GTC TCA AAC-3'. Thermal cycling parameters were as follows: an initial denaturing step (95° C for 10 min), followed by 40 cycles of denaturing-annealing and extending (95° C for 45 s, 58° C for 45 s and then 60° C for 1min) in a 96-well plate. The results were calculated by the comparative C_t method using β-actin as an endogenous reference gene, according to the Applied Biosystems ABI PRISM 7700 User Bulletin #2. A threshold cycle (C_t value) was obtained from each amplification curve. Each sample was run in duplicate to obtain average C_t values and a ΔC_t value for each sample was

first calculated by subtracting the C_t value of β-actin from the C_t value for the target gene of the same sample. The expression relative to β-actin was determined by calculating $2^{-\Delta C_t}$ and values for all experimental groups were averaged.

3.9 Statistical analysis

All data are expressed as mean ± standard error (SE). Student t-test was used to compare two independent groups (controls from two different strains, e.g. heterozygous 5-HTT vs C57BL/6J; wild type vs βless), One-way (e.g. CD1 controls vs CD1 Dom vs CD1 Sub; CD1 vs C57BL/6J vs 129SvEv) and Two-way repeated measure ANOVA using social status (controls or subordinates) and strains (e.g. heterozygous 5-HTT, C57BL/6J; CD1, C57BL/6J, 129SvEv; wild type vs βless) as between subjects followed by Tukey's multiple comparison tests. All analyses were performed with GraphPad Prism Software 5.0 (GraphPad, San Diego, CA, USA).

Part II

Chapter 1

Fat depot-specific expression of the NPY system, sympathetic-related and adipogenic markers in chronic psychosocially stressed heterozygous serotonin transporter knockout and C57BL6J mice

1.1 Introduction

It has been proposed that exposure to chronic stress stimulates the release of NPY and the expression of Y2Rs in the adipose tissue resulting in visceral obesity [Kuo et al., 2007]. The adipose organ is differentially innervated, sensitive to pro-adipogenic and lipolytic stimuli, and implicated in cardiovascular and metabolic diseases. To determine if exposure chronic stress per se (i.e. in absence of high fat diet) results in fat depot-specific regulation of neuronal and adipogenic gene expression wild type C57BL6J mice were exposed to an established paradigm of chronic subordination stress-induced weight gain and obesity [Bartolomucci et al., 2001, Bartolomucci et al., 2005, Bartolomucci et al., 2009]. Furthermore we also asked the question if exposure to chronic stress could interact with a known risk factor for depression and metabolic disorders, the serotonin transporter (5HTT) that have been shown to manifest increased vulnerability to subordination stress induced depression-like disorders in our model [Bartolomucci et al., 2010].

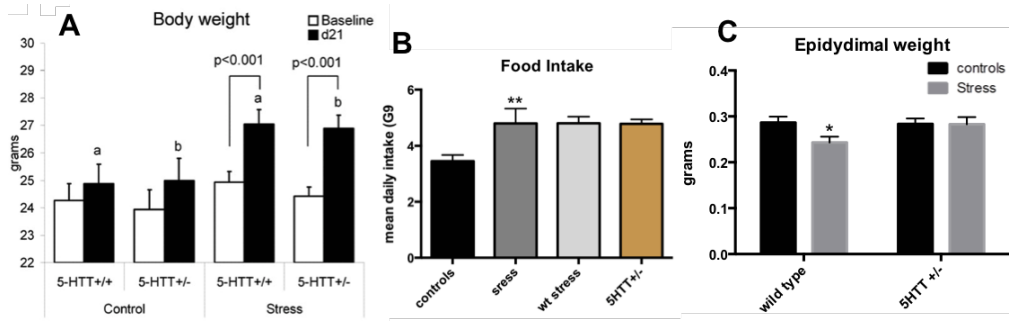


Figure 1.1: Metabolic consequences of C57BL6J and 5HTT$^{+}/^{-}$ under chronic psychosocial stress and standard diet (adapted from [Bartolomucci et al., 2010])

Following our validated and unique chronic subordination stress mouse based model, independent from the genotype, stressed induced hyperfagia (Figure 1.1, B) in C57BL6J and 5HTT$^{+}/^{-}$ subordinates, along with increased body weight gain (Figure 1.1, A) and fat mass (Figure 1.1, C).

The aim of this study was to whether chronic psychosocial stress alone would cause any alteration in the expression of NPY and its receptors along with sympathetic and adipogenic markers. To answer our question, after three of chronic psychosocial we investigated on the presence of any molecular changes only within white adipose tissues. White adipose depots are mainly divided in visceral adipose that surrounds the inner organs and is divided into perigonadal, perirenal, retroperitoneal, mediastinic, and mesenteric, while

the subcutaneous adipose depot is divided into interscapular, subscapular, axillary, cervical and inguinal [Cinti, 2012, Kissebah and Krakower, 1994]. These tissues are differently innervated by the sympathetic nervous system (SNS) and sensory innervation and have different molecular profiles therefore we kept them separate in our analysis [Bartness et al., 2010a, Bartness and Bamshad, 1998, Bamshad et al., 1998]. Our analysis focused on three visceral (perigonadal, perirenal, retroperitoneal) and two subcutaneous (inguinal and interscapular) fat depots.

1.2 Results

1.2.1 Effects of chronic psychological stress on the expression of the NPY system, sympathetic markers and PPARγ genes in the visceral fat depots

1.2.1.1 Epididymal Fat (Epi WAT)

We first compared the expression of all genes under analysis between the two genotypes to verify whether the deletion of one 5HTT allele would alter basal expression. No difference in gene expression was found between genotypes NPY [$t_{2,21} = 0.056$, $p = 0.95$]; Y1R [$t_{2,21} = 0.876$, $p = 0.4$]; Y2R [$t_{2,2} = 0.955, p = 0.4$]; Y5R [$t_{2,19} = 1.107, p = 0.29$]; DPPIV [$t_{2,19} = 0.835, p = 0.211$]; b3-AR [$t_{2,19} = 0.874$, $p = 0.39$]; PPARg [$t_{2,19} = 0.66$, $p = 0.52$] (Figure 1.2); moreover both genotypes did not express the UCP1 and TH gene under basal conditions (Figure 1.3).

Except the DPPIV gene that was expressed in all mice, independent form genotype and treatment, the other NPY system gene were not always expressed (Figure 1.3). We detected a lower frequency of Y5R expression in C57BL6J stressed mice compared to their controls ($p = 0.00027$) (Figure 1.3). No significant changes were detected in any of the NPY genes following chronic psychosocial stress, independent from the genetic background (NPY [$F(2.082) = 192339$, ns], Y1R [$F(2.724) = 262.5$, ns], Y2R [$F(1.796) = 254808$, ns], Y5R [$F(1.278) = 21929$, ns], DPPIV [$F(0.5693) = 71.54$, ns]).

Tyrosine hydroxylase (TH), key rate limiting enzyme for the production of catecholamines, is not produced by adipocytes but by sympathetic nerves and stored within vesicles at the terminals [Daubner et al., 2011], therefore used as a sympathetic marker. As predicted, EpiWAT being not highly innervated by the SNS [Bartness et al., 2010a] no TH was expressed in

any of the experimental animals either before or after stress (Figure 1.3). UCP1 mRNA, on the other hand, was not present in any of the control mice in both genotypes, but following chronic stress, some mice did show expression of this key brown adipocyte marker [Cannon and Nedergaard, 2004] [$F(0.8279) = 0.0004396$, ns)] (Figure 1.3, Figure 1.4). β3-AR, the most prominent β3-AR present in WAT that promotes lipolysis [Ferrer-Lorente et al., 2005], was expressed in mostly all animals (Figure 1.3) and its expression did not differ following stress [$F(0.6566) = 74.07$, ns] (Figure 1.4).

PPARγ mRNA, a pro-adipogenic and pro-diabetogenic marker, was observed in all animals (Figure 1.3). Chronic stress exposure did induce a significant up-regulation of this gene in both genotypes compared to their controls [$F(8.131) = 1719$, $p < 0.05$] (Figure 1.4, A). This finding could suggest development of impaired glucose tolerance (as found in our physiological data, not shown) rather than adipogenesis since these mice do not increase epidydimal mass (Figure 1.1, C).

	C57 Ctrl		5HTT+/- Ctrl	
	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	2E-03	9E-04	2E-03	8E-04
Y1R	3E-03	1E-03	1E-03	4E-04
Y2R	2E-03	2E-03	2E-04	1E-04
Y5R	4E-04	2E-04	1E-04	1E-04
DPPIV	9E-02	3E-02	9E-02	2E-02
β3-AR	5E-02	3E-02	2E-02	1E-02
TH	0E+00	0E+00	0E+00	0E+00
UCP1	0E+00	0E+00	0E+00	0E+00
PRARγ	4E-02	1E-02	3E-02	7E-03

*: C57 vs 5HTT+/-
No significant difference was found between the two genotypes

Figure 1.2: Absolute Expression of analyzed genes in Epidydimal Fat

	NPY	Y1R	Y2R	Y5R	DPPIV	β3-AR	TH	UCP1	PPARγ
C57 Ctrl	13/14	12/14	11/14	12/14	14/14	13/14	0/14	0/14	14/14
C57 Stress	15/16	16/16	15/16	5/16 **	16/16	16/16	0/16	8/16 **	16/16
5HTT+/- Ctrl	8/9	7/9	4/9	2/9	9/9	8/9	0/9	0/9	9/9
5HTT+/- Stress	16/20	20/20	15/20	11/20	20/20	20/20	0/20	14/20 ***	20/20

p= .0027 **

p= .0020 **
p= .0005 ***

Differences in frequency of gene expression between genotype.

*: C57 ctrl vs 5HTT+/- ctrl
*: C57 ctrl vs C57 Sub
*: 5HTT+/- Ctrl vs 5HTT+/- Sub

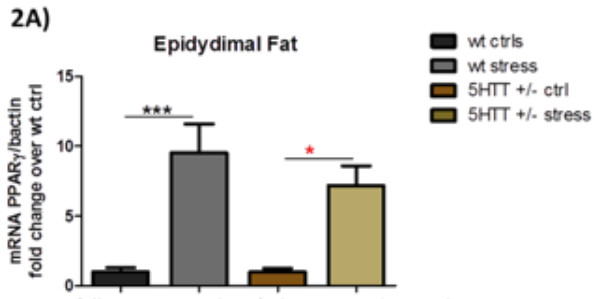
Stress decreased the frequency of Y5R expression in C57; while in both genotypes stress induce the expression of UCP1

Figure 1.3: Frequency of gene expression in Epididymal Fat

	NPY	Y1R	Y2R	Y5R	DPPIV	β3-AR	TH	UCP1	PPARγ
C57 Ctrl	1.0 ± 0.42	1.0 ± 0.5	1.0 ± 0.8	1.0 ± 0.5	1.0 ± 0.3	1.0 ± 0.5	0.0 ± 0.00	0.0 ± 0.00	1.0 ± 0.3
C57 Stress	51.1 ± 30.01	1.3 ± 0.4	3.0 ± 2.0	3.8 ± 3.2	0.6 ± 0.2	0.5 ± 0.1	0.0 ± 0.00	0.0 ± 0.00	9.5 ± 2.1 ***
5HTT+/- Ctrl	1.0 ± 0.4	1.0 ± 0.3	1.0 ± 0.5	1.0 ± 0.8	1.0 ± 0.2	1.0 ± 0.5	0.0 ± 0.00	0.0 ± 0.00	1.0 ± 0.3
5HTT+/- Stress	50.3 ± 29.6	2.8 ± 0.7	45.3 ± 25.8	13.0 ± 7.2	1.0 ± 0.3	0.8 ± 0.2	0.0 ± 0.00	0.0 ± 0.00	11.1 ± 4.2 *

Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.

*: C57 Ctrl vs C57 Sub
*: 5HTT +/- Ctrl vs 5HTT +/- Sub



A) The only significant increase following 4 weeks of chronic psychosocial stress was PPARγ expression in stressed mice of both strains compared to their controls [F(8.131)=1719, p<0.05].

Figure 1.4: Relative Expression of analyzed genes in Epididymal Fat

1.2.1.2 Retropritoneal Adipose Tissue (RetroWAT)

Also within RetroWAT no difference in terms of basal expression was detected between the two genotypes NPY [$t_{2,21} = 0.1798$, $p = 0.86$]; Y1R [$t_{2,20} = 0.077$, $p = 0.94$]; Y2R [$t_{2,21} = 0.905$, $p = 0.38$]; Y5R [$t_{2,2} = 1.169$, $p = 0.26$]; DPPIV [$t_{2,21} = 0.883$, $p = 0.3873$]; β3-AR [$t_{2,20} = 0.824$, $p = 0.23$]; PPARγ [$t_{2,19} = 0.296$, $p = 0.77$]; UCP1 [$t_{2,20} = 1.191$, $p = 0.247$]; TH

[$t_{2,1} = 0.115$, $p = 0.909$] (Figure 1.5). We observed a low frequency in the expression of Y2R, Y5R and the tyrosine hydroxylase (TH) genes (Figure 1.6).

Following the stress procedure no significant difference was in the expression of NPY [$F(3.257) = 11581$, ns] and its receptors Y1R [$F(0.2501) = 441.3$, ns] and Y5R [$F(1.522) = 14642$, ns] (Figure 1.7). The frequency of Y1R expression was significant different in the C57BL6J and 5HTT$^{+}/^{-}$ stressed mice compared to their controls, both decreased their expression following stress (Figure 1.6) ($p = 0.0001$). The opposite situation was observed in the frequency of the Y2R expression where all stressed mice expressed the receptor which a significant difference between the stressed 5HTT$^{+}/^{-}$ and their un-stressed counterparts (Figure 1.6) ($p = 0.002$). Moreover, along with the significant frequency of Y2R expression in the 5HTT$^{+}/^{-}$ stressed mice we also detected a significant up-regulation of the expression of the report as well [$F(5.529) = 3.122e + 006$, $p < 0.05$] (Figure 1.7, 3A). DPPIV, expressed in all mice (Figure 1.6) The expression of DPPIV, the enzymes that converts NPY1-36 to a shorter peptide NPY3-36 shifting the affinity from Y1R to Y2R was expressed in all mice (Figure 1.6) and was significantly up-regulated after the stress exposure in both genotypes [$F(12.43) = 711.4$, $p < 0.0001$] (Figure 1.7, 3B), which is in line with an increased Y2R in 5HTT$^{+}/^{-}$ stressed mice.

As expected, even though RetroWAT is more innervated by the SNS compared to EpiWAT depot, we found very few animals expressing (Figure 1.6) TH independent from genotype and stress with a very low expression TH [$F(0.896) = 7796$, ns]. On the other hand, B3AR was expressed in all animals, while only one stressed C57BL6J did not have UCP1 expression (Figure 1.6). Interestingly, UCP1 expression presented a tendency of increased expression in stressed (Figure 1.7, 3C), [$F(2.002) = 90447$, ns] indicating activation of brown adipocytes within this visceral tissue () . On the other hand we detected a very similar expression of β3-AR [$F(0.26) = 15.96$, ns] in all animals independent of treatment and genotype.

PPARγ, also expressed in most of the subjects (Figure 1.6) did not change its expression after stress (Figure 1.7).

	C57 Ctrl		5HTT+/- Ctrl	
	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	7E-05	3E-05	8E-05	4E-05
Y1R	3E-04	6E-05	3E-04	6E-05
Y2R	4E-03	3E-03	4E-05	2E-05
Y5R	2E-06	2E-06	3E-05	3E-05
DPPIV	9E-03	2E-03	7E-03	2E-03
β3-AR	8E-03	1E-03	7E-03	2E-03
TH	2E-06	2E-06	2E-06	2E-06
UCP1	2E-03	7E-04	1E-03	3E-04
PRARγ	5E-02	7E-03	5E-02	9E-03

*: C57 vs 5HTT+/-
No significant difference was found between the two genotypes

Figure 1.5: Absolute Expression of analyzed genes in Retroperitoneal Fat

	NPY	Y1R	Y2R	Y5R	DPPIV	β3-AR	TH	UCP1	PPARγ
C57 Ctrl	13/14	14/14	10/14	2/14	14/14	13/14	2/14	13/14	14/14
C57 Stress	6/9	2/9 ***	9/9	2/9	9/9	9/9	0/9	7/9	8/9
5HTT+/- Ctrl	9/9	9/9	5/9	2/9	9/9	9/9	1/9	9/9	9/9
5HTT+/- Stress	7/10	1/10 ***	10/10 *	3/10	10/10	8/10	1/10	10/10	10/10

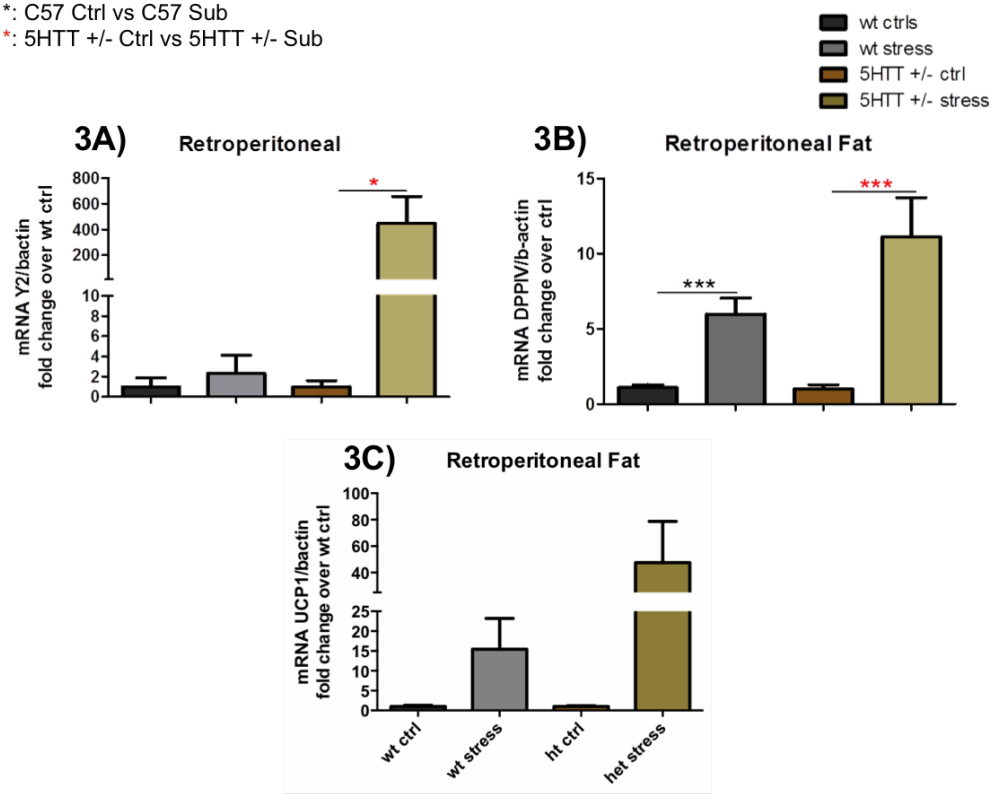
p= .0001 *** p= .002 *
p= .0001 ***

Frequency of gene expression in Brown Adipose Tissue. Differences in frequency of gene expression between genotype.
*: C57 ctrl vs 5HTT+/- ctrl
*: C57 ctrl vs C57 Sub
*: 5HTT+/- Ctrl vs 5HTT+/- Sub
Stress decreased the frequency of Y1R expression in both genotypes; while it increased the expression of Y2R in 5HTT+/- mice only.

Figure 1.6: Frequency of gene expression in Retroperitoneal Fat

	NPY	Y1R	Y2R	Y5R	DPPIV	β3-AR	TH	UCP1	PPARγ
C57 Ctrl	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.9	1.0 ± 1.0	1.0 ± 0.2 ***	1.0 ± 0.2	1.0 ± 0.9	1.0 ± 0.3	1.0 ± 0.1
C57 Stress	16.4 ± 8.2	1.0 ± 0.8	2.3 ± 1.8	17.4 ± 13.5	6.0 ± 1.1	0.8 ± 0.2	0.0 ± 0.00	15.4 ± 7.7	0.4 ± 0.1
5HTT+/- Ctrl	1.0 ± 0.5	1.0 ± 0.2	1.0 ± 0.6	1.0 ± 0.98	1.0 ± 0.3 ***	1.0 ± 0.3	1.0 ± 1.0	1.0 ± 0.2	1.0 ± 0.2
5HTT+/- Stress	19.1 ± 8.6	2.1 ± 2.1	782.0 ± 382.6 *	6.8 ± 3.9	11.1 ± 2.6	0.9 ± 0.2	9.2 ± 9.2	5.6 ± 1.5	1.4 ± 0.6

Relative mRNA expression in Retroperitoneal fat. Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.
*: C57 Ctrl vs C57 Sub
*: 5HTT +/- Ctrl vs 5HTT +/- Sub



A) Following stress Y2R expression was up regulated in 5HTT +/- mice F(5.529)=3.122e +006, p<0.05] ;

B): DPPIV expression was found to be increased in both genotypes after the stress protocol [F(12.43)=711.4, p<0.0001] .

C): UCP1 expression presented a tendency to be up-regulated stressed mice [F(2.002)=90447, ns]

Figure 1.7: Relative Expression of analyzed genes in Retroperitoneal Fat

1.2.1.3 Perirenal Adipose Tissue (PeriWAT)

We found a higher expression of β3-AR [$t_{2,19} = 2.29$, $p = 0.03$] (Figure 1.8, 4A) and PPARγ [$t_{2,21} = 2.465$, $p = 0.0224$] (Figure 1.8, 4B) in the 5HTT$^{+}/^{-}$ com-

pared to the C57BL6J mice (Figure 1.8) . All other genes under investigation did not differ between genotypes NPY [$t_{2,21} = 0.1727$, $p = 0.98$]; Y1R [$t_{2,21} = 1.708$, $p = 0.1031$]; Y2R ns; Y5R ns; DPPIV [$t_{2,20} = 0.3374$, $p = 0.7394$]; UCP1 [$t_{2,21} = 1.581$, $p = 0.129$]; TH [$t_{2,21} = 0.9625$, $p = 0.35$] (Figure 1.8). NPY, Y1R and DPPIV were expressed in most of the individuals under basal conditions; while Y2R was expressed only in C57BL6J and Y5R expression was not found in any animal (Figure 1.9). The sympathetic marker genes β3-AR and UCP1 were expressed in most of the individuals while TH had a low frequency of expression in general (Figure 1.9). The pro-adipogenic and diabetogenic gene PPARγ [Lehrke and Lazar, 2005] was expressed in all mice independent from their genetic background (Figure 1.9).

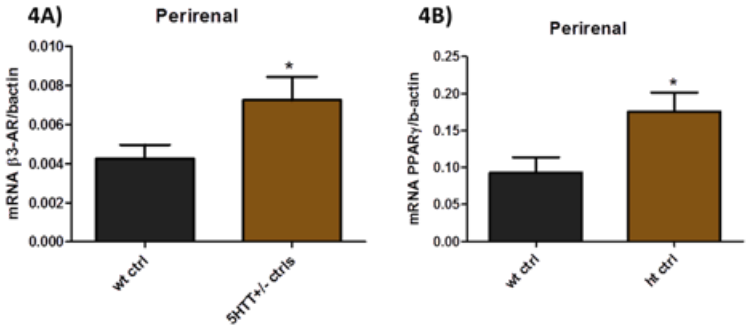
NPY frequency appeared lower in the stressed mice of both genotypes (Figure 1.9) and did not differ in expression before and after stress [$F(2.132) = 3128$, ns] (Figure 1.10). In general, it appeared that stress induce expanded the number of the 5HTT$^{+}/^{-}$ mice expressing the NPY receptors, where Y2R ($p = 0.004$) and Y5R ($p = 0.049$) frequency was significantly increased compared to their controls (Figure 1.9). This could suggest that deletion of one 5HTT allele was enough to increase the transcription of the NPY receptors within PeriWAT. Y1R [$F(0.6585) = 100.4$, ns], Y2R [$F(0.4122) = 0.001136$, ns] and DPPIV [$F(1.050) = 41.27$, ns] expression was very similar among the experimental groups (Figure 1.10).

Surprisingly TH was expressed in many more experimental subjects of both genotypes compared to the other visceral depots (Figure 1.9) but no significant changes were identified after the stress procedure [$F(0.393) = 1575, ns$]. UCP1 mRNA, expressed in all mice (Figure 1.9), did under an up-regulation in both genotypes after chronic stress, higher levels detected in the C57BL6J stressed mice, suggesting more brown like cells formation and more energy consumption [$F(10.13) = 4152$, $p < 0.0001$] (Figure 1.10, 5A). Moreover, the β3-AR gene expressed in all mice (Figure 1.9) also presented a trend in increased expression in the C57BL6J stressed animals only [$F(1.684) = 147.9$, ns] suggesting a higher lipid mobilization compared to their controls and the 5HTT$^{+}/^{-}$ stressed mice.

PPARγ expression was also boosted after stress in the C57BL6J stressed mice [$F(6.025) = 138.9$, $p < 0.001$] (Figure 1.10, 5B).

	C57 Ctrl		5HTT+/- Ctrl	
	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	2E-03	1E-03	6E-03	2E-03
Y1R	3E-03	1E-03	6E-03	1E-03
Y2R	1E-03	6E-04	0E+00	0E+00
Y5R	0E+00	0E+00	0E+00	0E+00
DPPIV	4E-02	1E-02	3E-02	1E-02
β3-AR	4E-03	7E-04	7E-03	1E-03
TH	1E-04	5E-05	3E-04	2E-04
UCP1	3E-01	6E-02	6E-01	1E-01
PRARγ	9E-02	2E-02	2E-01	3E-02

Absolute mRNA expression in Perirenal Fat.
*: C57 vs 5HTT+/-
No significant difference was found between the two genotypes



A) b3-AR mRNA [$t_{2,19}$ =2.29, p=0.03] and
B): the PPARγ gene was significantly higher in 5HTT+/- compared to the C57BL6J mice [$t_{2,21}$ =2.465, p=0.0224.

Figure 1.8: Absolute Expression of analyzed genes in Peritoneal Fat

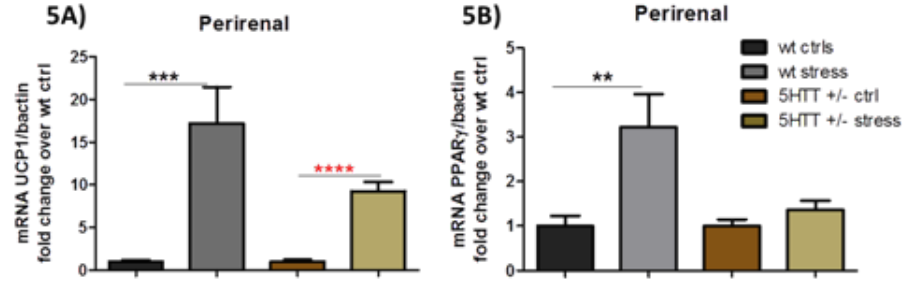
	NPY	Y1R	Y2R	Y5R	DPPIV	β3-AR	TH	UCP1	PPARγ
C57 Ctrl	13/14	14/14	6/14	0/14	13/14	13/14	7/14	14/14	14/14
C57 Stress	6/15	12/15	3/15	3/15	14/15	13/15	4/15	15/15	15/15
5HTT+/- Ctrl	9/9	8/9	0/9	0/9	9/9	8/9	3/9	9/9	9/9
5HTT+/- Stress	7/18	18/18	13/18 ***	6/18 *	18/18	18/18	11/18	18/18	18/18
			p= .0004 ***	p= .049 *					

Frequency of gene expression in Perirenal Fat. Differences in frequency of gene expression between genotype.
*: C57 ctrl vs 5HTT+/- ctrl
*: C57 ctrl vs C57 Sub
*: 5HTT+/- Ctrl vs 5HTT+/- Sub
Stress decreased the frequency of Y5R expression in C57; while in both genotypes stress induce the expression of UCP1

Figure 1.9: Frequency of gene expression in Peritoneal Fat

	NPY	Y1R	Y2R	Y5R	DPPIV	β3-AR	TH	UCP1	PPARγ
C57 Ctrl	1.0 ± 0.7	1.0 ± 0.3	1.0 ± 0.7	0.0 ± 0.0	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.2
C57 Stress	7.8 ± 3.5	1.5 ± 0.5	1.2 ± 1.0	0.0 ± 0.0	0.5 ± 0.1	2.2 ± 0.8	2.9 ± 2.4	17.2 ± 4.2 ***	3.2 ± 0.7 **
5HTT+/- Ctrl	1.0 ± 0.4	0.8 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.7	1.0± 0.2	1.0 ± 0.1
5HTT+/- Stress	5.2 ± 2.2	1.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.1	1.1 ± 0.2	2.1 ± 1.0	9.3 ± 1.1 ****	1.4 ± 0.2

Relative mRNA expression in Perirenal Fat. Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.
*: C57 Ctrl vs C57 Sub
*: 5HTT +/- Ctrl vs 5HTT +/- Sub



A) Following stress UCP1 expression was up regulated in wt and 5HTT +/- mice [F(10.13)=4152,p<0.0001] ;
B): PPARγ expression was increased in wt stressed [F(6.025)=138.9,p<0.001].

Figure 1.10: Relative Expression of analyzed genes in Peritoneal Fat

1.2.2 Effects of chronic psychological stress on the expression of the NPY system, sympathetic markers and PPARγ genes in subcutaneous adipose depots

1.2.2.1 Subcutaneous Inguinal (ScIngWAT)

We detected no difference in the expression of all genes under analysis within the ScIngWAT between the two genotypes NPY [$t_{2,21} = 0.431$, $p = 0.67$]; Y1R [$t_{2,21} = 0.8741$, $p = 0.392$]; Y2R [$t_{2,21} = 1.550$, $p = 0.136$]; Y5R [$t_{2,21} = 0.9303$, $p = 0.3628$]; DPPIV [$t_{2,21} = 0.7991$, $p = 0.4332$]; β3-AR [$t_{2,19} = 1.266$, $p = 0.2194$]; PPARγ [$t_{2,21} = 0.0160$, $p = 0.987$]; UCP1 [$t_{2,21} = 0.734$, $p = 0.471$]; TH [$t_{2,21} = 0.8101$, $p = 0.4270$] (Figure 1.11).

Our analysis showed that following stress more C57BL6J and 5HTT$^{+}/^{-}$ express the NPY genes with a significant increase in frequency of expression in NPY itself ($p = 0.0042$) and Y2R ($p = 0.0463$) in the 5HTT$^{+}/^{-}$ stressed and

Y5R ($p = 0.0271$) in C57BL6J stressed mice (Figure 1.12). NPY expression did not change after the stress exposure in both genotypes [$F(1.382) = 255, ns$] (Figure 1.13). Y1R was significantly down regulated [$F(2.505) = 32.42$, $p < 0.05$] (Figure 1.13, 6A) while Y2R mRNA was significantly was up-regulated [$F(4.680) = 5503$, $p < 0.05$] (Figure 1.13, 6B) in the C57BL6J stressed mice. Since both Y1R and Y2R promote adipogenesis [Kuo et al., 2007, Yang et al., 2008], the up-regulation of one and the simultaneous down regulation of the other could suggest a compensatory mechanism taking place within this tissue to limit the NPYergic pathway of tissue proliferation and expansion. Although, Y5R frequency of expression was increased following stress and its expression did not (Figure 1.12, Figure 1.13). DPPIV's expression resulted homogenous between genotypes as well as treatments [$F(0.8344) = 34.25$, ns].

Since the ScIngWAT tissue is highly responsive to cold induced differentiation of brown-like adipocytes under the control of the SNS [Cousin et al., 1992, Wu et al., 2012, Giordano et al., 2014] we investigated on the expression of β3-AR, expressed in almost all mice (Figure 1.12), and found a significant decreased expression of this sympathetic marker in the stressed subjects of both genotypes [$F(4.057) = 27.13$, $p < 0.05$]. Also UCP1 expression was detected in most of the mice (Figure 1.12) and a trend toward increased expression of this gene was observed in the heterozygous 5HTT stressed mice and a significant increase C57BL6J stressed mice [$F(2.920) = 2209$, $p < 0.05$] (Figure 1.13, 6D). The expression of the pro-adipogenic marker PPARγ appeared down-regulated in the both genotypes, with a significant decrease in the C57BL6J mice [$F(2.682) = 26.18$, $p < 0.05$].

	C57 Ctrl		5HTT+/- Ctrl	
	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	6E-04	2E-04	5E-04	3E-04
Y1R	9E-03	2E-03	6E-03	2E-03
Y2R	1E-03	6E-01	1E-02	7E-01
Y5R	8E-05	6E-05	3E-04	3E-04
DPPIV	4E-02	9E-03	3E-02	6E-03
β3-AR	1E-02	2E-03	2E-02	7E-03
TH	1E-03	7E-04	3E-03	3E-03
UCP1	3E-03	7E-04	4E-03	1E-03
PRARγ	2E-01	4E-02	2E-01	8E-02

Absolute mRNA expression in Sub Inguinal Fat.
*: C57 vs 5HTT+/-
No significant difference was found between the two genotypes

Figure 1.11: Absolute Expression of analyzed genes in SC Inguinal Fat

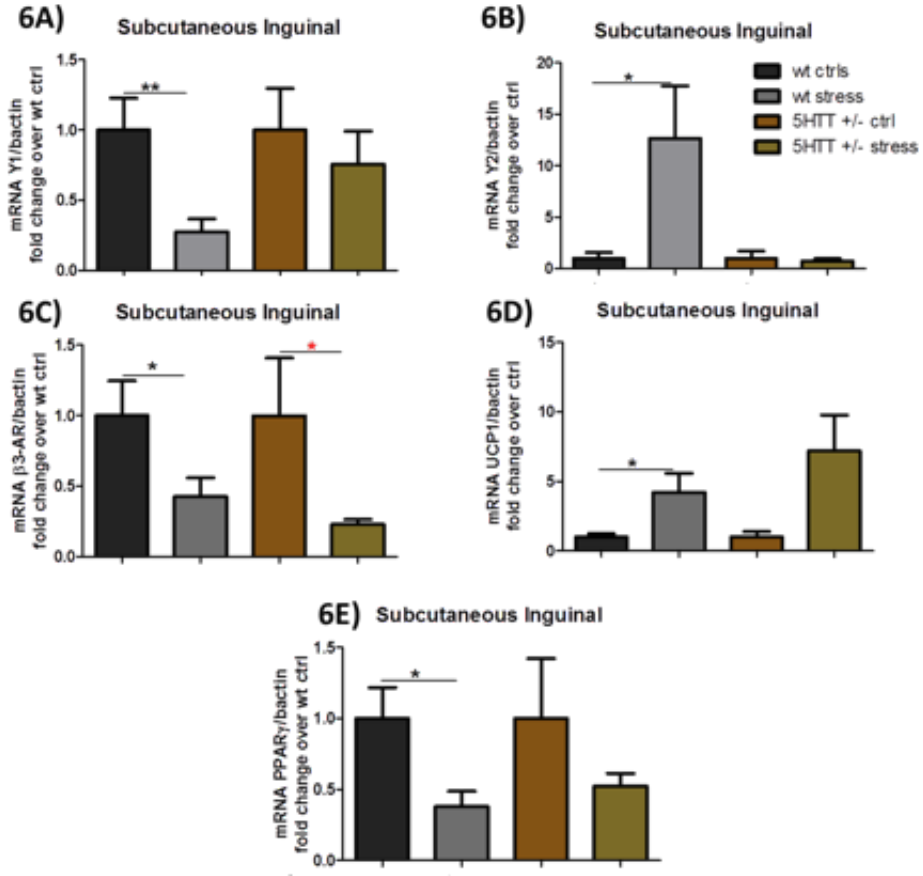
	NPY	Y1R	Y2R	Y5R	DPPIV	β3-AR	TH	UCP1	PPARγ
C57 Ctrl	7/14	13/14	8/14	2/14	14/14	13/14	3/14	13/14	14/14
C57 Stress	12/15	14/15	13/15	8/15	15/15	15/15	3/15	14/15	15/15
5HTT+/- Ctrl	4/9	8/9	4/9	3/9	9/9	7/9	1/9	6/9	9/9
5HTT+/- Stress	16/17	15/17	14/17	12/17	17/17	16/17	6/17	16/17	17/17
	p= .0042**		p= .0463*	p= .0271*					

Frequency of gene expression in Sub Inguinal Fat. Differences in frequency of gene expression between genotype.
*: C57 ctrl vs 5HTT+/- ctrl
*: C57 ctrl vs C57 Sub
*: 5HTT+/- Ctrl vs 5HTT+/- Sub
Stress increased the frequency of NPY and Y2R expression in 5HTT; while Y5R frequency increased in C57BL6J mice.

Figure 1.12: Frequency of gene expression in SC Inguinal Fat

	NPY	Y1R	Y2R	Y5R	DPPIV	β3-AR	TH	UCP1	PPARγ
C57 Ctrl	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.6	1.0 ± 0.7	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.7	1.0 ± 0.2	1.0 ± 0.2
C57 Stress	1.3 ± 0.6	** 0.3 ± 0.1	* 12.7 ± 5.1	30.3 ± 16.9	1.0 ± 0.2	* 0.4 ± 0.1	0.1 ± 0.1	* 4.2 ± 1.2	* 0.4 ± 0.1
5HTT+/- Ctrl	1.0 ± 0.7	1.0 ± 0.3	1.0 ± 0.7	1.0 ± 0.9	1.0 ± 0.2	1.0 ± 0.4	1.0 ± 1.0	1.0 ± 0.4	1.0 ± 0.4
5HTT+/- Stress	2.4 ± 0.6	0.8 ± 0.2	0.8 ± 0.2	16.3 ± 7.2	1.4 ± 0.2	* 0.2 ± 0.03	0.0 ± 0.0	7.2 ± 2.6	0.5 ± 0.1

Relative mRNA expression in Sub. Inguinal Fat. Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.
*: C57 Ctrl vs C57 Sub
*: 5HTT +/- Ctrl vs 5HTT +/- Sub



A) Following stress **Y1R expression was significantly down-regulated in** C57BL6J Y1R [F(2.505)=32.42, p<0.05];

B): while Y2R expression was significantly increased within the same mice Y2R [F(4.680)=5503, p<0.05] .

C) b3-AR expression was significantly increased in both genotypes after stress [F(4.057)=27.13, p<0.05];

D) UCP1 expression was upregulated too in both genotypes [F(2.920)=2209, p<0.05];

E) PPARγ expression was significantly decreased in wt stressed [F(2.682)=26.18, p<0.05].

Figure 1.13: Relative Expression of analyzed genes in SC Inguinal Fat

1.2.2.2 Subcutaneous Scapolar (ScSacpWAT)

We found a higher expression of DPPIV [$t_{2,19} = 3.251, p = 0.0042$] (Figure 1.14, 7A) and PPARγ [$t_{2,21} = 2.364$, $p = 0.03$] (Figure 1.14, 7B) in the 5HTT$^{+}/^{-}$ compared to the C57BL6J mice (Figure 1.14). All other genes under investigation did not differ between genotypes (NPY [$t_{2,19} = 1.444$, $p = 1.650$]; Y1R [$t_{2,21} = 0.065$, $p = 0.949$]; Y2R [$t_{2,20} = 1.018$, $p = 0.3207$]; Y5R [$t_{2,19} = 0.7055$, $p = 0.4891$]; β3-AR [$t_{2,19} = 0.4838$, $p = 0.6341$]; UCP1 [$t_{2,19} = 1.092$, $p = 0.2886$]; TH [$t_{2,19} = 1.641$, $p = 0.1173$]) (Figure 1.14). Except for DPPIV and Y1R that were expressed in most of the mice under basal condition, all other NPY system genes had a low frequency of expression (Figure 1.15). The sympathetic markers as well as PPARγ were highly detected in both genotypes under basal conditions (Figure 1.15).

The ScSacpWAT is closely located to the intrascapolar brown adipose tissue (iBAT) and it is the highest sympathetically innervated and vascularized WAT among the other fat pads analyzed [Cinti, 2012]. We observed no difference in the expression of NPY before and after stress in both genotypes NPY [$F(0.034) = 279.9$, ns]. Following stress the frequency and expression of Y1R was decreased in both genotypes, as significant decreased in the 5HTT$^{+}/^{-}$ stressed mice [$F(1.642) = 64.92$, $p < 0.05$] (Figure 1.16, 8A). NPY has been found to inhibit iBAT's activity through its Y1R, since ScSacpWAT surrounds IBAT the decreased Y1R could be suggestive of increased thremogenic activity within this tissue to cope with the stress perceived [Kushi et al., 1998, Billington et al., 1991]. Parallel to the decreased Y1R in the heterozygous 5HTT stressed group, we also detected a significant decrease in DPPIV's expression [$F(9.056) = 48.92$, $p < 0.05$] (Figure 1.16, 8B), the enzymes that cleaves NPY creating a smaller peptide more specific for Y2R, suggesting more available NPY that binds to the Y1R instead.

Very few animals, independent genotype and treatment did express Y2R and Y5R (Figure 1.15) and no difference was found before and after stress exposure (Y2R [$F(2.103) = 97.81$, ns], Y5R [$F(0.5378) = 2014$, ns]).

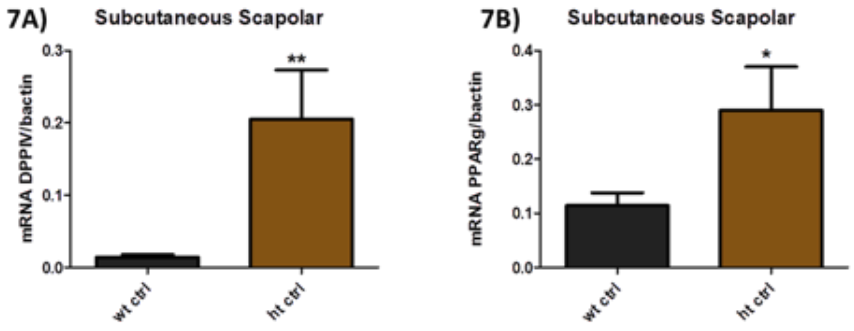
As expected, this white depots had the most mice that the sympathetic marker TH (Figure 1.15), and after stress 5HTT$^{+}/^{-}$ displayed a significant in their expression compared to their untreated littermates TH [$F(1.128) = 109.5$, $p < 0.05$] (Figure 1.16, 8D). This could suggest stress induced-less catecolaminergic innervation in the 5HTT$^{+}/^{-}$. β3-AR, except for one stressed C57BL6J mouse, and UCP1 were expressed in all experimental subject (Figure 1.15). While no difference in UCP1 expression was observed following stress [$F(0.946) = 178.8$, ns], the C57BL6J stressed mice presented

a significant down regulation of their β3-ARs [$F(2.006) = 28.80$, $p < 0.05$] (Figure 1.16, 8C).

Also PPARγ expression detected in all mice (Figure 1.15), was significantly decreased subsequently to stress in both genetic backgrounds [$F(9.163) = 12.72$, $p < 0.0001$] (Figure 1.16, 8E).

	C57 Ctrl		5HTT+/- Ctrl	
	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	1E-04	2E-04	3E-04	1E-04
Y1R	2E-02	1E-02	2E-02	7E-03
Y2R	5E-03	4E-03	6E-04	4E-04
Y5R	6E-04	4E-04	2E-04	2E-04
DPPIV	1E-02	4E-03	2E-01 *	7E-02
β3-AR	1E-02	4E-03	2E-02	3E-03
TH	3E-04	2E-04	1E-03	4E-04
UCP1	2E-01	1E-01	6E-01	3E-01
PRARγ	1E-01	2E-02	3E-01 **	8E-02

Absolute mRNA expression in Sub Scapolar Fat.
*: C57 vs 5HTT+/-
No significant difference was found between the two genotypes



A) DPPIV expression [$t_{2,19}$=3.251, p=0.0042] and
B): PPARγ expression were significantly higher in 5HTT+/- mice [$t_{2,21}$=2.364, p=0.03] compared to C57BL6J mice.

Figure 1.14: Absolute Expression of analyzed genes in Subcutaneous Scapolar Fat

	NPY	Y1R	Y2R	Y5R	DPPIV	β3-AR	TH	UCP1	PPARγ
C57 Ctrl	6/12	12/14	6/12	4/12	12/12	12/12	7/12	12/12	12/12
C57 Stress	7/15	9/15	3/15	3/15	15/15	14/15	9/15	14/15	15/15
5HTT+/- Ctrl	5/9	9/9	3/9	2/9	9/9	9/9	7/9	9/9	9/9
5HTT+/- Stress	11/18	7/10	2/18	1/18	17/18	17/18	8/18	17/18	17/18

Frequency of gene expression in Sub Inguinal Fat. Differences in frequency of gene expression between genotype.
*: C57 ctrl vs 5HTT+/- ctrl
*: C57 ctrl vs C57 Sub
*: 5HTT+/- Ctrl vs 5HTT+/- Sub
No difference was found in frequency of expression among groups.

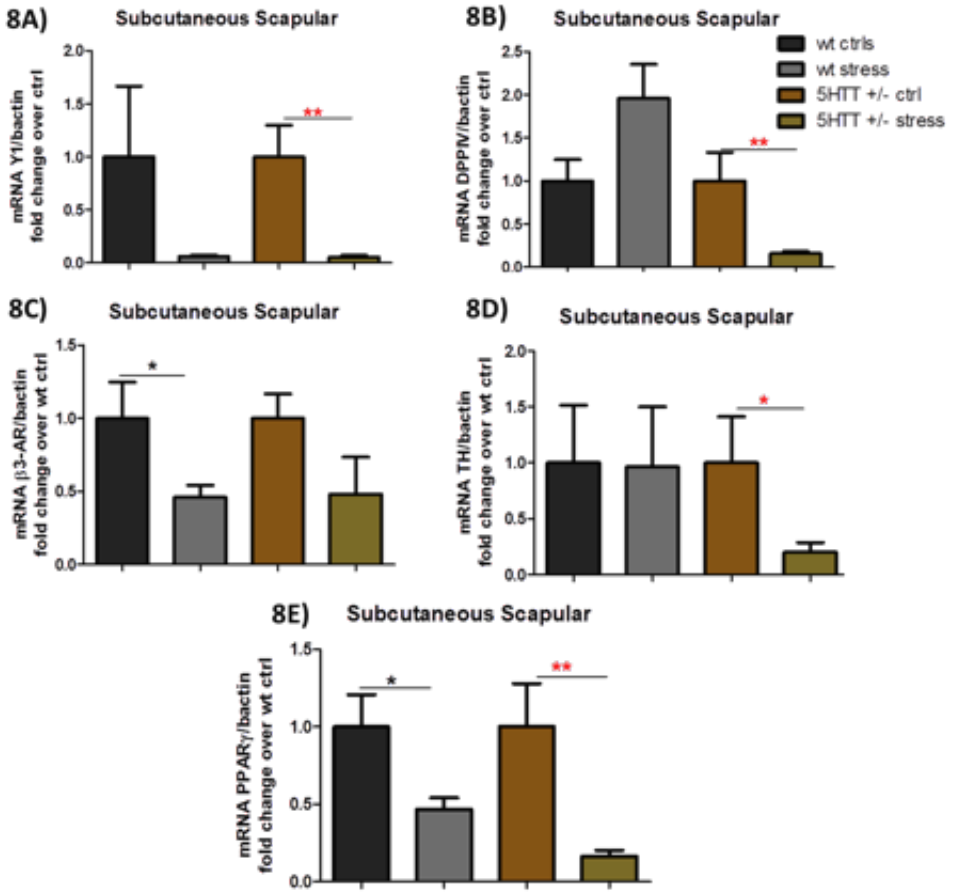
Figure 1.15: Frequency of gene expression in Subcutaneous Scapolar Fat

	NPY	Y1R	Y2R	Y5R	DPPIV	β3-AR	TH	UCP1	PPARγ
C57 Ctrl	1.0 ± 0.4	1.0 ± 0.7	1.0 ± 0.7	1.0 ± 0.7	1.0 ± 0.2	1.0 ± 0.2 *	1.0 ± 0.5	1.0 ± 0.5	1.0 ± 0.2 *
C57 Stress	1.1 ± 0.4	0.1 ± 0.1	0.0 ± 0.002	0.4 ± 0.3	2.0 ± 0.4	0.5 ± 0.1	1.0 ± 0.5	1.8 ± 0.8	0.5 ± 0.1
5HTT+/- Ctrl	1.0 ± 0.4	1.0 ± 1.0 **	1.0 ± 0.6	1.0 ± 0.8	1.0 ± 0.3 **	1.0 ± 0.2	1.0 ± 0.4 *	1.0 ± 0.5	1.0 ± 0.3 **
5HTT+/- Stress	1.2 ± 0.8	0.1 ± 0.02	0.0 ± 0.02	8.1 ± 8.1	0.2 ± 0.03	0.5 ± 0.25	0.2 ± 0.1	0.7 ± 0.2	0.2 ± 0.04

Relative mRNA expression in Sub Scapolar Fat. Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.

*: C57 Ctrl vs C57 Sub

*: 5HTT +/- Ctrl vs 5HTT +/- Sub



A) Following stress Y1R expression was down regulated in both genotypes, but significantly in the 5HTT +/- mice [F(1.642)=64.92, p<0.05],

B): DPPIV was decreased in 5HTT+/- mice [F(9.056)=48.92, p<0.05];

C): β#-AR was down regulated in both genotypes after stress, but a significant drop was found in the C57BL6J stressed mice [F(2.006)=28.80, p<0.05] ;

D): Stress induced a decease in TH mRNA in 5HTT+/- mice only [F(1.128)=109.5, p<0.05];

E); PPARγ expression was increased in both genotypes following stress [F(9.163)=12.72, p<0.0001].

Figure 1.16: Relative Expression of analyzed genes in Subcutaneous Scapolar Fat

1.2.3 Discussion

Chronic activation of the stress response which highly influences the hypothalamus-pituitary-adrencortical and the sympatho-adrenomedullary axis compromising the normal activity of several neurotransmitters and neuropeptides. Among these neurotransmitters neuropeptide Y (NPY) and serotonin (5-hydroxytryptamine;5-HT), are two fundamental molecules involved in chronic stress response and depression like behavior and have been shown to be linked [Karl et al., 2004]. Moreover, since NPY alters the expression and secretion of norepinephrine and serotonin [Shimizu and Bray, 1989] it has been hypothesized that NPY in the CNS may also modulate the serotonergic tone consequently influencing aggression and feeding behavior [Karl et al., 2004]. Having previously demonstrated that partial deletion of 5-HTT gene does increase the vulnerability to chronic psychological stress but maintained the same metabolic consequences as the C57BL6J stressed counterparts [Bartolomucci et al., 2010].

We observed that independent from the genotype chronic social stress did regulate and modulate adipose tissue molecular machinery and function to adequately respond to the development of stress induce obesity resulting in stressed induced fat depot specific gene expression. The main function of the white adipose tissue is to stores excess energy under the form of triglycerides that are released when required by other organs and it does regulate energy homeostasis [Fruhbeck et al., 2001]. The only differences detected between the heterozygous 5HTT mouse and the C57BL6J mice were a higher expression of the sympathetic marker β3-AR in the PeriWAT and ScScapWAT, the two most highly sympathetic innervated white depots. This could suggest that partial deletion of the 5HTT genes did induce a local up-regulation of the sympathetic innervation.

Following three weeks of chronic social stress we here demonstrated that stress alone was able to induce specific molecular changes in the different adipose tissue. Even though increase body weight and body fat percentage to a similar extent in C57BL6J and 5HTT$^{+}/^{-}$ mice (Figure 1.1, 1A, 1B), molecular changes induced by chronic stress were similar between the two genotypes but not identical in the different fat depots. Overall, we noted an increase in genes involved in energy breakdown such as UCP1, while the C57BL6J stressed mice went through more of these molecular changes within the ScIngWAT (Figure 1.13), the 5HTT$^{+}/^{-}$ mice presented more alteration in ScScapWAT (Figure 1.16). Except for the EpiWAT where no expression of UCP1 was detected before and after stress exposure, all other white depots, mainly in the RetroWAT, PeriWATA and ScIngWAT we observed stress induce up-regulation of this key brown adipocyte marker [Cannon and Nedergaard,

2004]. These data suggest that stress initiated a transdifferentiation process, from white-to-brown adipose cells allowing for more energy dissipation capable to breakdown more energy to cope with stress [Whittle and Vidal-Puig, 2013]. Overall we did not find a clear up-regulation of the NPYergic system within any visceral fat depots subsequently from chronic psychosocial stress. NPY regulates energy balance by decreasing energy expenditure as thermogenesis in BAT [Kotz et al., 2000] acting trough its Y1R [Kushi et al., 1998, Billington et al., 1991]. We found a significant decrease in expression of Y1R in both genetic backgrounds in ScScapWAT suggesting that chronic stress did limit the inhibitory effect of NPY [Kushi et al., 1998, Billington et al., 1991]. We detected an up-regulation of the PPARγ gene, which plays a role in the transcriptional regulation of metabolic pathways such as adipogenesis and β-oxidation of fatty acids is also [Tontonoz et al., 1994] in the visceral depots while a decreased expression in the subcutaneous tissue, suggesting less glucose sensitivity in visceral fat given by stress but at the same time this sensitivity was boosted in the ScWAT [Olefsky and Saltiel, 2000, Lehrke and Lazar, 2005]. Moreover, PPARγ is also known to induce UCP1 expression [Petrovic et al., 2010, Ryan et al., 2011], therefore the contemporary stress induction of PPARγ and UCP1 as found in PerWAT could support this hypothesis suggesting that within this depots chronic stress induced increased lipolysis and thermogenesis in C57BL6J stressed animals.

In conclusion, our findings indicate that chronic stress induced a complex profile of pro-adipogenic and lipolytic-markers' expression with a fat depot-specificity. Pro-adipogetic pathways, i.e. NPY, its Y2R and PPARγ were mostly over-expressed in the visceral depots of stressed mice, primarily in the 5HTT$^{+}/^{-}$ stressed mice. On the contrary, the subcutaneus inguinal depot did not show a clear increase of NPY genes together with a decreased expression of PPARγ, but it presented an increase in UCP1 expression (suggesting transdifferentiation of white into brown adipocytes), giving this tissue a mixed catabolic and anabolic profile. Sympathetic-responsive pads, such as retroperitoneal, perirenal and subcutaneus scapular, also presented a mixed profile with a significant increase in the sympathetic-related markers such as TH (rarely found in adipose as mRNA) and UCP1 in the stressed animals often in the presence of decreased NPYergic and PPARγ expression; a profile suggesting catabolic state and increased lipolysis within these depots. This is the first report showing chronic psychosocial stress-induced fat depot-specific expression of the NPY system, sympathetic-related and adipogenic markers. Our data suggest that partial deletion of the 5HTT gene does affect the molecular mechanism of the different fat depots but chronic stress alone plays a fundamental role. Moreover, we show that vulnerability vs. resilience to obesity depends upon the complex balance of pro-adipogenic/lipolytic and

neurogenic pathways' expression, occurring in different fat pads. Here we define a dynamic role for adipose tissue that can adapt to the environmental condition such as stress.

1.3 Supplementary figures

	Epidydimal							
	C57 Ctrl		C57 Sub		5HTT+/- Ctrl		5HTT+/- Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	2E-03	9E-04	1E-01	7E-02	2E-03	8E-04	2E-02	9E-03
Y1R	3E-03	1E-03	3E-03	1E-03	1E-03	4E-04	4E-03	8E-04
Y2R	2E-03	2E-03	7E-03	5E-03	2E-04	1E-04	1E-02	6E-03
Y5R	4E-04	2E-04	1E-03	1E-03	1E-04	1E-04	2E-03	9E-04
DPPIV	9E-02	3E-02	5E-02	2E-02	9E-02	2E-02	8E-02	3E-02
β3-AR	5E-02	3E-02	2E-02	4E-03	2E-02	1E-02	2E-02	3E-03
TH	0E+00	0E+00	0E+00	0E+00	0E+00	0E+00	0E+00	0E+00
UCP1	0E+00	0E+00	1E-03	9E-04	0E+00	0E+00	1E-03	8E-04
PRARγ	4E-02	1E-02	4E-01	8E-02	3E-02	7E-03	3E-01	4E-02

Figure 1.17: Absolute Expression of analyzed genes in Epidydimal Fat

	Retroperitoneal							
	C57 Ctrl		C57 Sub		5HTT+/- Ctrl		5HTT+/- Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	7E-05	3E-05	1E-03	6E-04	8E-05	4E-05	2E-03	7E-04
Y1R	3E-04	6E-05	3E-04	2E-04	3E-04	6E-05	6E-04	6E-04
Y2R	4E-03	3E-03	9E-03	7E-03	4E-05	2E-05	3E-02	1E-02
Y5R	2E-06	2E-06	4E-05	3E-05	3E-05	3E-05	2E-04	1E-04
DPPIV	9E-03	2E-03	5E-02	9E-03	7E-03	2E-03	7E-02	2E-02
β3-AR	8E-03	1E-03	6E-03	1E-03	7E-03	2E-03	6E-03	1E-03
TH	2E-06	2E-06	0E+00	0E+00	2E-06	2E-06	2E-05	2E-05
UCP1	2E-03	7E-04	7E-02	3E-02	1E-03	3E-04	6E-02	4E-02
PRARγ	5E-02	7E-03	2E-01	2E-01	5E-02	9E-03	3E-01	2E-01

Figure 1.18: Absolute Expression of analyzed genes in Retroperitoneal Fat

	Perirenal							
	C57 Ctrl		C57 Sub		5HTT+/- Ctrl		5HTT+/- Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	2E-03	1E-03	1E-02	6E-03	6E-03	2E-03	3E-02	1E-02
Y1R	3E-03	1E-03	6E-03	2E-03	6E-03	1E-03	7E-03	2E-03
Y2R	1E-03	6E-04	1E-03	1E-03	0E+00	0E+00	2E-03	2E-03
Y5R	0E+00	0E+00	2E-05	1E-05	0E+00	0E+00	5E-05	4E-05
DPPIV	4E-02	1E-02	2E-02	3E-03	3E-02	1E-02	2E-02	3E-03
β3-AR	4E-03	7E-04	9E-03	3E-03	7E-03	1E-03	8E-03	1E-03
TH	1E-04	5E-05	4E-04	4E-04	3E-04	2E-04	7E-04	3E-04
UCP1	3E-01	6E-02	6E+00	1E+00	6E-01	1E-01	5E+00	6E-01
PRARγ	9E-02	2E-02	3E-01	7E-02	2E-01	3E-02	2E-01	4E-02

Figure 1.19: Absolute Expression of analyzed genes in Perirenal Fat

	Subcutaneous Inguinal							
	C57 Ctrl		C57 Sub		5HTT+/- Ctrl		5HTT+/- Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	6E-04	2E-04	9E-04	4E-04	5E-04	3E-04	1E-03	3E-04
Y1R	9E-03	2E-03	2E-03	8E-04	6E-03	2E-03	5E-03	1E-03
Y2R	1E-03	6E-01	2E-02	5E+00	1E-02	7E-01	8E-03	2E-01
Y5R	8E-05	6E-05	2E-03	1E-03	3E-04	3E-04	5E-03	2E-03
DPPIV	4E-02	9E-03	4E-02	8E-03	3E-02	6E-03	4E-02	6E-03
β3-AR	1E-02	2E-03	4E-03	1E-03	2E-02	7E-03	4E-03	6E-04
TH	1E-03	7E-04	1E-04	1E-04	3E-03	3E-03	4E-05	2E-05
UCP1	3E-03	7E-04	1E-02	4E-03	4E-03	1E-03	3E-02	1E-02
PRARγ	2E-01	4E-02	7E-02	2E-02	2E-01	8E-02	9E-02	2E-02

Figure 1.20: Absolute Expression of analyzed genes in SC Inguinal Fat

	Subcutaneous Scapolar							
	C57 Ctrl		C57 Sub		5HTT+/- Ctrl		5HTT+/- Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	1E-04	2E-04	1E-04	5E-05	3E-04	1E-04	4E-04	2E-04
Y1R	2E-02	1E-02	1E-03	3E-04	2E-02	7E-03	1E-03	4E-04
Y2R	5E-03	4E-03	2E-05	1E-05	6E-04	4E-04	2E-05	1E-05
Y5R	6E-04	4E-04	2E-04	2E-04	2E-04	2E-04	2E-03	2E-03
DPPIV	1E-02	4E-03	3E-02	6E-03	2E-01	7E-02	3E-02	5E-03
β3-AR	1E-02	4E-03	7E-03	1E-03	2E-02	3E-03	8E-03	4E-03
TH	3E-04	2E-04	3E-04	2E-04	1E-03	4E-04	2E-04	8E-05
UCP1	2E-01	1E-01	5E-01	2E-01	6E-01	3E-01	4E-01	1E-01
PRARγ	1E-01	2E-02	5E-02	8E-03	3E-01	8E-02	5E-02	1E-02

Figure 1.21: Absolute Expression of analyzed genes in SC Scapolar Fat

Chapter 2

Characterization of key pro-adipogenic, pro-lipolytic, sympathetic and insulin signaling markers in Dominant and Subordinate mice under chronic stress and high fat diet

2.1 Introduction

Chronic exposure to stress has been associated with psychological, physiological, behavioral, neuroendocrine and immunological changes. We have previously shown that our model of chronic psychosocial stress that has a high ethological validity, associate to a high fat regime induces the development of obesity and the metabolic syndrome symptoms in CD1 subordinate (Sub) [Bartolomucci et al., 2009, Sanghez et al., 2013] (Figure 2.1). Dominant (Dom) mice, on the other hand, despite being hyperphagic, resulted resistant to the effects of chronic stress displaying a negative energy balance phenotype and healthy metabolic profile even when exposed to HFD [Bartolomucci et al., 2009, Sanghez et al., 2013] (Figure 2.1).

The aim of this study was to investigate the underlying changes of candidate genes involved in lipolysis, adipogenesis and sympathetic markers in three different fat depots (epididymal, subcutaneus inguinal and brown adipose tissue), as well as insulin signaling markers in skeletal muscle and liver tissue. Indeed we previously established that the metabolic changes observed in subordinate mice (Figure 2.1) were associated with molecular changes in liver and skeletal muscle downstream of insulin receptor substrates (IRS) and peroxisome proliferator-activated receptors (PPAR) pathways, indicative of insulin resistance ([Sanghez et al., 2013], unpublished).

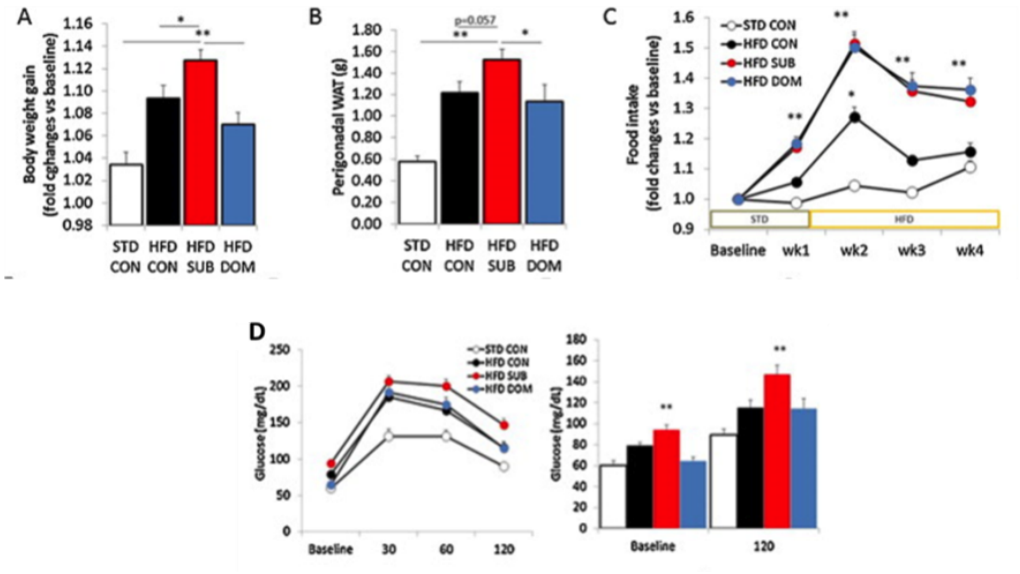


Figure 2.1: Metabolic consequences of chronic psychosocial stress in Dom and Sub CD1 male mice [Sanghez et al., 2013]

2.2 Results

2.2.1 Molecular consequences of stress and HFD in visceral and subcutaneous inguinal depots

After establishing the metabolic consequences of chronic stress in Dom and Sub mice the next question we asked was whether these stressed individuals did display any genetic changes that could be related to the metabolic and physiological profile.

Our data show that within the **epididymal fat** depot of CD1 mice, NPY as well as its Y1R and Y2R receptors and the main enzyme DPPIV, that cleaves NPY to a shorter peptide more specific to the Y2R receptor, are expressed in all control and Sub mice, while one out of five of the Dom mice did not express NPY, Y1R and Y2R (Figure 2.2). None of the above mentioned genes is affected by the experimental treatment (Figure 2.3), independent from their rank NPY [$F(0.4089) = 22.25$, ns], Y1R [$F(0.9136) = 9.848$, ns], Y2R [$F(0.5780) = 34.51$, ns], DPPIV [$F(1.310) = 5.872$, ns].

No significant difference was found in the β3-AR [$F(0.07622) = 5.330$, ns] and UCP1 [$F(1.316) = 9.168$, ns] expression among the experimental groups, while both genes were expressed in all mice (Figure 2.2 and Figure 2.3). Even though expressed in all mice, the PPARγ gene, a pro-adipogenic/diabetogenic marker [Auwerx, 1999], had a tendency of increased expression in Sub mice compared to controls and Dom mice suggesting differentiation and consequently accumulation of more adipocytes [$F(1.329) = 109.8$, ns]. Sub individuals had less frequency of α2-AR gene expression compared to controls; only one out of four animals expressed this anti-lipolytic gene ($p = 0.01$), (Figure 2.2) but without any difference in relative expression [F(1.776)=41.91,ns]. Finally, IRS1 and IRS2 were expressed in all mice (Figure 2.2) and the Sub mice showed a tendency in increased expression of both insulin signaling markers IRS1 [$F(1.179) = 429.7$, ns], IRS2 [$F(1.1776) = 41.91$, ns].

A similar profile to the epididymal fat was found in the **SC inguinal fat**. Starting with the frequency of expression, not all control mice expressed the NPY genes and following stress the frequency in expression did not differ among experimental groups (Figure 2.4). All Dom mice expressed NPY, Y1R and DPPIV, while all Sub mice expressed Y2R and DPPIV (Figure 2.4). No difference was in expression levels in any of the NPY system genes analyzed NPY [$F(0.8565) = 199.6$, ns], Y1R [$F(1.935) = 11.01$, ns], Y2R [$F(1.113) = 16.86$, ns], DPPIV [$F(0.7792) = 8.511$, ns] (Figure 2.5).

	NPY	Y1R	Y2R	DPPIV	b3-AR	a2-AR	IRS1	IRS2	UCP1	PPARg
CD1 Ctrl	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
CD1 Dom	4/5	4/5	4/5	5/5	5/5	4/5	5/5	5/5	5/5	5/5
CD1 Sub	4/4	4/4	4/4	4/4	4/4	1/4 *	4/4	4/4	4/4	4/4

p=0.01

Differences in frequency of gene expression between genotype and treatment.

*: CD1 Ctrl vs CD1 Dom
*: CD1 Ctrl vs CD1 Sub
*: CD1 Dom vs CD1 Sub

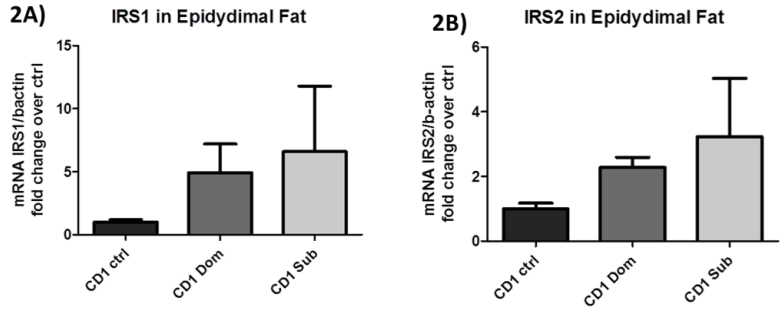
Sub mice have a significantly lower frequency of expression of the α2-AR gene compared to controls.

Figure 2.2: Frequency of gene expression in Epididymal Fat

	NPY	Y1R	Y2R	DPPIV	b3-AR	a2-AR	IRS1	IRS2	UCP1	PPARg
CD1 Ctrl	1.0 ± 0.9	1.0 ± 0.5	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.5	1.0 ± 0.3
CD1 Dom	0.1 ± 0.08	0.3 ± 0.2	2.2 ± 1.3	0.6 ± 0.1	2.2 ± 1.3	5.8 ± 4.1	4.9 ± 2.3	2.3 ± 0.3	0.4 ± 0.2	1.8 ± 0.3
CD1 Sub	0.6 ± 0.6	0.3 ± 0.2	1.5 ± 0.9	0.2 ± 0.1	0.9 ± 0.2	0.5 ± 0.5	5.3 ± 4.2	2.6 ± 1.5	0.2 ± 0.1	4.1 ± 3.0

Relative mRNA expression in Epididymal Fat. Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group. No significant difference among groups is found in any of the analyzed genes.

*: CD1 Ctrl vs CD1 Dom
*: CD1 Ctrl vs CD1 Sub
*: CD1 Dom vs CD1 Sub



A) Tendency of increased IRS1 [F(1.179)=429.7, ns] and
B) IRS2 expression in Sub individuals [F(1.1776)=41.91, ns].

Figure 2.3: Relative Expression of analyzed genes in Epididymal Fat

The β3-AR, did not significantly differ among the three experimental groups [$F(0.9914) = 11.17$, ns]. UCP1 was expressed in all subjects (Figure 2.4) and the Dom mice had a significantly increase compared to control and Sub groups suggesting that stress induces an increase in browning of SC inguinal white adipocytes toward beige/brite adipocytes [Guerra et al., 1998,Cao

et al., 2011] [F(6.427)=662.7, p<0.05] (Figure 2.5, 4A). PPARγ and the α2-AR gene were expressed in all stressed mice (Figure 2.4) and no differences in expression were detected among groups PPARγ [$F(1.236) = 71.62$, ns], α2-AR [$F(2.173) = 329.6$, ns].

The frequency of animals expressing IRS1 within the SC Inguinal was low (Figure 2.4) and no significant different in expression levels was found among groups [$F(0.6288) = 30.19$, ns]. IRS2 was expressed in all subjects did differ either in expression levels among groups [$F(0.8140) = 10.52$, ns] (Figure 2.4).

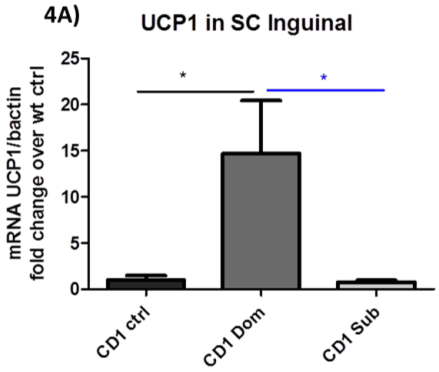
	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1 Ctrl	3/6	4/6	5/6	4/6	5/6	4/6	2/6	5/6	6/6	5/6
CD1 Dom	5/5	5/5	2/5	5/5	5/5	5/5	3/5	5/5	5/5	5/5
CD1 Sub	3/5	3/5	5/5	5/5	4/5	5/5	3/5	5/5	5/5	5/5

Differences in frequency of gene expression between genotype and treatment.
*: CD1 Ctrl vs CD1 Dom
*: CD1 Ctrl vs CD1 Sub
*: CD1 Dom vs CD1 Sub
No difference is found in the frequency of expression among the experimental groups.

Figure 2.4: Frequency of gene expression in SC Inguinal Fat

	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1 Ctrl	1.0 ± 0.9	1.0 ± 0.4	1.0 ± 0.6	1.0 ± 0.3	1.0 ± 0.6	1.0 ± 0.9	1.0 ± 1.0	1.0 ± 0.5	1.0 ± 0.5	1.0 ± 0.5
CD1 Dom	0.3 ± 0.2	1.5 ± 0.5	0.0 ± 0.0	1.5 ± 0.4	0.8 ± 0.2	0.1 ± 0.04	0.0 ± 0.01	0.3 ± 0.1	14.7 ± 5.7	1.6 ± 0.8
CD1 Sub	0.1 ± 0.1	0.3 ± 0.2	0.8 ± 0.5	0.9 ± 0.4	0.2 ± 0.1	0.7 ± 0.4	0.3 ± 0.2	0.5 ± 0.3	0.8 ± 0.2	3.2 ± 1.6

Relative mRNA expression in Sc Inguinal fat. Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.
*: CD1 Ctrl vs CD1 Dom
*: CD1 Ctrl vs CD1 Sub
*: CD1 Dom vs CD1 Sub



A) Following 4 weeks of chronic psychosocial UCP1 expression is significantly up-regulated in CD1 Dom compared to their controls and Sub mice [F(6.427)=662.7, p<0.05].

Figure 2.5: Relative Expression of analyzed genes in SC Inguinal Fat

2.2.2 Stress and HFD altered brown adipose tissue in subordinate mice

The **brown adipose tissue (BAT)** is highly innervated by the sympathetic nervous system and responsible for adaptive thermogenesis [Bartness et al., 2010b, Morrison et al., 2012]. In general, mice from all three experimental groups did express all the candidate genes analyzed (Figure 2.6).

The NPY system gene expression did undergo modification following three weeks of chronic, mainly in subordinate mice. More specifically, compared to the control group Y1R expression was significantly down regulated [Y1R: $F(5.135)=0.9992$, $p<0.05$] (Figure 2.7, 6A), while DPPIV expression was significantly up-regulated in the Sub mice [$F(5.825) = 0.8151$, ns] (Figure 2.7, 6B). In BAT, it has been shown that NPY acting through its Y1R inhibits the activity of the tissue; therefore a decreased expression of Y1R in Sub mice does suggest that stress induces an increase in thermogenic activity [Kushi et al., 1998]. NPY [$F(1.648) = 7.669$, ns] and Y2R [$F(0.6663) = 6.759$, ns] expression was not different among groups (Figure 2.7).

Based on increased temperature and energy expenditure in Dom mice

[Sanghez et al., 2013] we hypothesized an increased expression of UCP1 within this exponential group, but no significant difference was detected [$F(1.072) = 15.36$, ns]. It must be noted however that the UCP1 mRNA undergoes a rapid turnover and it is not infrequent to observe increased thermogenesis and unaltered gene expression. Unfortunately we have not quantified the UCP-1 protein level.

Also, β3-AR [$F(1.856) = 1.801$, ns], PPARγ [$F(1.468) = 9.497$, ns], α2-AR [$F(0.1971) = 1.930$, ns], IRS1 [$F(0.1826) = 2.610$, ns] and IRS2 [$F(1.790) = 3.221$, ns] were not different among the groups (Figure 2.7).

	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1 Ctrl	6/6	6/6	4/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
CD1 Dom	5/5	5/5	3/5	5/5	5/5	5/5	3/5	5/5	5/5	5/5
CD1 Sub	5/5	5/5	3/5	5/5	4/5	5/5	3/5	5/5	5/5	5/5

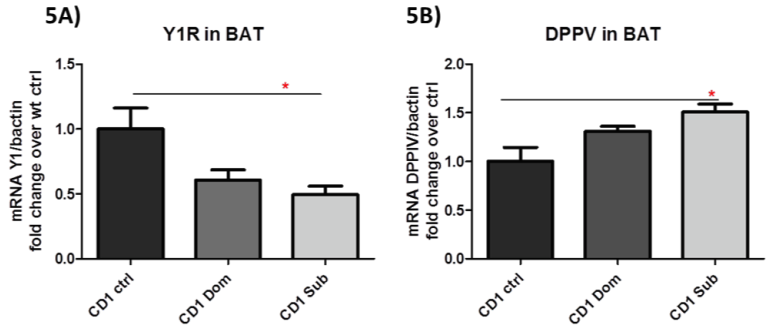
Differences in frequency of gene expression between genotype and treatment.
*: CD1 Ctrl vs CD1 Dom
*: CD1 Ctrl vs CD1 Sub
*: CD1 Dom vs CD1 Sub
No significant difference was found among experimental groups.

Figure 2.6: Frequency of gene expression in Brown Adipose Fat

	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1 Ctrl	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1
CD1 Dom	0.2 ± 0.04	0.6 ± 0.1	0.6 ± 0.3	1.3 ± 0.1	0.6 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	1.1 ± 0.2	1.2 ± 0.3	0.8 ± 0.1
CD1 Sub	0.6 ± 0.3	0.5 ± 0.1	0.5 ± 0.2	1.5 ± 0.1	0.8 ± 0.2	0.9 ± 0.1	0.8 ± 0.2	0.5 ± 0.2	1.9 ± 0.8	0.7 ± 0.1

Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.

*: CD1 Ctrl vs CD1 Dom
*: CD1 Ctrl vs CD1 Sub
*: CD1 Dom vs CD1 Sub



Following 4 weeks of chronic psychosocial **A)** Y1R expression is dowr-egulated in both stress group but significantly different in CD1 Sub mice [F(5.135)=1.789, p<0.05].

B): Compared to their controls, CD1 Sub mice display a significant increase in the expression of DPPIV F(5.825)=1.545, p<0.05].

Figure 2.7: Relative Expression of analyzed genes in Brown Adipose Fat

2.3 Chronic psychosocial stress induced insulin signaling impairment in subordinate mice

Dysregulation of Insulin receptor substrate (IRS) proteins signaling and function do lead to diabetes [Kubota et al., 2008]. All mice did express IRS1 and IRS2 in liver and muscle (Figure 2.8).

The expression of IRS1 in liver of Sub mice has a tendency of decreased expression compared to controls ($p = 0.0598$), while it is significantly down-regulated compared to Dom that show a clear up regulation of this protein following stress [$F(6.298) = 14.39$, $p < 0.05$] (Figure 2.9, 8A). The expression pattern of IRS2 was very similar to IRS1, up-regulated in Dom mice and down-regulated in Sub mice [$F(7.440) = 3.250$, $p < 0.05$] (Figure 2.9, 8B). These data suggest that stress induces insulin insensitivity in Sub mice.

No significant difference among groups was found in muscle tissue for both insulin signaling proteins in frequency and level of expression (IRS1

$[F(4.290) = 2.146, ns]$, IRS2 $[F(0.7338) = 3.262, ns]$ (Figure 2.8, Figure 2.9).

Liver

	IRS1	IRS2
CD1 Ctrl	4/4	4/4
CD1 Dom	4/4	4/4
CD1 Sub	5/5	5/5

Muscle

	IRS1	IRS2
CD1 Ctrl	6/6	6/6
CD1 Dom	5/5	5/5
CD1 Sub	5/5	5/5

Differences in frequency of gene expression between genotype and treatment.
*: CD1 Ctrl vs CD1 Dom
*: CD1 Ctrl vs CD1 Sub
*: CD1 Dom vs CD1 Sub
No significant difference is found among experimental groups.

Figure 2.8: Frequency of gene expression in Liver and Muscle

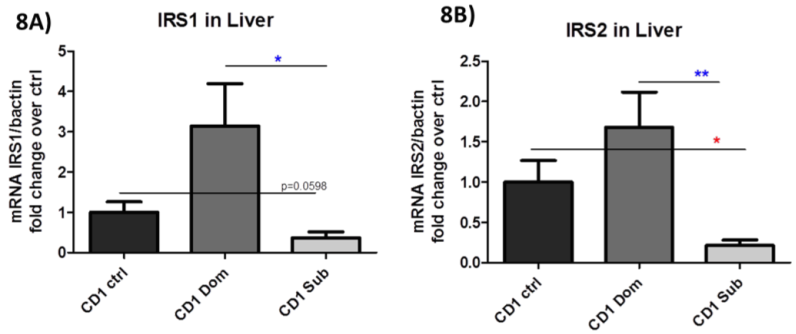
Liver

	IRS1	IRS2
CD1 Ctrl	1.0 ± 0.3	1.0 ± 0.3
CD1 Dom	3.1 ± 1.0	1.7 ± 0.4
CD1 Sub	* 0.4 ± 0.1	** * 0.2 ± 0.1

Muscle

	IRS1	IRS2
CD1 Ctrl	1.0 ± 0.2	1.0 ± 0.1
CD1 Dom	0.3 ± 0.1	0.9 ± 0.5
CD1 Sub	0.4 ± 0.2	0.6 ± 0.1

Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.
*: CD1 Ctrl vs CD1 Dom
*: CD1 Ctrl vs CD1 Sub
*: CD1 Dom vs CD1 Sub



A) Dom mice have an increased expression of IRS1 while Sub mice show a down regulation of IRS1 in liver F(2.986)=14.39, p<0.05].
B) Similar pattern to IRS1 is found for IRS2 in liver F(7.440)=3.250, p<0.05].

Figure 2.9: Relative Expression of analyzed genes in Liver and Muscle

2.4 Discussion

Our data suggest that exposure to chronic psychosocial stress and HFD does alter expression of key genes involved in adipogenesis, metabolic functions and sympathetic innervation within metabolically active tissues. In the visceral and subcutaneous adipose tissue we detected very low expression of NPY, its receptors Y1R and Y2R, and DPPIV independent from stress. The expression and function of NPY has been largely investigated within the central nervous system where NPY is the most potent orexigenic factor promoting hypephagia and obesity in a variety of experimental animal models of obesity [Kalra and Kalra, 2004]. Very little is known about the peripheral effects of neuropeptide Y. For instance, Kuo et al [Kumari et al., 2007] show that in the 129X1/SvJ mice strain chronic cold and chronic social defeat stress associated to a HFD does increase mRNA expression of NPY, its Y2R receptors and DPPIV in the subcutaneous (SC) abdominal/ inguinal depot inducing fat deposition. We did not observe any increase in the expression of these genes in CD1 adult male mice.

Following our model of chronic stress we did not observe any up-regulation of the NPY system genes in adult CD1 male mice, which could suggest that the genetic background may play a fundamental role for NPY system to exert it pro-adipogenic effects.

In addition, neither with the epidydimal nor in the SC inguinal fat depots we detected any alteration in the expression of β3-AR following chronic stress. Interestingly, the expression of UCP1 in the SC inguinal depot of Dom mice only was significantly up-regulated. UCP1, mitochondrial-uncoupling protein 1, that uncouples respiration and dissipates chemical energy as heat, is a key molecule of brown adipocytes [Cannon and Nedergaard, 2004, Wu et al., 2013]. It has been shown that there are some UCP1 positive cells within the white adipose depots that upon certain stimulation, such as chronic cold exposure, these cells could take on a BAT phenotype, adipocytes smaller in diameter filled with few lipid droplets and crowed with mitochondria concentration that express UCP1 [Cannon and Nedergaard, 2004, Lo and Sun, 2013]. Our data strongly indicates that a high rank (dominance) does induce browning in their SC inguinal tissue suggesting anti-obesity and antidiabetic effects [Wu et al., 2012, Cao et al., 2011] that could explain the resistance to diet and stress induced obesity and normal insulin sensitivity in spite of hyperphagia in the Dom mice under our model of stress [Sanghez et al., 2013].

On the other hand PPARγ expression Sub mice, a nuclear receptor highly expressed in white adipose tissues, primary responsible for adipocytes differentiation and metabolism [Lehrke and Lazar, 2005, Spiegelman, 1998] had a tendency of increased expression in visceral and subcutaneous adipose tissue

supporting the increase fat accumulation and larger adipocytes previously observed [Bartolomucci et al., 2009,Sanghez et al., 2013]. However, the insulin signaling markers IRS1 and IRS2 in epididymal fat of the Sub mice had a tendency of increased expression suggesting that this fat depot conserved its sensitivity to insulin allowing for lipid accumulation and cell expansion. Together these molecular data could in part explain the physiological data where as a result of chronic psychosocial stress and HDF, only the Sub mice displayed a positive energy balance phenotype; increasing body weight and fat mass [Sanghez et al., 2013]

Thermogenesis, primarily occurs in brown adipose tissue, and it is an essential process in regulating body temperature. BAT thermogenesis is tightly regulated by a neuronal network [Morrison et al., 2012]. One of the central pathways involved in the regulation of BAT's activity is direct projection of hypothalamic NPY/cathecolaminergic neurons to BAT to inhibit the sympathetic nervous activity in BAT consequently decreasing thermogenic activity [Egawa et al., 1991]. It has been shown that NPY acting through the Y1R inhibits BAT's activity [Kushi et al., 1998]. The significant decrease in Y1R expression detected in Sub mice only could suggest hyperactivity of BAT to compensate for the required energy to face the psychological and physical challenge. The expression of pro-adipogenic and anti-lipolytic markers in BAT did not differ either among groups. This could be a result of BAT being a highly metabolically active tissue.

Our analysis of insulin signaling markers IRS1 and IRS2 in peripheral tissue such as the liver show a significantly down-regulated in Sub mice and up-regulation in Dom mice, strongly in support with our physiological data where Sub mice being glucose intolerant have higher fasting glucose levels and an impaired ability of insulin to restore normal glucose levels following a glucose challenge, while Dom mice manifest normal glucose tolerance even though fed a HFD and being hyperphagic [Sanghez et al., 2013]. Decrease in IRS1 and iRS2 signaling is highly associated with the risk of developing peripheral insulin resistance and type 2 diabetes [Petersen and Shulman, 2006]. The tendency of increased expression of both IRS1 and IRS2 in the white adipose of Sub mice mechanistically supports the increased glucose uptake and adipogenesis observed.

In summary we observed that obesity in CD1 male mice was not associated with changes of the NPY system in various white fat depots. On the other hand, the NPY system undergoes modification in BAT, especially in Sub mice where it could indicate increase thermogenic activity. UCP1 increased expression in SC inguinal of Dom mice expression could explain the anti-obesity and anti-diabetic effects found in Dom mice. Lastly, we were able show that only three weeks of our paradigm of chronic stress has a huge molecular

impact on insulin signaling depending on the social rank, up-regulated in Dom mice while down-regulated in Sub mice.

2.5 Supplementary figures

Absolute Expression of analyzed genes in Epidydimal Fat

	CD1 Ctrl		CD1 Dom		CD1 Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	7E-03	5E-03	1E-02	1E-02	5E-03	5E-03
Y1R	3E-03	2E-03	1E-03	5E-04	1E-03	6E-04
Y2R	2E-04	6E-05	4E-04	2E-04	3E-04	2E-04
DPPIV	2E-02	7E-03	1E-02	2E-03	4E-03	5E-04
β3-AR	7E-02	3E-02	1E-01	8E-02	7E-02	9E-03
α2-AR	3E-04	4E-05	2E-03	1E-03	2E-04	2E-04
IRS1	2E-03	3E-04	8E-03	4E-03	1E-02	8E-03
IRS2	2E-02	3E-03	4E-02	5E-03	5E-02	3E-02
UCP1	3E-02	3E-02	1E-02	6E-02	4E-03	1E-01
PRARγ	1E-02	1E-02	7E-03	1E-02	3E-03	1E-01

Supplementary Table 1) Absolute mRNA expression in Epidydimal fat. Absolute values are reported for each experimental.

Absolute Expression of analyzed genes in SC Inguinal Fat

	CD1 Ctrl		CD1 Dom		CD1 Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	4E-04	4E-04	1E-04	8E-05	4E-05	3E-05
Y1R	3E-03	1E-03	4E-03	1E-03	9E-04	5E-04
Y2R	1E-02	7E-03	7E-05	5E-05	9E-03	5E-03
DPPIV	5E-03	2E-03	8E-03	2E-03	5E-03	2E-03
β3-AR	2E-02	1E-02	2E-02	5E-03	5E-03	2E-03
α2-AR	7E-03	7E-03	8E-04	3E-04	5E-03	3E-03
IRS1	2E-03	2E-03	5E-05	2E-05	6E-04	5E-04
IRS2	3E-02	2E-02	1E-02	4E-03	2E-02	9E-03
UCP1	2E-01	1E-01	3E+00	1E+00	2E-01	5E-02
PRARγ	1E-02	6E-03	2E-02	1E-02	4E-02	2E-02

Supplementary Table 2) Absolute mRNA expression in Sc Inguinal fat. Absolute values are reported for each experimental.

Absolute Expression of analyzed genes in Brown Adipose Fat

	CD1 Ctrl		CD1 Dom		CD1 Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	1E-05	4E-06	2E-06	4E-07	6E-06	3E-06
Y1R	6E-04	9E-05	3E-04	4E-05	3E-04	4E-05
Y2R	1E-06	5E-07	8E-07	4E-07	7E-07	3E-07
DPPIV	1E-04	2E-05	2E-04	7E-06	2E-04	1E-05
β3-AR	9E-02	2E-02	5E-02	1E-02	8E-02	2E-02
α2-AR	5E-05	1E-05	5E-05	1E-05	5E-05	6E-06
IRS1	7E-03	1E-03	6E-03	2E-03	6E-03	1E-03
IRS2	4E-02	8E-03	4E-02	9E-03	2E-02	7E-03
UCP1	3E+01	5E+00	3E+01	8E+00	5E+01	2E+01
PRARγ	2E-02	3E-03	2E-02	3E-03	2E-02	3E-03

Supplementary Table 3) Absolute mRNA expression in Brown Adipose fat. Absolute values are reported for each experimental.

Absolute Expression of analyzed genes in Liver and Muscle

	Liver					
	CD1 Ctrl		CD1 Dom		CD1 Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
IRS1	1E-08	3E-09	3E-08	1E-08	4E-09	2E-09
IRS2	3E-08	9E-09	5E-08	1E-08	7E-09	2E-09

	Muscle					
	CD1 Ctrl		CD1 Dom		CD1 Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
IRS1	2E-08	4E-09	1E-08	8E-09	7E-09	3E-09
IRS2	2E-08	2E-09	6E-08	4E-08	1E-08	3E-09

Supplementary Table 4) Absolute mRNA expression in Liver and Muscle. Absolute values are reported for each experimental.

Chapter 3

Basal and stressed induced modification in pro-adipogenic, pro-lipolytic, sympathetic and insulin signaling markers' expression among CD1, 129SvEv, C57BL/6J male mice under high fat diet

3.1 Introduction

The link between chronic stress exposure and its deleterious consequences have been well established and described in several studies [Bjorntorp, 2001,Brunner et al., 2007], yet individuals greatly differ in their vulnerability to stress [Bornstein et al., 2006, Uchida et al., 2011]. Indeed, it has been observed that some individuals can cope better than others to stress, clearly suggesting different vulnerability to stress. Uncovering the molecular mechanisms underlying the individual susceptibility to stress will allow us to better understand why some individuals can cope better than others. Many animal models, mostly rodent models, are used to study the behavioral, neuroendocrinal and physiological consequences to stress. Individual genetic susceptibility to stress has been largely investigated mainly comparing inbred strain of mice under chronic social stress [Crawley et al., 1997, Parmigiani et al., 1999]. The choice of inbred mouse strains is because they are isogenic (genetically identical) and have low genetic variability [Beck et al., 2000]; while outbred mice have high heterozigosity [Silver, 1981]. These strains of mice, often used as experimental subjects in stress studies, do have high behavioral and metabolic variability that become hard to interpret since different genetic background does affects gene expression levels and originate different outcomes [Francis et al., 2003].

The aim of this study was to focus on the genetic component that causes strain-dependent vulnerability to our ethological model of chronic psychosocial stress using three very commonly used mouse strains, the CD1, the C57BL6J and the 129SvEv. We have shown the metabolic changes in all three stains under a high fat diet (HFD) regime and chronic psychosocial in subordinate (Sub) mice ([Bartolomucci et al., 2005, Bartolomucci et al., 2009, Dadomo et al., 2011, Sanghez et al., 2013] unpublished) (Figure 3.1). As described in chapter two, subordination stress induces hyperphagia, body weight and fat mass gain and glucose intolerance in CD1 Sub mice (Figure 3.1, chapter 2). The inbred subordinate mice undergo different metabolic changes compared to the outbred CD1strain as well as between them. Subordinates from both strains, C57BL6J and 129SvEv, increase body weight and fat mass (Figure 3.1 B, C), however C57BL6J Sub mice do not have increased epidydimal fat weight and food intake contrarily to the 129SvEv Sub mice (Figure 3.1 A, D). Moreover, we observed glucose impairment in C57BL6J Sub mice while 129SvEv Sub mice maintained normal glucose tolerance (Figure 3.1 E). These metabolic data do strongly suggest that genetic background could predispose to the development of metabolic and physiological changes following stress and HFD.

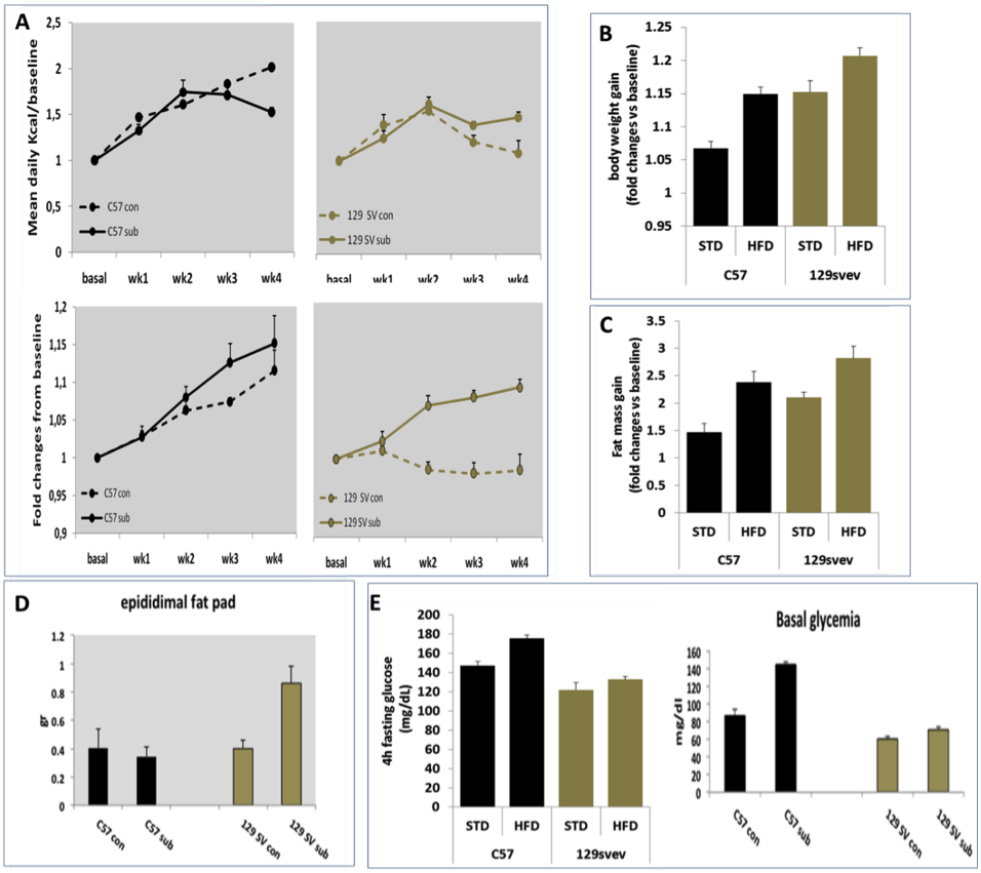


Figure 3.1: Metabolic consequences of chronic psychosocial stress in Sub C57BL6J and 129SvEv male mice. All data in this figue are unpublished

We determined gene expression profiles analyzing metabolically active tissues such as adipose, muscle and liver tissue using the quantitative real time PCR technique. We first wanted to establish the basal expression of the NPY system genes, key pro-lipolytic and insulin signaling markers in genetically different mice under a high fat diet to detect if genetic background affected basal expression. Secondly, we investigated whether and to what extent prolonged psychosocial stress associated with a high fat diet modified the gene expression with the attempt to understand how these tissues physiologically and molecularly respond to our murine model of chronic stress. Mice fed on a standard diet were not included into our analysis since the study focused on the effects of stress paired with a HFD.

3.2 Results

3.2.1 Genetic differences in visceral fat among strains under HFD

We first investigated the basal expression of our genes of interest among different strains.

Within the ***epididymal fat*** depot, the genes part of the NPY system were expressed in all CD1, except for the one mouse that did not express Y1R, the expression of all other genes were detected in all 129SvEv and C57BL/6J mice (Figure 3.2). The C57BL/6J strain did have the highest expression of NPY which was significantly different from the 129SvEv strain NPY [$F(4.276) = 0.0012$, $p < 0.05$] (Figure 3.3, 2A), as well as its receptor Y2R [$F(6.557) = 3.554e-005$, $p < 0.05$] (Figure 3.3, 2B) compared to the 129SvEv and the CD1 strains. The expression of the other two genes part of the NPY system, Y1R and DPPIV did not differ among strains Y1R [$F(2.879) = 0.052$, ns], DPPIV [$F(0.2646) = 0.0015$, ns] (Figure 3.3).

C57BL/6J did also show a significant larger expression of the β3-AR, expressed in all experimental mice, compared to the other two strains but a significant increase in expression compared to the outbred CD1 strain [$F(4.332) = 0.4063$, $p < 0.05$] (Figure 3.3, 2C). On the other hand, UCP1 was expressed in only one out of the three C57BL/6J under investigation (Figure 3.2) and its expression was not different among strains [$F(2.574) = 39.45$, ns].

All mice expressed PPARγ (Figure 3.2), a pro-adipogenic/diabetogenic and the expression was not different among strains [$F(1.917) = 1.547e-005$, ns]. Compared to the 129SvEv and CD1 strains that all expressed the α2-AR anti-lipolytic gene, only two out of the three C57BL/6J expressed α2-AR (Figure 3.2) and the expression was not significantly different among strains [$F(0.2905) = 10.18$, ns].

The same C57BL/6J subject that did not have the expression of Y1R, α2-AR and UCP1 did also lack in expressing IRS1 and IRS2 in the epidydimal fat; while all 129SvEv and CD1 mice displayed the expression of IRS1 and IRS2 (Figure 3.2). No significant difference in expression of IRS1 and IRS2 was detected among strains IRS1 [$F(1.155) = 0.061$, ns], IRS2 [$F(1.895) = 0.006$, ns], but the 129SvEv mice did show a tendency of increased expression of both insulin signaling markers.

	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
C57	3/3	1/3	3/3	3/3	3/3	2/3	2/3	2/3	1/3	3/3
Sv129	6/6	5/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6

Table 1. Frequency of gene expression in Epididymal Fat. Differences in frequency of gene expression between genotype and treatment.
*: CD1 Ctrl vs CD1 Sub
*: C57 Ctrl vs C57 Sub
*: Sv129 Ctrl vs Sv129 Sub
No difference is found in the frequency of expression among strains.

Figure 3.2: Frequency of gene expression in Epididymal Fat

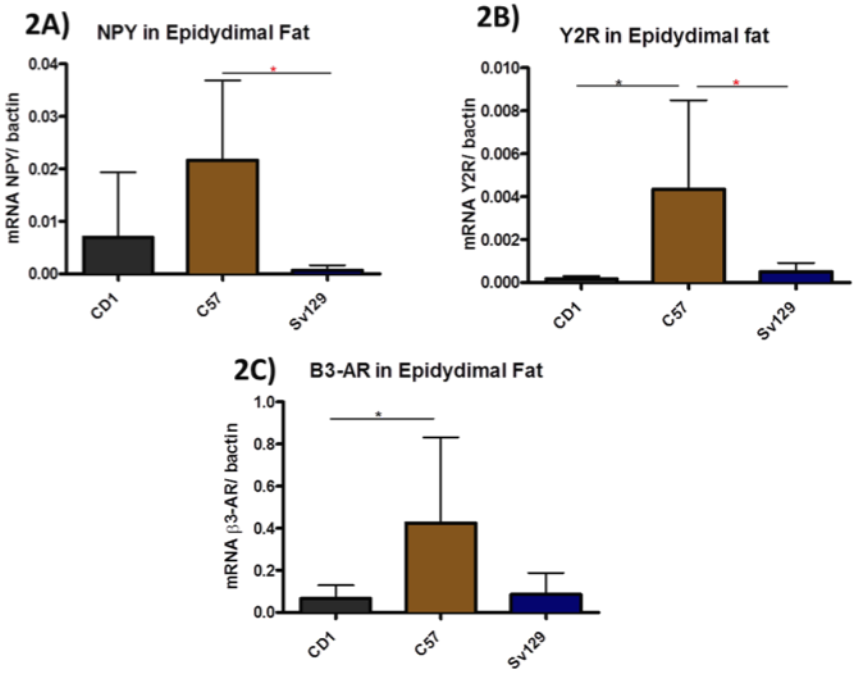
	CD1		c57		Sv129	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	7E-03	5E-03	2E-02	9E-03	7E-04	4E-04
Y1R	3E-03	2E-03	5E-04	5E-04	4E-03	1E-03
Y2R	2E-04	6E-05	4E-03	2E-03	5E-04	2E-04
DPPIV	2E-02	7E-03	1E-02	2E-03	1E-02	2E-03
β3-AR	7E-02	3E-02	4E-01	2E-01	9E-02	4E-02
α2-AR	3E-04	4E-05	2E-03	2E-03	6E-04	1E-04
IRS1	2E-03	3E-04	6E-03	3E-03	1E-02	2E-03
IRS2	2E-02	3E-03	4E-02	2E-02	4E-02	8E-03
UCP1	3E-02	3E-02	6E-04	6E-04	2E+00	1E+00
PRARγ	1E-02	1E-02	9E-02	3E-02	9E-02	4E-02

Absolute mRNA expression in Epididymal Adipose Tissue.

*: CD1 vs C57

*: C57 vs Sv129

*: Sv129 vs CD1



2A) C57 mice have a significantly lower expression of NPY compared to Sv129 [F(4.276)=0.00124, p<0.05].

2B) and the highest expression of Y2R compared to CD1 and Sv129 mice [F(6.557)=3.554e-005, p<0.05];

2C) b3-AR mRNA is significantly higher in C57 mice compared to CD1 F(4.332)=0.4063, p<0.05].

Figure 3.3: Absolute Expression of analyzed genes in Epididymal Tissue

3.2.2 Genetic differences in subcutaneous fat among strains under HFD

The NPY system genes were expressed in all 129SvEv and C57BL/6J mice, besides the one C57BL/6J mouse that did not show expression of Y2R (Figure 3.4). The lack of Y2R expression within this tissue compared to the lack of Y1R in epididymal fat does indicate a different pattern of expression for the NPY receptor genes in different visceral depots of this specific strain. Not all CD1 individuals presented the NPY genes; we detected a much lower frequency of expression compared to the epididymal fat (Figure 3.4). In terms of level of expression, compared to the visceral epididymal depot, the expression of the NPY system genes within the ***SC inguinal fat*** did not differ among strains NPY [$F(3.420) = 0.0003, ns$], Y1R [$F(0.6844) = 4.261e-006, ns$], Y2R [$F(1.550) = 0.0015, ns$], DPPIV [$F(2.928) = 9.608e-005, ns$].

All mice expressed the sympathetic marker β3-AR and the expression did not significantly differ among the three strains [$F(0.5852) = 0.96, ns$] (Figure 3.4). UCP1 mRNA, also expressed in all mice (Figure 3.4) was significantly higher in the inbred, two fold higher compared to the C57BL/6J strain and one fold higher compared to the CD1 mice [$F(6.342) = 24.50, p < 0.05$] (Figure 3.5, 4A). These data suggest the presence of a larger pool of UCP1 positive cells present in the SC inguinal white adipose compared to the visceral fat.

We did not find any difference in frequency and level of expression of PPARγ [$F(0.5573) = 0.0015, ns$] and α2-AR [$F(1.546) = 4.018e-006, ns$] among strains (Figure 3.4, Figure 3.5).

All inbred mice expressed IRS1 and IRS2 (Figure 3.4), while not all CD1 mice expressed these genes, where IRS1 was expressed only by two out of six experimental CD1 mice and one mouse did not have the expression of IRS2 (Figure 3.4). No significant difference in terms of gene expression was detected among the three strains for the insulin signaling markers IRS1 [$F(0.4306) = 0.0001, ns$], IRS2 [$F(0.7727) = 0.008, ns$] (Figure 3.5).

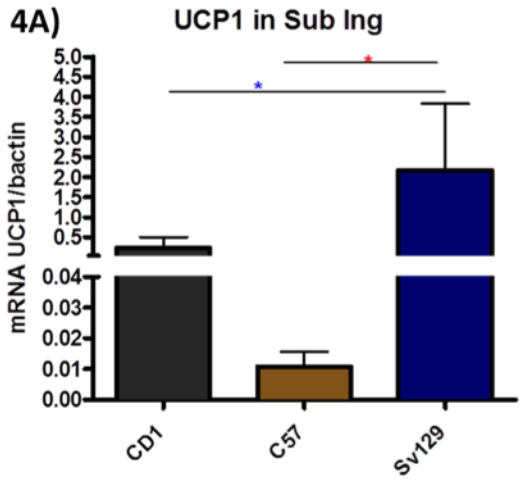
	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1	3/6	4/6	5/6	4/6	5/6	4/6	2/6	5/6	6/6	5/6
C57	3/3	3/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Sv129	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5

Table 3. Frequency of gene expression in SC Inguinal Fat Tissue. Differences in frequency of gene expression between genotype and treatment.
*: CD1 vs C57
*: C57 vs Sv129
*: Sv129 vs CD1
No difference is found in the frequency of expression among strains.

Figure 3.4: Frequency of gene expression in SC Inguinal Fat

	CD1		C57		Sv129	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	4E-04	4E-04	7E-06	1E-06	1E-05	7E-05
Y1R	3E-03	1E-03	4E-04	1E-04	4E-04	2E-03
Y2R	1E-02	7E-03	3E-06	2E-06	2E-05	7E-05
DPPIV	5E-03	2E-03	4E-03	1E-03	1E-03	4E-03
β3-AR	2E-02	1E-02	1E-02	2E-03	9E-03	2E-02
α2-AR	7E-03	7E-03	7E-05	1E-05	1E-04	4E-04
IRS1	2E-03	2E-03	3E-04	3E-05	4E-04	6E-04
IRS2	3E-02	2E-02	1E-02	4E-03	1E-02	2E-02
UCP1	2E-01	1E-01	1E-02	3E-03	2E+00	7E+00
PRARγ	1E-02	6E-03	4E-03	2E-03	8E-03	1E-02

Absolute mRNA expression in SC Inguinal Fat Tissue.
*: CD1 vs C57
*: C57 vs Sv129
*: Sv129 vs CD1



A) Sv129 mice have a significantly higher expression of UCP1 compared to CD1 and C57 mice [F(6.342)=11.38, p<0.05].

Figure 3.5: Absolute Expression of analyzed genes in SC Inguinal Fat Tissue

3.2.3 Genetic differences in brown adipose tissue among strains under HFD

With the exception of Y2R that was not always expressed in the ***brown adipose tissue (BAT)*** of all three strains under investigation, we detected the expression all the other genes (Figure 3.6). Only one out of five 129SvEv, one out of three C57BL/6J and four out of six CD1 mice presented the expression of Y2R; the frequency of this gene's expression was not significant among

strains (Figure 3.6). These results suggest that in general the Y2R gene is not frequently and abundantly expressed within the BAT independently from the genetic background.

The expression of NPY and Y1R differ among strains. NPY expression was significantly higher in the 129SvEv strain; four times higher compared to the C57BL/6J and almost three fold higher compared to CD1 individuals [$F(137.8) = 0.0003$, $p < 0.0001$] (Figure 3.7, 6A). Y1R expression was highly expressed in the CD1 and 129SvEv strain compared to the C57BL/6J mice, with a significant difference only between the CD1 and C57BL/6J strain [$F(5.697) = 3.277e-007$, $p < 0.05$] (Figure 3.7, 6B). The high expression of NPY together with its Y1R could suggest low BAT activity since NPY has been shown to inhibit BAT's temogenic activity via the Y1Rs [Kushi et al., 1998]. DPPIV expression was very similar across strains [$F(1.738) = 2.828e-008$, ns] (Figure 3.7). Together, these data demonstrates a complex and variable strain-dependent expression of the NPY system genes among the strains investigated.

We also detected no significant difference in the expression of β3-AR [$F(3.428) = 0.0113$, ns] and UCP1 [$F(0.1105) = 3059$, ns] among strains (Figure 3.7).

CD1 mice displayed a tendency of increased expression of PPARγ, not significantly different from the C57BL/6J and 129SvEv strain [$F(3.655) = 0.0004$, ns] (Figure 3.7). The 129SvEv mice presented the lowest expression of α2-AR, significantly lower compared to the CD1 individuals [$F(4.851) = 3.663e-009$, $p < 0.05$] (Figure 3.7, 6C) suggesting that the 129SvEv subjects may have the lowest inhibitory effects of α2-AR on lipolysis within BAT.

The insulin signaling markers expression were very similar across strains IRS1 [$F(0.5430) = 7.761e-005$, ns], IRS2 [$F(0.3424) = 0.004$, ns] with no difference detected (Figure 3.7).

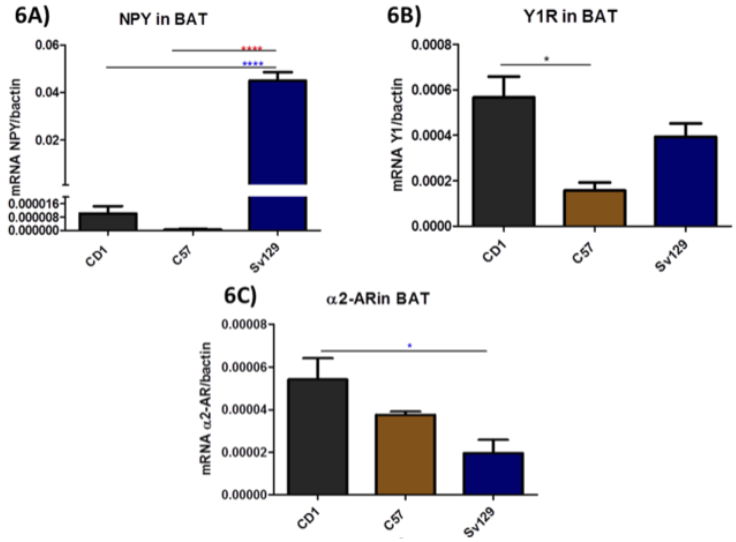
	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1	6/6	6/6	4/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
C57	3/3	3/3	1/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Sv129	5/5	5/5	1/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5

Table 5. Frequency of gene expression in Brown Adipose Tissue. Differences in frequency of gene expression between genotype and treatment.
*: CD1 vs C57
*: C57 vs Sv129
*: Sv129 vs CD1
No difference is found in the frequency of expression among strains.

Figure 3.6: Frequency of gene expression in Brown Adipose Tissue

	CD1		C57		Sv129	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	1E-05	4E-06	6E-07	2E-07	2E-05	7E-06
Y1R	6E-04	9E-05	2E-04	4E-05	4E-04	6E-05
Y2R	1E-06	5E-07	1E-07	1E-07	6E-07	6E-07
DPPIV	1E-04	2E-05	2E-04	4E-05	1E-04	2E-05
β3-AR	9E-02	2E-02	9E-02	2E-02	4E-02	4E-03
α2-AR	5E-05	1E-05	4E-05	1E-06	2E-05	6E-06
IRS1	7E-03	1E-03	9E-03	8E-04	8E-03	9E-04
IRS2	4E-02	8E-03	5E-02	2E-02	5E-02	6E-03
UCP1	3E+01	5E+00	3E+01	5E+00	3E+01	1E+01
PRARγ	2E-02	3E-03	1E-02	1E-03	1E+00	3E-03

Absolute mRNA expression in Brown Adipose Tissue.
*: CD1 vs C57
*: C57 vs Sv129
*: Sv129 vs CD1



6A) Sv129 mice have a significantly higher expression of NPY compared to CD1 and C57 mice [F(137.8)=0.00026, p<0.05].

6B) C57 mice display the lowest expression of Y1R, significantly lower compared to CD1 miceY1R[F(5.697)=3.277e-007, p<0.05];

6C) α2-AR expression was the lowest in Sv129 mice [F(4.851)=3.663e-009, p<0.05], significantly lower compared to CD1 mice

Figure 3.7: Absolute Expression of analyzed genes in Brown Adipose Tissue

3.2.4 Insulin signaling markers' expression in muscle and liver among strains under HFD

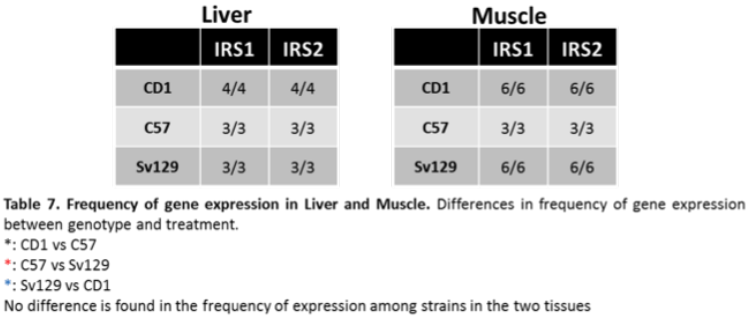
Our analysis showed that mice from all strains under analysis do express the insulin signaling markers IRS1 and IRS2 in liver and muscle (Figure 3.8).

We observed that the C57BL/6J displayed the highest expression of both IRS1 (Figure 3.9, 8A) and IRS2 (Figure 3.9, 8B) compared to 129SvEv

and CD1 mice IRS1 $[F(7.689) = 1.33e-015,\ p < 0.05]$, IRS2 $[F(6.734) = 4.003e-015,\ p < 0.05]$ (Figure 3.9).

Within muscle we found that the CD1 mice presented the lowest expression of IRS2 compared to the other two strains and was significantly lower compared to the 129SvEv mice $[F(5.263) = 1.483e-015, p < 0.05]$ (Figure 3.9, 8C). IRS1 expression was very similar among strains $[F((0.1299) = 9.824e-016,\ ns]$ (Figure 3.9).

Our molecular data suggested that among the strains investigated the C57BL/6J strain should be the one having the best glucose homeostasis capacity. This conclusion however is not supported by experimental data where C57BL/6J Sub mice are glucose impaired [Dadomo et al., 2011] (Figure 3.1 E, unpublished).



Liver

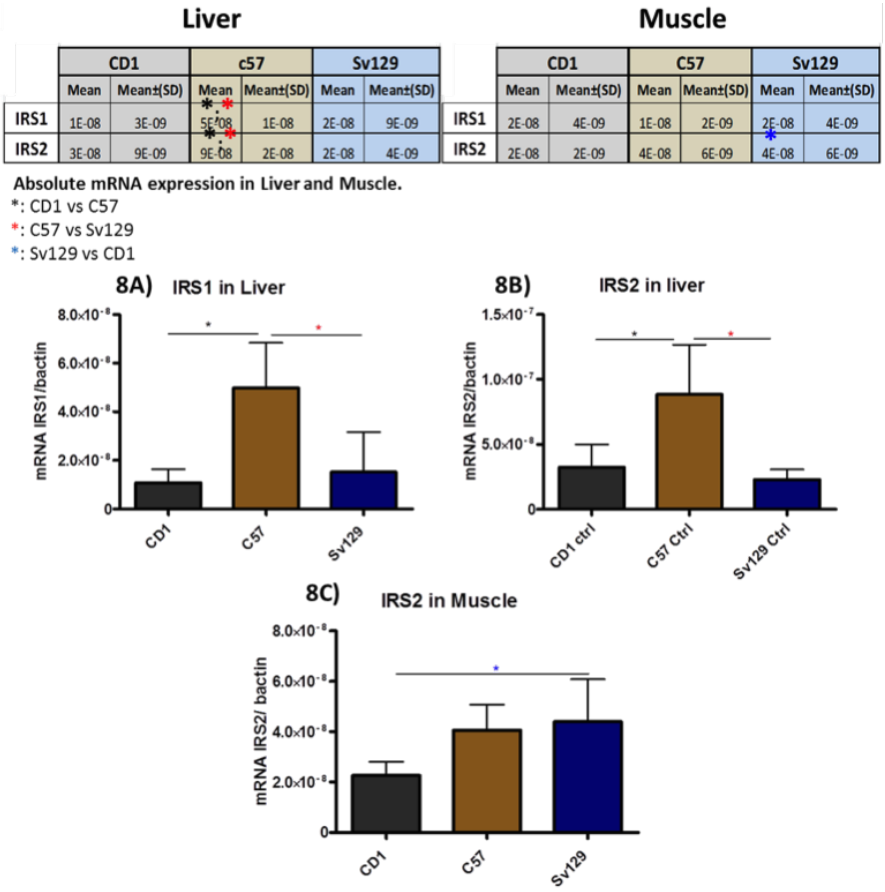
	IRS1	IRS2
CD1	4/4	4/4
C57	3/3	3/3
Sv129	3/3	3/3

Muscle

	IRS1	IRS2
CD1	6/6	6/6
C57	3/3	3/3
Sv129	6/6	6/6

Table 7. Frequency of gene expression in Liver and Muscle. Differences in frequency of gene expression between genotype and treatment.
*: CD1 vs C57
*: C57 vs Sv129
*: Sv129 vs CD1
No difference is found in the frequency of expression among strains in the two tissues

Figure 3.8: Frequency of gene expression in Liver and Muscle



Liver

	CD1		c57		Sv129	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
IRS1	1E-08	3E-09	5E-08	1E-08	2E-08	9E-09
IRS2	3E-08	9E-09	9E-08	2E-08	2E-08	4E-09

Muscle

	CD1		C57		Sv129	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
IRS1	2E-08	4E-09	1E-08	2E-09	2E-08	4E-09
IRS2	2E-08	2E-09	4E-08	6E-09	4E-08	6E-09

8A) C57 mice have a significantly higher expression of IRS1 [F(7.689)=1.33e-015, p<0.05]

8B) IRS2 [F(6.734)=4.003e-015, p<0.05] compared to CD1 and C57 mice.

8C) Sv129 mice have a significantly higher expression of IRS2 in muscle compared to CD1 and C57 mice [F(5.263)=1.483e-015, p<0.05].

Figure 3.9: Absolute Expression of analyzed genes in Liver and Muscle

3.2.5 No significant gene alterations were induced by chronic social stress and HFD in epididymal fat in all three strains

We found that three weeks of chronic psychosocial stress did not cause any significant modification in the expression of any genes under analysis when comparing stressed mice to their control littermates in all three strains (Figure 3.11).

The expression of the NPY system gene did not differ between stressed and controls individuals across strains NPY $[F(0.4124) = 424.0,\ ns]$, Y1R

[$F(0.6466) = 26.67$, ns], Y2R [$F(0.7939) = 103.2$, ns], DPPIV [$F(1.436) = 10.23$, ns] (Figure 3.11). Only one C57BL/6J control mouse expressed Y1R, while only out of six 129SvEv stressed mice did not have any expression of the Y2R gene, while all CD1 mice independent from stress expressed all the NPY genes (Figure 3.10).

The sympathetic marker β3-AR was expressed in all mice (Figure 3.10) and did not change in its expression following stress was encountered [$F(0.6224) = 47.20$, ns]. In general our analysis show that CD1 and 129SvEv mice independent from being under stress did have more UCP1 positive cells within the epididymal fat compared to the C57BL/6J strain where only one of three control mice and two out of four stress mice display expression of UCP1 in this depot (Figure 3.10). No variation was found in terms of the expression of this pro-lipolytic gene following stress [$F(0.2379) = 25.71$, ns] (Figure 3.11).

PPARγ, expressed in all mice (Figure 3.10) and no significant distinction in terms of expression was detected after stress [$F(0.9971) = 130.6$, ns]. The frequency of expression of anti-lipoltytic gene α2-AR was significantly lower in the CD1 stressed individuals (Figure 3.10, p>0.01) but no significant difference was found in the expression itself between stress and controls among strains [$F(0.2905) = 10.18$, ns] (Figure 3.11).

Not all C57BL/6J mice expressed the insulin signaling markers IRS1 and IRS2 compared to the CD1 and 129SvEv mice that all expressed these two insulin proteins (Figure 3.10). No difference was found in terms of gene expression IRS1 [$F(0.7495) = 377.9$, ns], IRS2 [$F(0.1316) = 86.84$, ns] among strains following stress (Figure 3.11).

	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1 Ctrl	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
CD1 Sub	4/4	4/4	4/4	4/4	4/4	1/4 *	4/4	4/4	4/4	4/4
C57 Ctrl	3/3	1/3	3/3	3/3	3/3	2/3	2/3	2/3	1/3	3/3
C57 Sub	4/4	4/4	4/4	4/4	4/4	4/4	3/4	4/4	2/4	4/4
Sv129 Ctrl	6/6	5/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
Sv129 Sub	6/6	6/6	5/6	6/6	6/6	4/6	6/6	6/6	6/6	6/6

Table 9. Frequency of gene expression in Epididymal Fat. Differences in frequency of gene expression between genotype and treatment.
*: CD1 Ctrl vs CD1 Sub
*: C57 Ctrl vs C57 Sub
*: Sv129 Ctrl vs Sv129 Sub
Only α2-AR frequency of expression in CD1 Sub mice is significantly different (p=0.01) from their control group.

Figure 3.10: Frequency of gene expression in Epididymal Fat

	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1 Ctrl	1.0 ± 0.9	1.0 ± 0.5	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.5	1.0 ± 0.3
CD1 Sub	0.6 ± 0.6	0.3 ± 0.2	1.5 ± 0.9	0.2 ± 0.1	0.9 ± 0.2	0.5 ± 0.5	5.3 ± 4.2	2.6 ± 1.5	0.2 ± 0.1	4.1 ± 3.0
C57 Ctrl	1.0 ± 0.4	1.0 ± 1.0	1.0 ± 0.6	1.0 ± 0.2	1.0 ± 0.6	1.0 ± 0.9	1.0 ± 0.5	1.0 ± 0.6	1.0 ± 1.0	1.0 ± 0.3
C57 Sub	1.3 ± 1.0	1.4 ± 0.4	0.2 ± 0.1	1.3 ± 0.3	0.4 ± 0.1	0.4 ± 0.1	1.0 ± 0.4	1.6 ± 0.8	0.8 ± 0.5	0.6 ± 0.1
Sv129 Ctrl	1.0 ± 0.6	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.5	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.5	1.0 ± 0.5
Sv129 Sub	4.1 ± 3.6	1.0 ± 0.5	3.0 ± 2.0	0.8 ± 0.3	2.0 ± 1.0	1.0 ± 0.3	1.5 ± 0.7	2.1 ± 1.0	0.4 ± 0.2	1.0 ± 0.3

Table 10. Relative mRNA expression in Epididymal fat. Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.
*: CD1 Ctrl vs CD1 Sub
*: C57 Ctrl vs C57 Sub
*: Sv129 Ctrl vs Sv129 Sub

No difference is found in the expression among strains following stress.

Figure 3.11: Relative Expression of analyzed genes in Epididymal Fat

3.2.6 The 129SvEv strain underwent profound changes in the NPY system and anti-lipoltytic genes within the subcutaneous inguinal depot following chronic psychosocial stress and HFD

Examining the basal expression of genes under investigation in the Sc Inguinal we found that the 129SvEv strain had the largest expression of UCP1 compared to the C57BL/6J and CD1 (Figure 3.5, 4A). Following our model of psychosocial stress this fat depot showed the greatest change in gene expression mainly in the 129SvEv strain.

Staring with the NPY system genes, chronic stress did alter the gene expression of NPY [$F(3.067) = 100.9$, $p < 0.05$] (Figure 3.13, 12A), its cleaving enzyme DPPIV [$F(6.533) = 32.56$, $p < 0.001$] (Figure 3.13, 12C) and its receptor Y1R [$F(4.479) = 47.14$, $p < 0.05$] (Figure 3.13, 12B) only within the 129SvEv strain, where the stressed subjects significantly increased their expression compared to their controls. We also observed a tendency of increased Y2R mRNA in the 129SvEv strain only, but was not significant [$F(0.7892) = 354.2$, ns] (Figure 3.13). Analyzing the frequency of expression we observed that while all 129SvEv stressed mice expressed the all NPY system genes under analysis, not all the C57BL/6J and CD1 stressed mice did (Figure 3.12). The increase in NPY, Y1R, Y2R and DPPIV in chronically stressed 129SvEv mice does strongly suggest a local activation of the NPYergic system within the Sc Inguinal depot. NPY is known to promote adipogeneis

through the action of its Y1R [Yang et al., 2008, Zhang et al., 2010, Shi and Baldock, 2012] and Y2R [Kuo et al., 2007], consequently an increases in these two receptors are indicators of NPY's direct effect on promoting adipocytes proliferation, differentiation and consequently fat accretion. The molecular changes observed in the stressed 129SvEv mice were very similar to Kuo et al. that show similar increase in NPY, Y2R and DPPIV in Sc abdominal fat following intermittent chronic social stress in 129X1/SvJ. This similarity does strongly suggest that NPY activity is tissue specific as well as strain specific.

Except for one CD1 stressed mice, all experimental subjects expressed the sympathetic marker β3-AR (Figure 3.12) and the expression did not differ between stress and controls [$F(3.276) = 25.73$, ns] in any strain.

UCP1, expressed in all mice (Figure 3.12), as predicted from our basal analysis, we determined a significant increase, almost three fold increased, in the stressed inbred 129SvEv compared to their controls [$F(2.687) = 48.42, p < 0.05$] (Figure 3.13, 12D). This outcome did confirm our hypothesis that under stressful events the large pool of UCP1 positive cells would even further be increased and could indicate more sympathetic innervation within the Sc Inguinal of these specific mice causing elevated energy consumption and lipid breakdown [Carriere et al., 2013].

The anti-lipolytic gene α2-AR, expressed in all inbred mice and CD1 stressed mice (Figure 3.12) and the pro-adipogenic gene PPARγ, expressed in all mice (Figure 3.12), did not differ in level of expression before and after the stress procedure [α2-AR [$F(1.363) = 90.20$, ns], PPARγ [$F((1.307) = 69.35$, ns] in any strain (Figure 3.13).

The insulin signaling markers were expressed in both inbred strains, while IRS1 was not expressed in all CD1 independent from stress, on the contrary to IRS2 that was expressed in all CD1 Sub mice (Figure 3.12). Our expression analysis did not show any significant difference given by stress among the three strains IRS1 [$F(0.8331) = 47.36$, ns], IRS2 [$F(0.5675) = 17.70$, ns] (Figure 3.13).

	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1 Ctrl	3/6	4/6	5/6	4/6	5/6	4/6	2/6	5/6	6/6	5/6
CD1 Sub	3/5	3/5	5/5	5/5	4/5	5/5	3/5	5/5	5/5	5/5
C57 Ctrl	3/3	3/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
C57 Sub	4/4	4/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
Sv129 Ctrl	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Sv129 Sub	7/7	7/7	6/7	7/7	7/7	7/7	7/7	7/7	7/7	7/7

Table 11. Frequency of gene expression in SC Inguinal Fat. Differences in frequency of gene expression between genotype and treatment.
*: CD1 Ctrl vs CD1 Sub
*: C57 Ctrl vs C57 Sub
*: Sv129 Ctrl vs Sv129 Sub
No difference is found in the frequency of expression among strains following stress.

Figure 3.12: Frequency of gene expression in SC Inguinal Fat

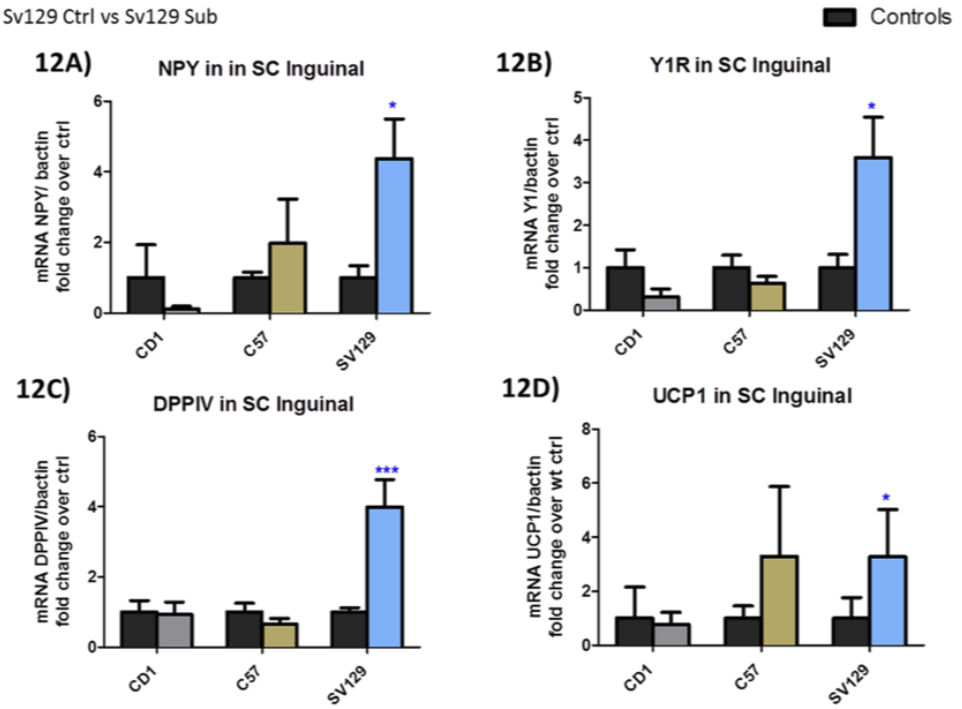
	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1 Ctrl	1.0 ± 0.9	1.0 ± 0.4	1.0 ± 0.6	1.0 ± 0.3	1.0 ± 0.6	1.0 ± 0.9	1.0 ± 1.0	1.0 ± 0.5	1.0 ± 0.5	1.0 ± 0.5
CD1 Sub	0.1 ± 0.1	0.3 ± 0.2	0.8 ± 0.5	0.9 ± 0.4	0.2 ± 0.1	0.7 ± 0.4	0.3 ± 0.2	0.5 ± 0.3	0.8 ± 0.2	3.2 ± 1.6
C57 Ctrl	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.5	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.6
C57 Sub	2.0 ± 1.3	0.6 ± 0.2	4.9 ± 4.5	0.7 ± 0.2	0.9 ± 0.2	1.2 ± 0.6	1.9 ± 0.4	0.7 ± 0.1	3.3 ± 1.3	0.6 ± 0.3
Sv129 Ctrl	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.8	1.0 ± 0.4	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.3	1.0 ± 0.5
Sv129 Sub	* 4.4 ± 1.1	* 3.6 ± 1.0	4.1 ± 1.4	*** 2.4 ± 0.5	4.0 ± 0.8	3.3 ± 1.4	1.3 ± 0.5	1.3 ± 0.3	* 3.3 ± 0.7	1.4 ± 0.3

Relative mRNA expression in SC Inguinal fat tissue. Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.

*: CD1 Ctrl vs CD1 Sub

*: C57 Ctrl vs C57 Sub

*: Sv129 Ctrl vs Sv129 Sub



Following 4 weeks of chronic psychosocial stress Sv129 stress mice display a significantly higher expression of

12A) NPY [$F(3.067)=100.9$, $p<0.05$];

12B) Y1R [$F(4.479)=47.14$, $p<0.05$];

12C) DPPIV [$F(6.533)=32.56$, $p<0.001$]; and

12D) UCP1 [$F(2.687)=48.42$, $p<0.05$].

Figure 3.13: Relative Expression of analyzed genes in SC Inguinal Fat

3.2.7 129SvEv stressed mice sustained a complex modification of their metabolic genes in the brown adipose tissue

With the exception of the Y2R gene that was not expressed in all mice independent from strain and stress, all the other genes under investigation were expressed in mice belonging to all three strains (Figure 3.14).

After exposure to chronic psychosocial stress we detected an elevation, but not significant in NPY mRNA of the two inbred strains, C57BL/6J and 129SvEv while CD1 stressed mice did not differ much from their controls [$F(2.646) = 27.46$, ns] (Figure 3.15). Also the DPPIV enzyme expression was slightly, but not significantly elevated, in the stressed mice [$F(0.1995) = 8.245$, ns]. Y1R expression was decreased in all mice following stress with a significant change in the CD1 mice [$F(0.9752) = 1.834$, $p < 0.05$] (Figure 3.15, 14A). NPY, a sympathetic neurotransmitter that does increase under stress and co-stored and co-released with noradrenaline, is known to suppress intrascapolar BAT activity via the Y1R, therefore a decreased expression of Y1R in stressed mice could suggest increased thermogenic activity in BAT mainly in the CD1 stressed mice [Billington et al., 1991, Kushi et al., 1998]. Y2R expression did not differ between control and stress subjects [$F(0.5788) = 238.3$, ns] (Figure 3.15).

We detected a significant increase of the sympathetic marker β3-AR in the 129SvEv stressed mice only [$F(6.248) = 14.41$, $p < 0.001$] (Figure 3.15, 14B) suggesting that this strain could be undergoing more chronic stress induced lipolysis compared to the other two strains. No significant difference was found in the expression of UCP1 between our control and stressed groups in all three strains [$F(0.5871) = 23.61$, ns] (Figure 3.15).

The α2-AR gene, that mediate anti-lipolytic activity, was significantly increased in the 129SvEv stressed mice [$F(2.488) = 11.77, p < 0.05$] suggesting a counteracting mechanism to β3-AR mediated lipolysis (Figure 3.15, 14C). No difference was found for PPARγ [$F(1.473) = 3.754$, ns] before and after stress in all strains (Figure 3.15). Our analysis of IRS1, required for BAT differentiation [Tseng et al., 2004] did not display any significant difference in expression IRS1 [$F(0.7665) = 3.454$, ns] following stress (Figure 3.15); while IRS2 expression, whose role is not fully understood in brown fat [Tseng et al., 2004], appeared to have a tendency to decrease after stress with a significant drop in the 129SvEv stressed mice only [$F(0.2505) = 3.471$, $p < 0.05$] (Figure 3.15, 14D).

	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1 Ctrl	6/6	6/6	4/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
CD1 Sub	5/5	5/5	3/5	5/5	4/5	5/5	3/5	5/5	5/5	5/5
c57 Ctrl	3/3	3/3	1/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
c57 Sub	4/4	4/4	1/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
Sv129 Ctrl	5/5	5/5	1/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Sv129 Sub	7/7	7/7	3/7	7/7	7/7	7/7	7/7	7/7	7/7	7/7

Table 13. Frequency of gene expression in Brown Adipose Tissue. Differences in frequency of gene expression between genotype and treatment.
*: CD1 Ctrl vs CD1 Sub
*: C57 Ctrl vs C57 Sub
*: Sv129 Ctrl vs Sv129 Sub
No difference is found in the frequency of expression among strains following stress.

Figure 3.14: Frequency of gene expression in Brown Adipose Tissue

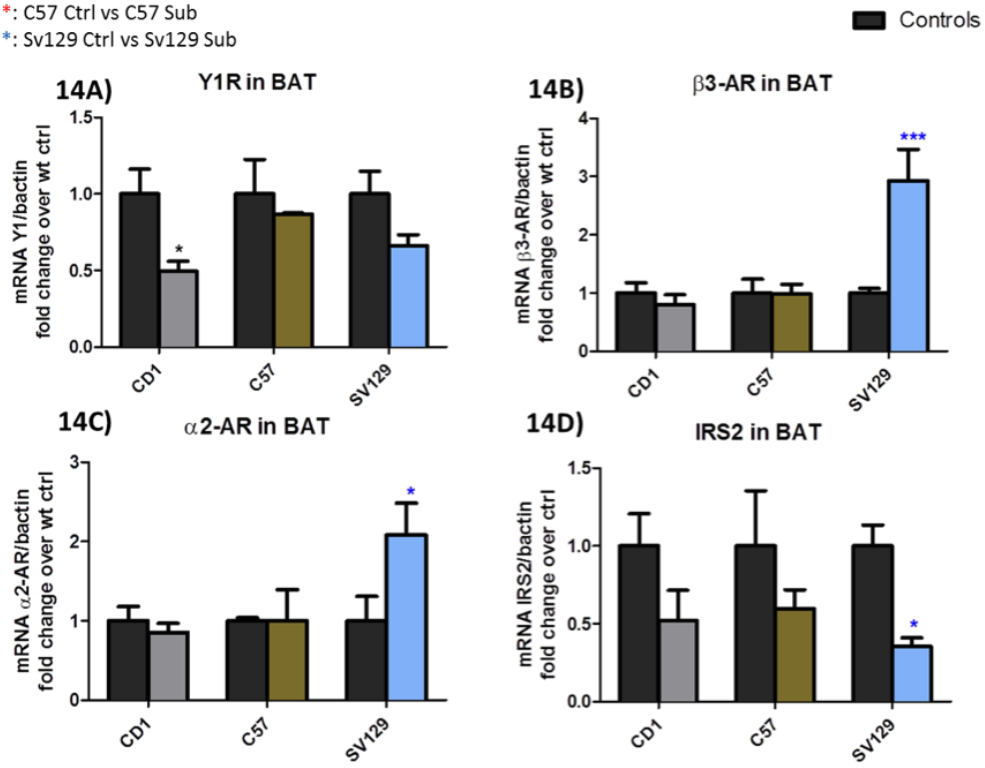
	NPY	Y1R	Y2R	DPPIV	β3-AR	α2-AR	IRS1	IRS2	UCP1	PPARγ
CD1 Ctrl	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1
CD1 Sub	0.6 ± 0.3	0.5 ± 0.1	0.5 ± 0.2 *	1.5 ± 0.1	0.8 ± 0.2	0.9 ± 0.1	0.8 ± 0.2	0.5 ± 0.2	1.9 ± 0.8	0.7 ± 0.1
C57 Ctrl	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 1.0	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.04	1.0 ± 0.1	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.1
C57 Sub	2.9 ± 0.6	0.9 ± 0.01	3.8 ± 3.8	1.4 ± 0.6	1.0 ± 0.2	1.0 ± 0.4	1.0 ± 0.1	0.6 ± 0.1	1.0 ± 0.4	1.0 ± 0.2
Sv129 Ctrl	1.0 ± 0.5	1.0 ± 0.1	1.0 ± 1.0	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.3	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.4	1.0 ± 0.2
Sv129 Sub	1.8 ± 0.3	0.7 ± 0.07	1.5 ± 0.9	1.7 ± 0.2	2.9 ± 0.5 ***	2.1 ± 0.4 *	1.2 ± 0.2	0.4 ± 0.1 *	1.2 ± 0.4	1.3 ± 0.2

Relative mRNA expression in Brown Adipose Tissue. Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.

*: CD1 Ctrl vs CD1 Sub

*: C57 Ctrl vs C57 Sub

*: Sv129 Ctrl vs Sv129 Sub



14A) Following 4 weeks of chronic psychosocial stress CD1 Stress mice have a significantly higher expression of Y1R [F(0.9752)=1.834, p<0.05]. **13B)**

14B) Compared to their contols, Sv129 Sub mice display a significant increase in the expression of β3-AR [F(6.248)=14.41, p<0.001]; 13C);

14C) α2-AR b3-AR [F(2.488)=11.77, p<0.05] and

14D) IRS2 [F(0.2505)=3.471, p<0.05].

Figure 3.15: Relative Expression of analyzed genes in Brown Adipose Tissue

3.2.8 Insulin signaling markers' expression altered following chronic stress and HFD

Liver and skeletal muscle are major target organs of insulin actions and play an essential role in insulin-induced glucose uptake, therefore inefficient response to insulin can lead to insulin resistance and diabetes [Kubota et al., 2008, Kubota et al., 2011]. All the experimental mice did express IRS1 and IRS2 in liver and muscle (Figure 3.16).

Stress resulted in a decreased, but not significant, of IRS1 and IRS2 expression in liver of CD1 and C57BL/6J stressed mice (Figure 3.17, 16A, 16B). The 129SvEv stressed mice displayed a tendency to decrease IRS1 (Figure 16A) and increased IRS2 in liver (Figure 16B) IRS1 [$F(0.1510) = 4.196$, ns], IRS2 [$F(3.426) = 3.918$, ns].

In muscle, CD1 stressed mice presented a very similar profile to what we found in the liver; the C57BL/6J stressed mice did not differ from their controls while the 129SvEv stressed had a tendency of increased IRS1 [$F(1.684) = 23.60, ns$] (Figure 3.17, 16C) while no difference was found in IRS2 (Figure 3.17, 16D) IRS2 [$F(0.5527) = 7.20$, ns] (Figure 3.17). The tendency to increasing the insulin signaling molecules in the 129SvEv stressed mice could suggest that this strain has a better glucose coping mechanism compared to the other two strains which is in line with our physiological data where the Sub 129SvEv mice presented a similar basal and fasting glucose levels to their controls (Figure 3.1, E).

Liver

	IRS1	IRS2
CD1 Ctrl	4/4	4/4
CD1 Sub	5/5	5/5
c57 Ctrl	3/3	3/3
c57 Sub	4/4	4/4
Sv129 Ctrl	3/3	3/3
Sv129 Sub	4/4	4/4

Muscle

	IRS1	IRS2
CD1 Ctrl	6/6	6/6
CD1 Sub	5/5	5/5
c57 Ctrl	3/3	3/3
c57 Sub	4/4	4/4
Sv129 Ctrl	6/6	6/6
Sv129 Sub	7/7	7/7

Table 15. Frequency of gene expression in Liver. Differences in frequency of gene expression between genotype and treatment.
*: CD1 Ctrl vs CD1 Sub
*: C57 Ctrl vs C57 Sub
*: Sv129 Ctrl vs Sv129 Sub
No difference is found in the frequency of expression among strains following stress in liver and muscle.

Figure 3.16: Frequency of gene expression in Liver and Muscle

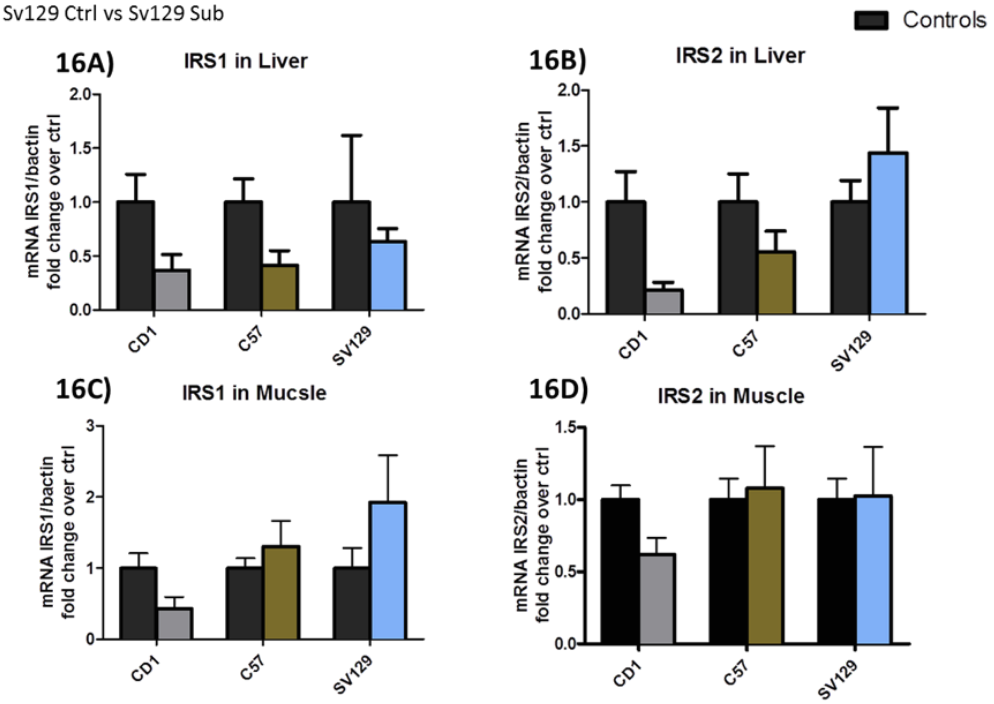
Liver

	IRS1	IRS2
CD1 Ctrl	1.0 ± 0.3	1.0 ± 0.3
CD1 Sub	0.4 ± 0.1	0.2 ± 0.1
c57 Ctrl	1.0 ± 0.2	1.0 ± 0.3
c57 Sub	0.4 ± 0.1	0.6 ± 0.2
Sv129 Ctrl	1.0 ± 0.6	1.0 ± 0.2
Sv129 Sub	0.6 ± 0.1	1.4 ± 0.4

Muscle

	IRS1	IRS2
CD1 Ctrl	1.0 ± 0.2	1.0 ± 0.1
CD1 Sub	0.4 ± 0.2	0.6 ± 0.1
c57 Ctrl	1.0 ± 0.1	1.0 ± 0.1
c57 Sub	1.3 ± 0.4	1.1 ± 0.3
Sv129 Ctrl	1.0 ± 0.3	1.0 ± 0.1
Sv129 Sub	2.5 ± 0.8	1.3 ± 0.4

Relative mRNA expression in Muscle Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.
*: CD1 Ctrl vs CD1 Sub
*: C57 Ctrl vs C57 Sub
*: Sv129 Ctrl vs Sv129 Sub



No significant difference in gene expression of IRS1 and IRS2 was found following stress in different strains in liver and muscle.

16A) In liver IRS1 [F(0.1510)=4.196, ns];

16B) IRS2 [F(3.426)=3.918, ns].

16C) In muscle IRS1 [F(1.684)=23.60, ns];

16D) IRS2 [F(0.5527)=7.20, ns].

Figure 3.17: Relative Expression of analyzed genes in Liver and Muscle

3.3 Discussion

Both environmental and genetic factors influence exposure to stressful life events [Kendler, 2001] and a great interest in individual differences that influence stress susceptibility even trying to localize individual susceptibility genes is being done [Xie et al., 2013]. Despite the active research, the molecular mechanisms of susceptibility and adaptation to chronic stress are poorly understood. This study used three genetically different mice, obesity-prone and impaired glucose tolerance C57BL/6J [Chatzigeorgiou et al., 2009, Plum et al., 2005]and CD1 mice [Mathews et al., 2004], and the obesity-resistant 129Sv mice, all commonly used in stress studies [Chuang et al., 2010a, Mathews et al., 2004]. Under conditions of our chronic psychosocial stress associated to a high fat diet, not only the CD1 and C57BL/6J Sub mice develop obesity and metabolic syndrome like phenotype, but also the 129Sv Sub mice that are normally considered obesity resistant [Bartolomucci et al., 2005, Bartolomucci et al., 2009, Dadomo et al., 2011, Sanghez et al., 2013]. Subsequent to our physiological data, we wanted to investigate on genes involved in the metabolic response to stress within metabolically active tissues such adipose, skeletal muscle and liver, before and after three week exposure to our validated naturalistic model of chronic subordination stress associated to a high caloric diet to put the influence of differential gene expression due to genetic background in perspective.

Under HFD the C57BL/6J in their visceral epididymal fat presented an increased expression of NPY and Y1R genes as well as β3-AR that could indicate that diet alone can alter the expression of pro-adipogenic genes which was counteracted by an increased expression of anti-lipolytic genes, such as α2-AR, to keep the organism in balance. Our analysis showed that the epididymal tissue was the least responsive to stress in all three strains, no difference were detected in gene expression between control and stressed subjects.

Evidence of white adipocytes within the SC inguinal fat depot expressing the UCP1, a specific brown adipocyte marker that uncouples the oxidation of fuel, mainly fatty acids, independent from the production of ATP releasing the energy associated with the fuel oxidation as heat, has been demonstrated [Cousin et al., 1992, Wu et al., 2012]. In SC inguinal fat, we detected significantly high expression of UCP1 in the 129SvEv and CD1 mice compared to the C57BL/6J mice suggesting that these two strains carry higher basal levels of this uncoupling protein. No other genes differed within this tissue among strains. Perhaps the genetic predisposition of increased UCP1 expression could be one of the reasons why the 129SvEv strain was considered obese resistant, but not under our chronic social stress model [Dadomo et al.,

2011]. Following stress, the 129SvEv strain following stress was the only strain to display an increase in the expression of the NPY genes, with a significant increase in NPY, Y1R and its cleaving enzyme DPPIV suggesting a local activation of the NPYergic system in the SC inguinal fat. The molecular obtained in this specific strain within the SC inguinal fat could be strongly related to previous findings where NPY mRNA has been shown to increase under stressful condition [Zukowska-Grojec, 1995,Morales-Medina et al., 2012]. In addition to the increased neuropeptide levels the augmented expression of Y1R and Y2R could also be indicative of local NPY's action considering that NPY acts through its Y1R [Zhang et al., 2010, Shi and Baldock, 2012] and Y2R [Kuo et al., 2007] to increase adipocytes proliferation, differentiation and fat mass accumulation, in line with the physiological data [Dadomo et al., 2011] (Figure 3.1, unpublished). Moreover, our expression data obtained within subcutaneous abdominal fat of the 129SvEv Sub mice could be strongly related to the finding of Kuo et al [Kuo et al., 2007] where an increase in NPY, Y2R and DPPIV was observed following intermittent social defeat in stressed 129X1/SvJ mice that share similar genetic background. These finding indicate increased NPYerigic activity in SC inguinal could be strain specific and tissue specific. Furthermore, also a significant increase of UCP1 mRNA level was found in the 129SvEv stressed mice compared to their controls suggesting that chronic psychosocial stress robustly increased the transcription of the thermogenically functional gene in white adipocytes causing these white adipocytes to turn into brown-like adipocytes, reasonably a counteracting allowing for increased clearance and combustion of triglycerides to have ready energy required to cope and handle stressand prevent the development of metabolic diseases [Carriere et al., 2013].

Regarding the brown adipose tissue, the 129SvEv mice displayed the highest NPY expression compared to the other two strains, but did not differ following stress. Under HFD conditions alone the CD1 mice presented the most expression of Y1R that could suggest a less active tissue since NPY acts through these specific receptors in BAT to suppress thermogenic activity [Billington et al., 1991, Kushi et al., 1998]. Following three weeks of chronic stress, a decrease in Y1R expression was identified in all three strains, with a significant decrease in the CD1 stressed mice only. These data suggest that chronic social stress had a strong influence on the activity of NPY in BAT, mainly in CD1 Sub mice, where stress decreased the inhibition of NPY augmenting the capability of BAT to promote thermogenesis as a counterbalance [Egawa et al., 1991, Egawa et al., 1990, Kushi et al., 1998]. Following stress we also encountered an up-regulation of both adrenergic receptors under analysis in 129SvEv mice, a two-fold β3-AR and a one-fold α2-AR increased expression, strengthening our hypothesis that this

strain's intrascaplor BAT was the most sympathetically innervated and most responsive to chronic stress. Importantly, the two adrenergic receptors have distinct functions. On one hand, β3-ARs are the most abundant of the three subtypes of β-adrenergic receptors and are the main receptors activated by noradrenaline to increase intracellular lipolysis and consequently increase FFA to be combusted by the mitochondria to produce heat and promote UCP1 expression [Cannon and Nedergaard, 2004, Bachman et al., 2002, Scarpace and Matheny, 1998]. On the other hand, α2-AR inhibits thermogenesis by inhibiting the activity of adenylyl cyclase [Cannon and Nedergaard, 2004], therefore decreased free fatty acids released and UCP1 expression. These data strongly support a fine balance taking place in the 129SvEv mice tightly regulating lipolysis within BAT. The same strain of mice also displayed a decreased expression of IRS2 following stress suggesting a direct influence of stress in lowering insulin sensitivity in BAT even though the role of this insulin signaling molecule is not fully understood [Tseng et al., 2004].

Hyperglycemia is caused by insulin resistance not only in the brain and adipose tissue, but also in the liver and skeletal muscle, which are central targets in controlling blood glucose and lipid homeostasis [Tomas et al., 2002]. Reduced capacity for insulin to uptake glucose and induce glycogen synthesis is a common feature recognized in obese and type 2 diabetic patients. Insulin signaling molecules IRS1 and IRS2 play a fundamental role in promoting insulin's intracellular signaling. In the liver the C57BL/6J mice presented the highest expression of IRS1 and IRS2, while in muscle the 129SvEv had the highest expression of IRS2 suggesting that in response to the high fat diet these mice were the most insulin sensitive. Succeeding stress, a boost in the expression of both IRS1 and IRS2 in muscle and liver of 129SvEv Sub mice was revealed implying the presence of a much more functional insulin signaling system compared to the other two stains allowing the 129SvEv stressed to be less prone in developing insulin resistance and type 2 diabetes. Indeed, as shown by our physiological data (Figure 3.1 E), the 129SvEv stressed maintained the same levels of basal and fasting glucose as their control littermates, allowing them to have a better peripheral insulin sensitivity preventing the development of type 2 diabetes, contrarily to the CD1 and C57BL/6J Sub mice that were glucose intolerant [Sanghez et al., 2013].

In conclusion this study does show that genetic background does influence individual variability to chronic psychosocial stress. Undoubtedly, among the three strains under investigation, the 129SvEv strain was the most affected by our stress protocol. Our data suggested that the genotype of the 129SvEv strain was the most versatile in restricting the consequences of high fat diet associated with chronic psychosocial stress. Moreover the increment of NPY system in the subcutaneous inguinal of 129SvEv mice only does indicate

regional and strain specific mediation of NPY in augmenting the effects of stress and diet. Globally the 129SvEv strain resulted being the most metabolically active, increasing sympathetic activity in white adipose tissue to burn the excess calories given by the high fat diet and stress, as well as in brown adipose tissue increasing thermogenesis to allow for the energy required to encounter stress.

3.4 Supplementary figures

	Epidydimal											
	CD1 Ctrl		CD1 Sub		c57 Ctrl		c57 Sub		Sv129 Ctrl		Sv129 Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	7E-03	5E-03	5E-03	5E-03	2E-02	9E-03	3E-02	2E-02	7E-04	4E-04	3E-03	2E-03
Y1R	3E-03	2E-03	1E-03	6E-04	5E-04	5E-04	7E-04	2E-04	4E-03	1E-03	4E-03	2E-03
Y2R	2E-04	6E-05	3E-04	2E-04	4E-03	2E-03	8E-04	4E-04	5E-04	2E-04	2E-03	1E-03
DPPIV	2E-02	7E-03	4E-03	5E-04	1E-02	2E-03	1E-02	3E-03	1E-02	2E-03	1E-02	4E-03
β3-AR	7E-02	3E-02	7E-02	9E-03	4E-01	2E-01	2E-01	3E-02	9E-02	4E-02	2E-01	9E-02
α2-AR	3E-04	4E-05	2E-04	2E-04	2E-03	2E-03	7E-04	1E-04	6E-04	1E-04	7E-04	2E-04
IRS1	2E-03	3E-04	1E-02	8E-03	6E-03	3E-03	6E-03	2E-03	1E-02	2E-03	2E-02	7E-03
IRS2	2E-02	3E-03	5E-02	3E-02	4E-02	2E-02	6E-02	3E-02	4E-02	8E-03	8E-02	4E-02
UCP1	3E-02	3E-02	4E-03	1E-01	6E-04	6E-04	5E-04	3E-04	2E+00	1E+00	8E-01	5E-01
PRARγ	1E-02	1E-02	3E-03	1E-01	9E-02	3E-02	5E-02	1E-02	9E-02	4E-02	9E-02	3E-02

Figure 3.18: Absolute Expression of analyzed genes in Epidydimal Fat

	Subcutaneous Inguinal											
	CD1 Ctrl		CD1 Sub		c57 Ctrl		c57 Sub		Sv129 Ctrl		Sv129 Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	4E-04	4E-04	4E-05	3E-05	7E-06	1E-06	1E-05	9E-06	1E-05	7E-05	5E-06	2E-05
Y1R	3E-03	1E-03	9E-04	5E-04	4E-04	1E-04	3E-04	6E-05	4E-04	2E-03	1E-04	4E-04
Y2R	1E-02	7E-03	9E-03	5E-03	3E-06	2E-06	1E-05	1E-05	2E-05	7E-05	1E-05	3E-05
DPPIV	5E-03	2E-03	5E-03	2E-03	4E-03	1E-03	3E-03	7E-04	1E-03	4E-03	1E-04	8E-04
β3-AR	2E-02	1E-02	5E-03	2E-03	1E-02	2E-03	1E-02	3E-03	9E-03	2E-02	3E-03	5E-03
α2-AR	7E-03	7E-03	5E-03	3E-03	7E-05	1E-05	9E-05	4E-05	1E-04	4E-04	3E-05	1E-04
IRS1	2E-03	2E-03	6E-04	5E-04	3E-04	3E-05	7E-04	2E-04	4E-04	6E-04	2E-04	2E-04
IRS2	3E-02	2E-02	2E-02	9E-03	1E-02	4E-03	9E-03	1E-03	1E-02	2E-02	5E-03	3E-03
UCP1	2E-01	1E-01	2E-01	5E-02	1E-02	3E-03	4E-02	1E-02	2E+00	7E+00	7E-01	1E+00
PRARγ	1E-02	6E-03	4E-02	2E-02	4E-03	2E-03	2E-03	1E-03	8E-03	1E-02	4E-03	3E-03

Figure 3.19: Absolute Expression of analyzed genes in SC Inguinal Fat

	Brown Adipose Tissue											
	CD1 Ctrl		CD1 Sub		C57 Ctrl		C57 Sub		Sv129 Ctrl		Sv129 Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	1E-05	4E-06	6E-06	3E-06	6E-07	2E-07	2E-06	3E-07	2E-05	7E-06	4E-05	1E-05
Y1R	6E-04	9E-05	3E-04	4E-05	2E-04	4E-05	1E-04	1E-06	4E-04	6E-05	3E-04	3E-05
Y2R	1E-06	5E-07	7E-07	3E-07	1E-07	1E-07	5E-07	5E-07	6E-07	6E-07	9E-07	5E-07
DPPIV	1E-04	2E-05	2E-04	1E-05	2E-04	4E-05	2E-04	1E-04	1E-04	2E-05	2E-04	3E-05
β3-AR	9E-02	2E-02	8E-02	2E-02	9E-02	2E-02	8E-02	1E-02	4E-02	4E-03	1E-01	2E-02
α2-AR	5E-05	1E-05	5E-05	6E-06	4E-05	1E-06	4E-05	1E-05	2E-05	6E-06	4E-05	8E-06
IRS1	7E-03	1E-03	6E-03	1E-03	9E-03	8E-04	9E-03	9E-04	8E-03	9E-04	1E-02	1E-03
IRS2	4E-02	8E-03	2E-02	7E-03	5E-02	2E-02	3E-02	6E-03	5E-02	6E-03	2E-02	3E-03
UCP1	3E+01	5E+00	5E+01	2E+01	3E+01	5E+00	3E+01	1E+01	3E+01	1E+01	4E+01	1E+01
PRARγ	2E-02	3E-03	2E-02	3E-03	1E-02	1E-03	1E-02	2E-03	1E+00	3E-03	1E+00	3E-03

Figure 3.20: Absolute Expression of analyzed genes in Brown Adipose Fat

	Liver											
	CD1 Ctrl		CD1 Sub		c57 Ctrl		c57 Sub		Sv129 Ctrl		Sv129 Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
IRS1	1E-08	3E-09	4E-09	2E-09	5E-08	1E-08	2E-08	7E-09	2E-08	9E-09	1E-08	2E-09
IRS2	3E-08	9E-09	7E-09	2E-09	9E-08	2E-08	5E-08	2E-08	2E-08	4E-09	3E-08	9E-09

Figure 3.21: Absolute Expression of analyzed genes in Liver

	Muscle											
	CD1 Ctrl		CD1 Sub		c57 Ctrl		c57 Sub		Sv129 Ctrl		Sv129 Sub	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
IRS1	2E-08	4E-09	7E-09	3E-09	1E-08	2E-09	3E-08	2E-08	2E-08	4E-09	3E-08	1E-08
IRS2	2E-08	2E-09	1E-08	3E-09	4E-08	6E-09	4E-08	1E-08	4E-08	6E-09	4E-08	1E-08

Figure 3.22: Absolute Expression of analyzed genes in Muscle

Chapter 4

Basal and stressed induced gene expression alteration of pro-adipogenic, pro-lipolytic, sympathetic and insulin signaling markers' between wild type and βless mice under high fat diet

4.1 Introduction

The role of the sympathetic nervous system (SNS) has been well established in the stress response as well as in the control of energy homeostasis and glucose metabolism [Kalsbeek et al., 2010], therefore a functional and intact SNS network is crucial to prevent stress related disorders [Chrousos and Gold, 1992, Johnson et al., 1992]. Studies strongly show that altered SNS activation, characteristic of the stress response does induce metabolic syndrome and cardiovascular diseases [Bjorntorp, 2001, Chandola et al., 2006].

βless mice globally lack all three β-adrenergic receptors (β-AR), β_1-AR, β_2-AR and β_3-AR, and are incapable of adaptive thermogenesis and diet induced thermogenesis due to down-regulation of UCP1 and impaired brown adipose tissue (BAT) activity; indeed these mice can not survive when kept at cold temperatures and become massively obese when fed a high fat diet [Bachman et al., 2002, Lowell and Bachman, 2003, Asensio et al., 2005]. We have shown that subordination following our chronic psychosocial stress model associated to a hypercaloric diet does induce obesity and metabolic syndrome in the most common mouse strains used in stressed studies [Bartolomucci et al., 2009, Bartolomucci et al., 2010, Dadomo et al., 2011, Sanghez et al., 2013]. Given the importance of the SNS in the stress response and thermogenesis, we wanted to explore the phenotype, physiological and molecular consequences in βless mice and their wild type (wt) under the same experimental conditions as previously described [Bartolomucci et al., 2009].

We hypothesized that chronic stress within this genetically modified strain of mice would overall cause a positive energy balance phenotype with deleterious metabolic consequences.

Our physiological data show that as the other strains described above also stressed wt mice displayed hyperphagia (Figure 4.1, B), increased body weight (Figure 4.1, A), fat mass (Figure 4.1, C) and fating glucose levels compared to their wt control littermates (Figure 4.1, D). We detected a completely opposite and unexpected phenotype in βless stressed mice that significantly decreased body weight (Figure 4.1, A), body fat composition (Figure 4.1, C) and lower fasting glucose levels (Figure 4.1, D) despite being hyperphagic (Figure 4.1, B). Overall, the physiological data indicate that chronic psychosocial stress induces a catabolic phenotype in βless mice preventing the development of obesity and glucose intolerance.

Moreover in support to the physiological data, our immunohistochemistry analysis showed that chronic stress induced profound morphological and molecular changes within the brown adipose tissue of βless mice. Unexpectedly, βless stressed mice display a BAT that has a significantly smaller adipocytes compared to their controls, comparable to wt control mice; with a significant

increase in sympathetic innervation and UCP1 expression (Figure 4.1, E). These data strongly illustrate that chronic psychosocial stress induces a normalization of BAT of βless stress mice, although the mechanisms are yet uncovered.

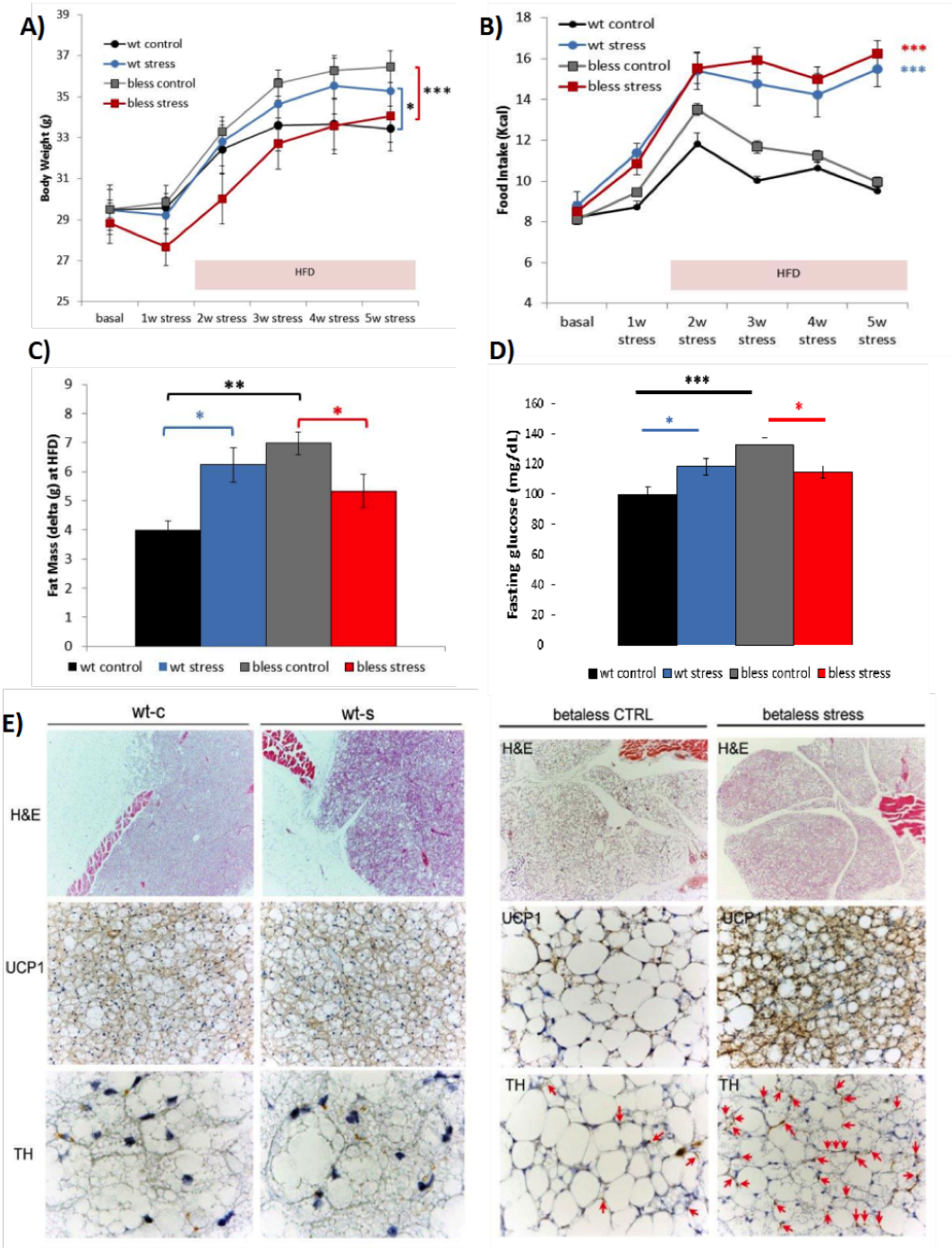


Figure 4.1: Metabolic consequences of chronic psychosocial stress in Control and Sub βless and wt male mice. From Razzoli et al. ms in preparation

The aim of this study was to understand some of the mechanism involved in preventing the development of an anabolic phenotype, excessive fat storage and normalization of BAT in βless stressed mice. In this study we first investigated the differences in lipoltytic, adipogenic and sympathetic gene markers using the quantitative real time PCR technique in visceral (epidydimal), subcutaneous (SC Inguinal) and brown adipose tissue, as well as inulin signaling markers in liver between wt and βless under a HFD. Secondly, we performed the same analysis following three weeks of chronic social stress to verify whether prolonged psychosocial stress associated with a high fat diet modifies the gene expression in the metabolically active tissues described above.

4.2 Results

4.2.1 Genetic differences in visceral epidydimal fat between βless and wt under HFD

We first investigated the basal expression of our genes of interest among wt and βless mice to determine the molecular differences in the two distinct genetic backgrounds. Experimental mice of both genetic backgrounds, wt and βless, did express all genes under investigation within the ***epidydimal fat*** depot (Figure 4.2). Very similar expression of NPY [$t_{2,13} = 0.1080$, $p = 0.92$] was detected in the wt and βless mice, while NPY's receptor Y1R [$t_{2,13} = 1.213$, $p = 0.25$] and the enzyme DPPIV that cleaves NPY to a shorter peptide more specific for Y2R, [$t_{2,13} = 1.104$, $p = 0.3$] presented a tendency to be decreased in the βless mice suggesting less NPY activity in visceral fat of βless mice [$t_{2,13} = 1.104$, $p = 0.3$] (Figure 4.3).

UCP1, a key brown adipocyte marker but expressed by some cells within white adipose tissue [Cousin et al., 1992], could be barley detected in βless mice that globally lack β-AR compared to wt mice [$t_{2,13} = 1.557$, $p = 0.1$] (Figure 4.3).

PPARγ, a key transcription regulator highly expressed in white adipose tissue where it regulates adipocytes' differentiation [Lehrke and Lazar, 2005], commonly used as a pro-adipogenic/diabetogenic marker was significantly decreased in βless mice [$t_{2,13} = 2.901$, $p = 0.01$] which could be a counteracting mechanism in limiting the development of diabetes in mice that are massively obese compared to the wild type mice due to impaired diet induced thermogenesis (Figure 4.3, 3A).

The expression of α_2-AR, also showed a tendency to be decreased in βless mice indicating low inhibitory effects of α_2-AR on lipolysis [$t_{2,13} = 2.901$, $p = 0.1$] (Figure 4.3).

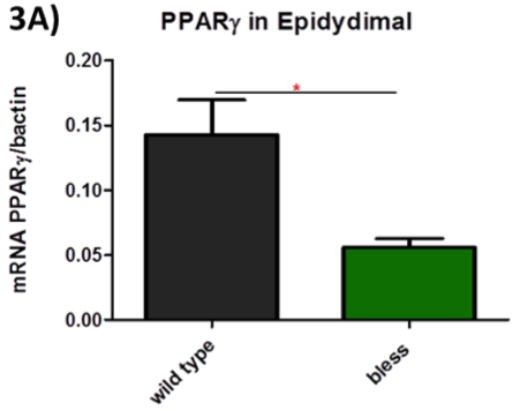
	NPY	Y1R	DPPIV	α2-AR	UCP1	PPARγ
wt Ctrl	8/8	8/8	8/8	8/8	8/8	8/8
βless Ctrl	7/7	7/7	7/7	7/7	7/7	7/7

Differences in frequency of gene expression between genotype.
*: wild type (wt) vs bless
No significant difference was found between experimental groups.

Figure 4.2: Frequency of gene expression in Epidydimal Fat

	wild tpe		βless	
	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	1.2E-04	5.5E-05	1.1E-04	1.9E-05
Y1R	2.0E-04	9.2E-05	8.3E-05	1.8E-05
DPPIV	1.5E-03	4.7E-04	8.9E-04	1.9E-04
a2-AR	2.1E-03	9.3E-04	3.4E-04	8.6E-05
UCP1	2.1E-02	1.3E-02	1.7E-04	4.0E-05
PRARg	* 1.4E-01	2.7E-02	5.6E-02	6.9E-03

*: wild type (wt) vs bless



3A) wt mice have a significantly higher expression of PPARγ compared to bless mice [$t_{2,13}$ =2.901, p=0.01].

Figure 4.3: Absolute Expression of analyzed genes in Epidydimal Fat

4.2.2 Genetic differences in subcutaneous fat between βless and wt under HFD

All mice express the genes of interest in the ***SC inguinal fat*** (Figure 4.4). Our molecular analysis indicate a tendency of decreased of NPY [$t_{2,16} = 1.89$, $p = 0.3$] and DPPIV expression [$t_{2,16} = 1.978$, $p = 0.0654$] (Figure 4.5, 5A) in βless mice compared to the wt experimental group, while Y1R mRNA was very similar in the two strains [$t_{2,16} = 0.57$, $p = 0.6$]. The decreased expression of the NPY system genes within the SC Inguinal fat pat of βless mice could be indicative of a depressed NPYergic activity in mediating adipocytes proliferation and differentiation in a depot that is hypertrophic.

Also α_2-AR expression was detected lower in βless mice compared to wt mice [$t_{2,16} = 1.391$, $p = 0.2$] while PPARγ expression [$t_{2,17} = 1.038$, $p = 0.3$] was similar between the strains (Figure 4.5), suggesting impaired α_2-AR dependent anti-lipolysis and less adipocytes differentiation within this tissue of βless mice.

As identified in the visceral fat depot, UCP1 expression in SC inguinal of βless mice was significantly lower compared to the wt mice [$t_{2,17} = 2.396$, $p = 0.0284$] (Figure 4.5, 5B), mainly due to the lack of the β-ARs.

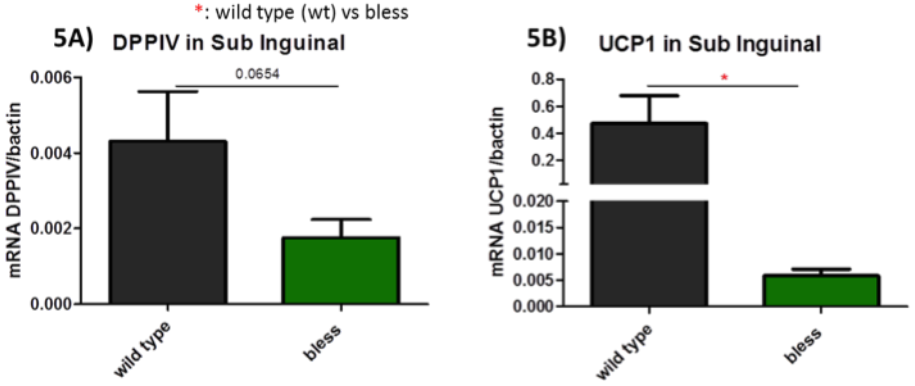
	NPY	Y1R	DPPIV	a2-AR	UCP1	PPARg
wt Ctrl	8/8	8/8	8/8	8/8	9/9	9/9
βless Ctrl	10/10	10/10	10/10	10/10	10/10	10/10

Differences in frequency of gene expression between genotype.
*: wild type (wt) vs bless
No significant difference was found between experimental groups.

Figure 4.4: Frequency of gene expression in Sub Inguinal Fat

	wild tpe		βless	
	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	2.1E-04	1.2E-04	8.3E-05	3.5E-05
Y1R	7.1E-04	1.1E-04	6.2E-04	1.2E-04
DPPIV	4.3E-03	1.3E-03	1.8E-03	4.8E-04
a2-AR	3.6E-03	1.7E-03	1.2E-03	7.7E-04
UCP1	* 4.7E-01	2.1E-01	5.8E-03	1.3E-03
PRARg	1.4E-01	2.4E-02	1.1E-01	1.3E-02

Absolute mRNA expression in Sc Iguinal Fat.
*: wild type (wt) vs bless



5A) bless mice have a tendency of lower DPPIV expression [$t_{2,16}$ =1.978, p=0.0654] and
5B) a significantly lower UCP1 expression compared to wt mice [$t_{2,17}$ =2.396, p=0.0284].

Figure 4.5: Absolute Expression of analyzed genes in Sub Inguinal Fat

4.2.3 Genetic differences in brown adipose tissue between βless and wt under HFD

Among the other adipose tissue the ***brown adipose tissue (BAT)*** presented the most differences between genotypes in the expression of our genes of interest even if no differences were found in frequency of expression (Figure 4.6). It is important to specify that even though wt and βless mice share the same localization of BAT, within the intrascapolar region, BAT of βless mice is morphologically and functionally more similar to white adipose tissue due to the lack of βARs and consequently proper UCP1 expression that causes incapability to increase BAT thermogenesis [Bachman et al., 2002]. Indeed our molecular analysis show a significantly lower expression of UCP1 [$t_{2,17} = 4.466$, $p = 0.0003$] (Figure 4.7, 7C) in βless mice compared to their wt.

Also PPARγ mRNA, the transcription factor involved in promoting UCP1 expression in brown adipocytes [Ohno et al., 2012] and brown adipocytes differentiation [Nedergaard et al., 2005] was significantly higher in wt mice that have intact and functional BAT compared to the β-AR deficient mice [$t_{2,18} = 4.744$, $p = 0.0002$] (Figure 4.7, 7D). Our investigation on the NPY system genes showed a significant higher NPY mRNA [$t_{2,15} = 2.307$, $p = 0.04$] (Figure 4.7, 7A) and a significant lower expression of Y1R [$t_{2,17} = 3.540$, $p = 0.0025$] (Figure 4.7, 7A) in βless mice compared to wt littermates. DPPIV expression was very similar between genotypes [$t_{2,14} = 0.7067$, $p = 0.5$]. The elevated NPY expression in BAT of βless mice could be indicative of increased NPY production from adipocytes themselves [Yang et al., 2008], while the down-regulation of Y1R could be a consequence of over-stimulated adipocytes by NPY.

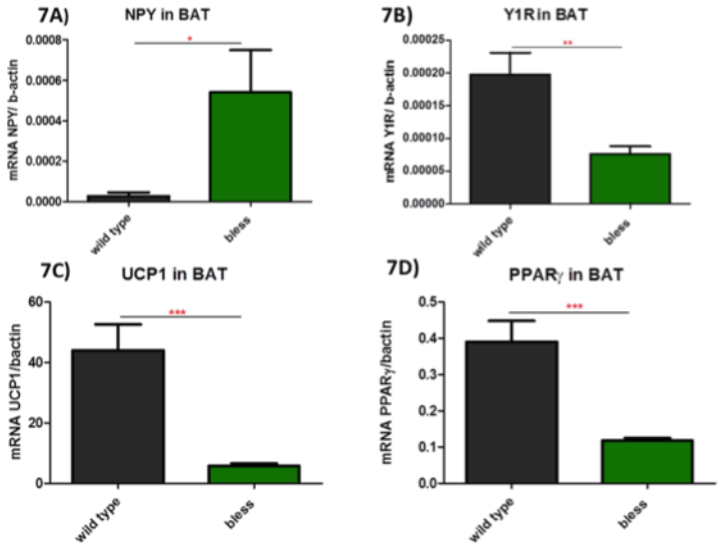
	NPY	Y1R	DPPIV	α2-AR	UCP1	PPARγ
wt Ctrl	8/8	9/9	8/8	8/8	10/10	10/10
βless Ctrl	9/9	10/10	8/8	8/8	10/10	10/10

Frequency of gene expression in Brown Adipose Tissue. Differences in frequency of gene expression between genotype.
*: wild type (wt) vs bless
No significant difference was found between experimental groups.

Figure 4.6: Frequency of gene expression in Brown Adipose Tissue

	wild tpe		βless	
	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	2.7E-05	1.9E-05	* 5.4E-04	2.1E-04
Y1R	2.0E-04	3.4E-05	** 7.6E-05	1.2E-05
DPPIV	3.3E-04	5.4E-05	5.4E-04	3.0E-04
a2-AR	9.5E-04	3.6E-04	1.5E-04	1.1E-04
UCP1	4.4E+01	8.5E+00	*** 5.7E+00	9.5E-01
PRARg	3.9E-01	5.7E-02	*** 1.2E-01	6.7E-03

*: wild type (wt) vs bless



7A) Bless mice have a significantly higher expression of NPY compared to wt [$t_{2,15}$=2.307, p=0.04],

7B) and a significantly lower Y1R expression ($t_{2,17}$=3.540, p=0.0025).

7C) Wt mice display a significantly higher expression of UCP1 [$t_{2,17}$=4.466, p=0.0003] and

7D) PPARγ [$t_{2,18}$ =4.744, p=0.0002] compared to the bless strain.

Figure 4.7: Absolute Expression of analyzed genes in Brown Adipose Tissue

4.2.4 Insulin signaling markers' expression in liver between βless and wt under HFD

Our investigation on insulin signaling markers IRS1 and IRS2 in liver showed that they were expressed in all mice (Figure 4.8). IRS1 and IRS2 mRNA was very similar between genotypes (IRS1: $[t_{2,12} = 1.551,\ p = 0.15]$; IRS2: $[t_{2,12} = 1.185,\ p = 0.26]$) (Figure 4.9). This result suggests that the hyperglycemia observed in βless mice is not due to impaired insulin signaling in liver, but perhaps other peripheral insulin sensitive tissue.

	IRS1	IRS2
wt Ctrl	7/7	7/7
βless Ctrl	7/7	7/7

Differences in frequency of gene expression between genotype.
*: wild type (wt) vs bless
No significant difference was found among experimental groups.

Figure 4.8: Frequency of gene expression in Liver

	wild tpe		βless	
	Mean	Mean±(SD)	Mean	Mean±(SD)
IRS1	2.5E-03	4.4E-04	1.6E-02	8.6E-03
IRS2	5.8E-03	1.6E-03	1.9E-02	1.1E-02

*: wild type (wt) vs bless
No significant difference was found between genotypes

Figure 4.9: Absolute Expression of analyzed genes in Liver

4.2.5 Chronic psychosocial stress associated with HFD did restore some of the epididymal fat functions in βless mice

All mice presented the expression of genes under investigation (Figure 4.10). NPY expression appeared decreased in βless stressed mice compared to their controls, but no difference was observed between controls and stressed wt mice under the same conditions $[F(0.6953) = 20.32, ns]$. Similar to NPY, Y1R and DPPIV mRNA did not differ between wt stress and their controls, whereas βless stressed subjects presented a tendency of increased expression of both genes (NPY:$[F(1.031) = 16.55, ns]$; DPPIV:$[F(1.249) = 10.96, ns]$).

In control mice, we detected a tendency of lower expression of the NPY system genes in βless mice compared to the wt (Figure 4.3), but following stress it appeared that in βless mice only a local up-regulation of the same genes took place suggesting increased NPYeric activity.

Also, α_2-AR expression presented a tendency to be increased in βless stressed mice suggesting that chronic psychosocial stress rescued some of α_2-AR dependent anti-lipolysis $[F(1.152) = 214.2, ns]$. We observed a significant increment of UCP1 expression in βless stress mice only $[F(3.473) =$

394.8, $p = 0.09$] (Figure 4.11, 11A) indicating that chronic stress augmented sympathetic activity within this tissue of these mice inducing improvement of the metabolic activity and tissue responsiveness to sympathetic input. Furthermore PPARγ expression was also significantly increased in βless stressed mice only, suggesting a resetting of the activities of this transcription factor [$F(3.673) = 12.19$, $p < 0.05$] (Figure 4.11, 11B).

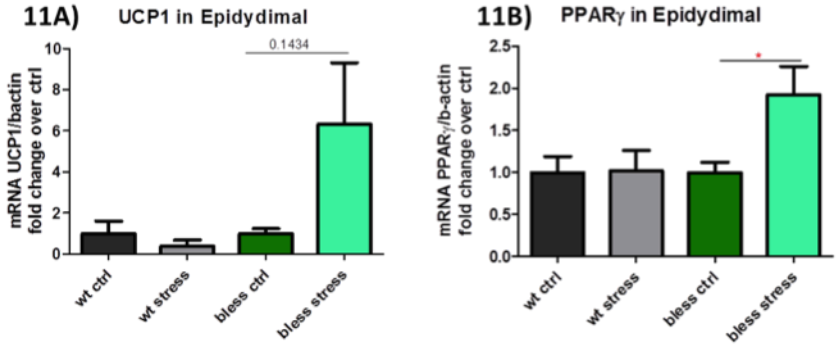
	NPY	Y1R	DPPIV	a2-AR	UCP1	PPARg
wt Ctrl	8/8	8/8	8/8	8/8	8/8	8/8
wt Stress	8/8	8/8	8/8	8/8	8/8	8/8
βless Ctrl	7/7	7/7	7/7	7/7	7/7	7/7
βless Stress	8/8	8/8	8/8	8/8	8/8	8/8

Differences in frequency of gene expression between genotype and treatment.
*: wt Ctrl vs wt stress
*: bless Ctrl vs bless stress
No significant difference was found among experimental groups.

Figure 4.10: Frequency of gene expression in Epidydimal Fat

	NPY	Y1R	DPPIV	α2-AR	UCP1	PPARγ
wt Ctrl	1.0 ± 0.4	1.0 ± 0.5	1.0 ± 0.3	1.0 ± 0.4	1.0 ± 0.6	1.0 ± 0.2
wt Stress	0.6 ± 0.3	0.7 ± 0.2	1.1 ± 0.2	1.2 ± 0.8	0.4 ± 0.6	1.0 ± 0.2
βless Ctrl	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.1
βless Stress	0.5 ± 0.2	1.4 ± 0.2	1.5 ± 0.2	3.2 ± 1.8	6.3 ± 3.0	* 1.9 ± 0.3

Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.
*: wt Ctrl vs wt stress
*: bless Ctrl vs bless stress



11A) 4 weeks of chronic psychosocial stress did induced a tendency in UCP1 expression [F(3.473)=394.8, p=0.09] and
11B) a significant increase in PPARγ [F(3.673)=12.19, p<0.05].

Figure 4.11: Absolute Expression of analyzed genes in Epidydimal Fat

4.2.6 βless mice augmented sympathetic and metabolic activities in subcutaneous inguinal fat following chronic psychosocial stress and HFD

Following chronic social stress, no differences were detected in the frequency of gene expression (Figure 4.12). We found a tendency of decreased expression of NPY and no difference in Y1R mRNA in wt stressed mice compared to their controls, whereas a slight increase of both genes was observed in βless stressed mice compared to their controls within the SC Inguinal depot (NPY:$[F(1.506) = 401.3, ns]$; Y1R:$[F(1.241) = 56.78, ns]$). In our basal analysis, DPPIV expression, major NPY cleaving enzyme, was much lower in βless mice compared to wt mice (Figure 4.5, A), however chronic stress did strongly up-regulate the expression of this enzyme in βless mice, a three fold increased was detected between βless stress compared to their control littermates as well as the wt stress and unstressed group (Figure 4.13, 13A).

The anti-lipolytic gene α_2-AR, also appeared to be elevated following

stress in both experimental groups, although the βless stressed mice conferred the most expression (Figure 4.13) [$F(1.324) = 898.5$, ns]. The pro-adipogenic gene PPARγ also appeared increased in βless mice after the stress procedure, while no difference in level of expression was detected before and after stress in wt mice [$F(2.735) = 7.430$, ns].

UCP1 expression was significantly increased in βless mice following stress [$F(4.286) = 4624$, $p < 0.05$], a 16 fold increased was observed comparing the stress group to the control group (Figure 4.13, 13B). These data strongly suggest not only an increase in sympathetic activity in βless stressed mice within the SC inguinal depots, nevertheless "browing" of white adipocytes [Cousin et al., 1992]. Together, these molecular changes could be responsible for an elevated energy consumption and lipid breakdown causing the decreased body weight gain established in βless stressed mice and alleviating metabolic dysfunction [Carriere et al., 2013].

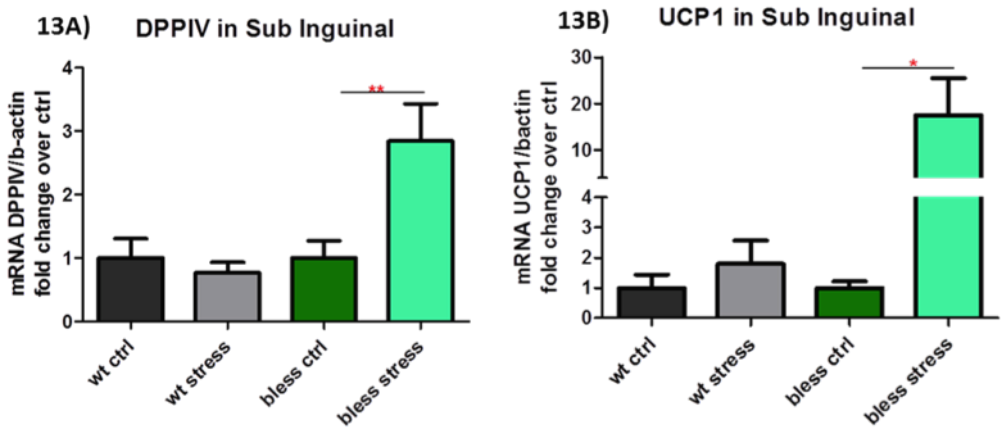
	NPY	Y1R	DPPIV	a2-AR	UCP1	PPARg
wt Ctrl	8/8	8/8	8/8	8/8	9/9	9/9
wt Stress	8/8	8/8	8/8	8/8	9/9	9/9
βless Ctrl	10/10	10/10	10/10	10/10	10/10	10/10
βless Stress	10/10	10/10	10/10	10/10	9/9	9/9

Differences in frequency of gene expression between genotype and treatment.
*: wt Ctrl vs wt stress
*: bless Ctrl vs bless stress
No significant difference was found among experimental groups.

Figure 4.12: Frequency of gene expression in Sub Inguinal Fat

	NPY	Y1R	DPPIV	α2-AR	UCP1	PPARγ
wt Ctrl	1.0 ± 0.6	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.2
wt Stress	0.2 ± 0.03	1.0 ± 0.2	0.8 ± 0.2	1.9 ± 1.1	1.8 ± 0.8	0.9 ± 0.1
βless Ctrl	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.7	1.0 ± 0.2	1.0 ± 0.1
βless Stress	3.5 ± 2.0	1.9 ± 0.7	2.8 ± 0.6 *	5.1 ± 3.0	17.5 ± 8.0 **	1.5 ± 0.2

Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.
*: wt Ctrl vs wt stress
*: bless Ctrl vs bless stress



13A) Following 4 weeks of chronic psychosocial stress bless displayed a significant increase in DPPIV mRNA [F(6.463)=44.20, p=0.09] and
13B) a significant increase in UCP1 expression [F(4.286)=4624, p<0.05].

Figure 4.13: Absolute Expression of analyzed genes in Sub Inguinal Fat

4.2.7 βless stressed mice sustained an up-regulation of their sympathetic and metabolic genes within the brown adipose tissue following chronic stress and HFD

All genes under investigation were expressed in mice belonging to both strains and treatment (Figure 4.14). NPY expression that was significantly higher in βless mice compared to wt mice under basal conditions (Figure 4.7, A) did remarkably drop after exposure to chronic psychosocial stress suggesting less white adipocytes that produce NPY and more brown-like adipocytes [$F(1.004) = 40.59$, ns] (Figure 4.15). Y1R mRNA was significantly increased in βless stress mice suggesting the re-establishment of the activity of these

receptors compared to basal condition; while a slight tendency of decreased expression was found in wt stress mice compared to their controls [$F(6.037) = 13.99$, $p < 0.05$] (Figure 4.15, 15A). As under basal condition, even following stress we did not observe any difference in DPPIV expression between stains and treatment [$F(0.2370) = 22.51$, ns].

Opposite to the basal conditions, we observed a significant elevation of PPARγ expression in βless stressed mice suggesting more activity of this transcription factor [$F(6.783) = 5.607$, $p < 0.05$] (Figure 4.15, 15B). Indeed, increased PPARγ expression was paralleled by a significant increase in UCP1 expression in the βless subordinate mice only [$F(1.834) = 18.15$, $p < 0.05$] (Figure 4.15, 15C). These molecular data are in agreement with the physiological and morphological data where we observe a normalization of pseudo-BAT to BAT in stressed βless with functional brown adipocytes capable of thermogenic activity generating heat in response to chronic stress. α_2-AR expression, main gene that mediates anti-lipolytic activity, was not different between the two experimental group following stress [$F(0.4778) = 176.9$, ns].

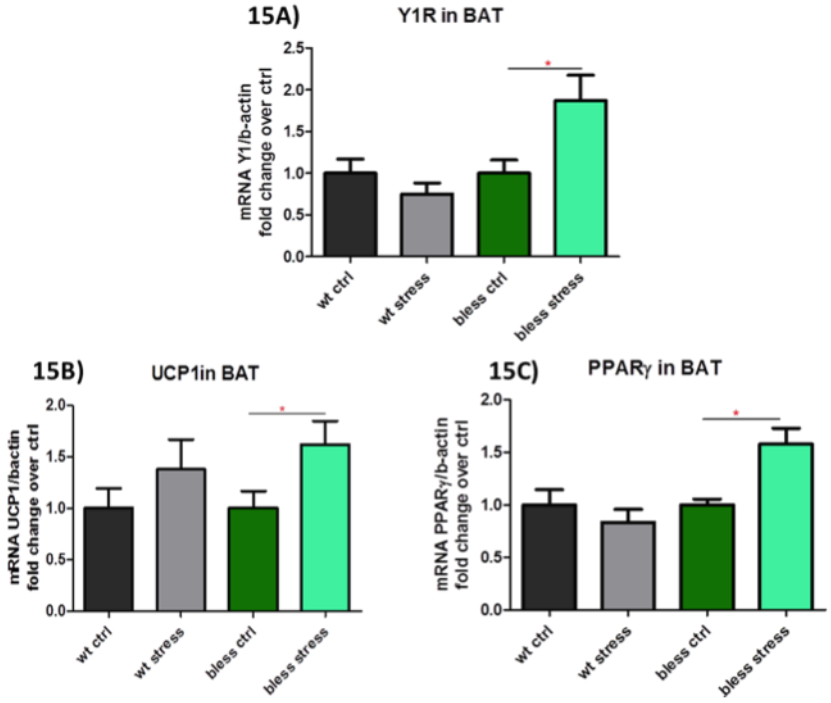
	NPY	Y1R	DPPIV	α2-AR	UCP1	PPARγ
wt Ctrl	8/8	9/9	8/8	8/8	10/10	10/10
wt Stress	8/8	10/10	8/8	8/8	10/10	10/10
βless Ctrl	9/9	10/10	8/8	8/8	10/10	10/10
βless Stress	8/8	10/10	8/8	8/8	10/10	10/10

Differences in frequency of gene expression between genotype and treatment.
*: wt Ctrl vs wt stress
*: bless Ctrl vs bless stress
No significant difference was found among experimental groups.

Figure 4.14: Frequency of gene expression in Brown Adipose Tissue

	NPY	Y1R	DPPIV	α2-AR	UCP1	PPARγ
wt Ctrl	1.0 ± 0.7	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.2
wt Stress	0.6 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	1.4 ± 0.8	1.4 ± 0.3	0.8 ± 0.1
βless Ctrl	1.0 ± 0.4	1.0 ± 0.2 *	1.0 ± 0.6	1.0 ± 0.7	1.0 ± 0.2 *	1.0 ± 0.06 *
βless Stress	0.1 ± 0.03	1.9 ± 0.3	0.7 ± 0.2	2.3 ± 1.4	1.6 ± 0.2	1.6 ± 0.2

Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.
*: wt Ctrl vs wt stress
*: bless Ctrl vs bless stress



Following 4 weeks of chronic psychosocial stress bless significantly increase their expression of
15A) Y1R [F(6.037)=13.99, p<0.05];

15B) UCP1 [F(1.834)=18.15, p<0.05] and

15C) PPARγ [F(6.783)=5.607, p<0.05].

Figure 4.15: Absolute Expression of analyzed genes in Brown Adipose Tissue

4.2.8 No stressed induced differences detected in insulin signaling markers in liver between βless and wild type mice

Hepatic insulin resistance, given by impairment of the organ to respond to insulin, plays a central role for development of type 2 diabetes and obesity. Chronic stress is known to compromise the normal functions of this organ increasing the risk of insulin resistance and glucose intolerance [Sanghez et al., 2013].

Our data showed that following 4 weeks of chronic psychosocial stress, all mice expressed IRS1 and IRS2, two critical nodes in the insulin signaling pathways (Figure 4.16). No significant difference was found in the expression of IRS1 [$F(1.121) = 30.7$, ns] (Figure 4.17, 17A) and IRS2 [$F(0.5259) = 405.49$, ns] (Figure 4.17, 17B) in the two genotypes before and after stress.

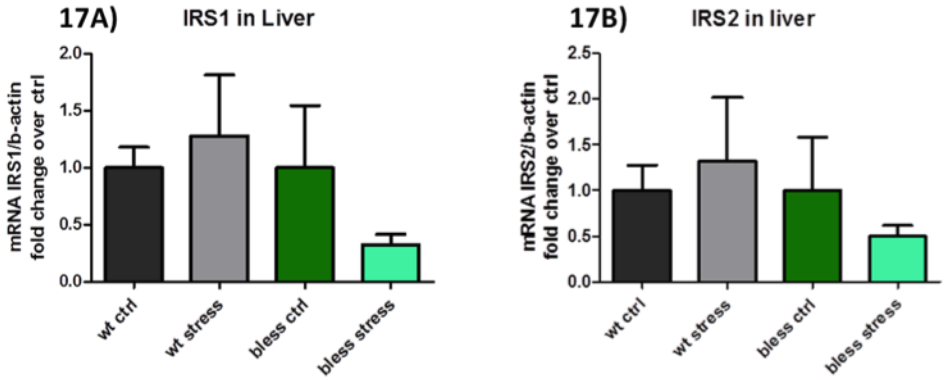
	IRS1	IRS2
wt Ctrl	7/7	7/7
wt Stress	8/8	8/8
βless Ctrl	7/7	7/7
βless Stress	8/18	8/18

Differences in frequency of gene expression between genotype and treatment.
*: wt Ctrl vs wt stress
*: bless Ctrl vs bless stress
No significant difference was found among experimental groups.

Figure 4.16: Frequency of gene expression in Liver

	IRS1	IRS2
wt Ctrl	1.0 ± 0.2	1.0 ± 0.3
wt Stress	1.3 ± 0.5	1.3 ± 0.7
βless Ctrl	1.0 ± 0.5	1.0 ± 0.6
βless Stress	0.3 ± 0.1	0.5 ± 0.1

Data are normalized to beta-actin and relative abundance determined for experimental group are expressed as fold-differences relative to the control group.
*: wt Ctrl vs wt stress
*: bless Ctrl vs bless stress



17A) IRS1 expression has a tendency of decreased expression in bless mice [F(1.121)=30.7, ns]
17B) same profile found for IRS2 [F(0.5259)=405.49, ns]

Figure 4.17: Absolute Expression of analyzed genes in Liver

4.3 Discussion

Following our model of chronic psychosocial stress subordinate mice from different strains develop obesity and metabolic syndrome like phenotype [Bartolomucci et al., 2009, Bartolomucci et al., 2010, Dadomo et al., 2011, Sanghez et al., 2013]. We wanted to investigate the outcome and consequences in a mouse whose genetic background predisposes a dysfunctional sympathetic activity causing excessive fat accumulation and metabolic dysfunction. For this purpose we used genetically modified mouse, global β_1, β_2 and β_3 adrenergic receptor knock out mice that have impaired adaptive thermogenesis [Bachman et al., 2002, Lowell and Bachman, 2003]. Bless mice and their wild type control littermates were subjected to our ethological stress procedure associated to a high fat diet regime. We predicted to find detrimental physiological and metabolic consequences in βless mice compared to any other strain previously

used in our stress model due to the inability of their sympathetic system to properly regulate metabolic function. Unexpectedly, although under HFD, βless mice subjected to chronic social stress resulted to be the solely strain to manifest a catabolic phenotype (Figure 4.1). Moreover, our protocol of chronic social stress induced normalization of the brown adipose tissue of βless stressed mice, from a white adipose tissue phenotype, large adipocytes packed with lipid droplets, very few mitochondria, limited sympathetic innervation and a low UCP1 expression that counter for the lack of function the tissue [Lowell and Bachman, 2003], to a regular and fully active brown adipose tissue, highly SNS innervated, restored UCP1 production and heat generation (Figure 4.1, E).

We investigated on the expression of key genes involved in lipolysis, adipogenesis, sympathetic innervation and insulin signaling markers in highly metabolically active tissues before and after three week exposure to our validated naturalistic and ethological model of chronic subordination stress associated with a HFD to tack any molecular modifications that could explain the unpredicted catabolic phenotype found in the βless stressed mice.

Under HFD alone βless mice presented a marked positive energy phenotype with increased body weight, fat mass accumulation and impaired glucose tolerance compared to the wt mice (Figure 4.1). Our molecular analysis in mice without stress showed that within the visceral epididymal depot βless individuals held low NPY activity, impaired α_2-AR-dependent anti-lipolysis and almost undetectable UCP1 mRNA compared to the wt indicative a depressed sympathetic-adrenergic control of lipolysis and dramatic alteration in adipocyte sensitivity to catecholamines. Moreover, the lack of β-AR prevented the tissue to undergo lipolysis and consequently massive amount of lipids were stored within the tissue, which could explain the down-regulation of PPARγ. The PPARγ gene, that stimulates several genes linked to adipocyte differentiation and glucose homeostasis [Auwerx, 1999] could be dysfunctional causing less adipogenesis and less insulin sensitivity. A comparable molecular profile to the visceral depot in βless mice was identified within the subcutaneous inguinal fat too where low NPYergic activity, inhibited anti-lipolysis and pro-adipogeneis were observed. The lower expression of PPARγ and α_2-AR in both tissues could be counteracting molecular mechanisms to somehow keep the βless organism somehow balanced by activating mechanisms that would limit and prevent formation of new adipocytes and therefore lipid storage and in depots that are already massively enlarged and hypertrophic.

As expected, the major differences between wt and βless mice under a HFD were detected in the intrascapolar BAT (iBAT). βless mice do not have regular BAT, rather a fat depot that is more comparable to white adipose tissue in morphologically and functionally due to the lack of β-ARs ergo a

defective NE-βAR-UCP1 axis, crucial for the regulation of whole body energy and metabolism [Cannon and Nedergaard, 2004, Collins et al., 2004] enables these mice to have diet induced thermogenesis [Lowell and Bachman, 2003]. NE-induced lipolysis promotes the expression of the UCP1 gene [Cannon and Nedergaard, 2004], key brown adipocyte marker which was found significantly decreased in βless mice compared to their controls. Moreover, PPARγ that with BAT is involved in promoting UP1 expression and lipolysis [Teruel et al., 2005, Wilson-Fritch et al., 2004] was also significantly decreased in βless mice compared to their controls as expected. We identified a significantly higher expression of NPY in the βless mice, similar expression levels to what was observed in visceral and subcutaneous fat. These data suggest that the NPY found in the intrascapolar region βless was actually produced by the white-like adipocytes that know to secrete NPY [Yang et al., 2008, Kos et al., 2007] within this dysfunctional tissue. Therefore we attribute NPY in BAT of βless mice as a marker for white adipocytes rather than a pro-adipogenic marker since Y1R expression was significantly decreased within the same mice, potentially as a counteracting mechanism as a result of overstimulation of these receptors by NPY. We could exclude the hypothesis of neuropeptide Y being released by SNS terminals since these mice have a significantly lower sympathetic BAT innervation [Lowell and Bachman, 2003].

Insulin signaling markers in liver tissue was similar between genotypes, opposite finding to our physiological data where βless under HFD do have a significantly higher fasting glucose levels compared to the wild type strain (Figure 4.1, D) and their glucose intolerant phenotype [Asensio et al., 2005]. The similar insulin signaling found between the two strains under HFD conditions suggest that βless mice have a greater glucose oxidation with a reciprocal decreased lipid oxidation since glucose and lipid oxidation are inversely related [Randle et al., 1963].

Following three weeks of chronic psychosocial stress associated with HFD we encountered significant physiological and molecular changes in mostly all the genes in all tissues we were investigating in βless mice. Chronic social stress induced an up-regulation of the NPY system in visceral and subcutaneous white adipose depots, specifically NPY's receptor Y1 and DPPIV. NPY has been found to localize with NE in sympathetic nerve terminals innervating white adipose tissue [Giordano et al., 2005], thereby the increase NPY genes' expression in both white adipose depots could suggest a selective increase of sympathetic drive innervation. Moreover, we also detected in both tissues an increase in α_2-AR expression in βless stress mice only, strongly indicating that within this strain of mice, chronic social stress induced an improvement of the otherwise defective ability to respond to catecholamines [Bachman et al., 2002, Asensio et al., 2005], indicating

more sympathetic responsiveness leading to less impaired α_2-AR dependent anti-lipolysis. In agreement with chronic psychosocial stress induced gain of sympathetic activity within the white adipose tissue of βless mice, we found a significant increase in UCP1 expression suggesting that stress activated adaptive thermogenesis in white adipose tissue prompting lipid breakdown and combustion into heat production [Lowell and Bachman, 2003]. These data greatly support the evidence that chronic stress in βless mice stimulates browning of white adipose tissue [Wu et al., 2012, Cousin et al., 1992] and would explain the decreased body weigh, fat mass and tissue weight of βless stressed mice. Also, PPARγ expression was markedly increased in βless stressed mice suggesting the restoration of the activities of this transcription factor in regulating lipid and glucose metabolism [Olefsky and Saltiel, 2000].

Most molecular were detected in BAT βless mice induced by stress. Stress induced a dramatic decrease in NPY expression of βless mice indicating less white adipocytes and more brown-like adipocytes, in agreement with our immunohistochemistry data (Figure 4.1, E). We believe that the increased Y1R expression detected following stress βless mice is not indicative of the normal inhibitory effects of NPY on its Y1R on BAT's activity [Kushi et al., 1998, Billington et al., 1991] since NPY expression was extremely low. We suppose that the increased Y1R expression in brown adipocytes of in βless stressed mice could be indicative of increased cathecolaminergic innervation, which is proven by the increased positive TH staining within their BAT (Figure 4.1, E). As discussed above, cathecolmainergic nerve terminals do accommodate both neurotransmitters vesicles, NE and NPY, [Giordano et al., 2005], and NPY released by these terminals could act through its Y1R to activate the transcription of UCP1 responsible for the browning processes of the tissue promoting normal phenotype and functionality. Indeed, our morphological and physiological data clearly showed a gain in function of BAT induced by stress in βless stressed mice to a degree that could be correlates an unstressed wild type BAT (Figure 4.1, E). By the same token, also PPARγ expression was increased within this strain which could have promoted BAT's lipolytic, oxidative, and also its thermogenic machineries [Teruel et al., 2005, Wilson-Fritch et al., 2004, Festuccia et al., 2012]. No difference was detected in α_2-AR expression between the two experimental group following stress.

No significant difference was detected in the insulin signaling markers in liver, in line with the physiological data where we showed a similar glucose tolerance between the two stressed groups [Kubota et al., 2008]. In conclusion this is the first study that shows that our model of chronic social stress triggers mechanisms within the βless strain of mice that promotes normalization of their obese phenotype by restoring the functionally of their brown adipose

tissue allowing them to have a normalized thermogenic response.

4.4 Supplementary figures

	Epidydimal							
	wt Ctrl		wt Stress		βless Ctrl		βless Stress	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	1.2E-04	5.5E-05	6.9E-05	3.9E-05	1.1E-04	1.9E-05	5.7E-05	2.5E-05
Y1R	2.0E-04	9.2E-05	1.5E-04	3.4E-05	8.3E-05	1.8E-05	1.2E-04	1.5E-05
DPPIV	1.5E-03	4.7E-04	1.6E-03	2.6E-04	8.9E-04	1.9E-04	1.4E-03	1.6E-04
a2-AR	2.1E-03	9.3E-04	2.4E-03	1.4E-03	3.4E-04	8.6E-05	1.1E-03	6.1E-04
UCP1	2.1E-02	1.3E-02	8.7E-03	6.0E-03	1.7E-04	4.0E-05	1.1E-03	4.9E-04
PRARg	1.4E-01	2.7E-02	1.5E-01	3.4E-02	5.6E-02	6.9E-03	1.1E-01	1.9E-02

Figure 4.18: Absolute Expression of analyzed genes in Epidydimal Fat

	Sc Inguinal Fat							
	wt Ctrl		wt Stress		βless Ctrl		βless Stress	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	2.1E-04	1.2E-04	3.7E-05	6.3E-06	8.3E-05	3.5E-05	2.9E-04	1.7E-04
Y1R	7.1E-04	1.1E-04	6.9E-04	1.3E-04	6.2E-04	1.2E-04	7.7E-04	1.4E-04
DPPIV	4.3E-03	1.3E-03	3.3E-03	6.9E-04	1.8E-03	4.8E-04	5.0E-03	1.0E-03
a2-AR	3.6E-03	1.7E-03	7.0E-03	3.8E-03	1.2E-03	7.7E-04	6.0E-03	3.5E-03
UCP1	4.7E-01	2.1E-01	8.5E-01	3.7E-01	5.8E-03	1.3E-03	1.0E-01	4.6E-02
PRARg	1.4E-01	2.4E-02	1.2E-01	2.0E-02	1.1E-01	1.3E-02	1.6E-01	2.1E-02

Figure 4.19: Absolute Expression of analyzed genes in SC Inguinal Fat

	Brown Adipose Tissue							
	wt Ctrl		wt Stress		βless Ctrl		βless Stress	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
NPY	2.7E-05	1.9E-05	1.6E-05	4.6E-06	5.4E-04	2.1E-04	7.0E-05	1.6E-05
Y1R	2.0E-04	3.4E-05	1.5E-04	2.5E-05	7.6E-05	1.2E-05	1.4E-04	2.3E-05
DPPIV	3.3E-04	5.4E-05	3.4E-04	6.6E-05	5.4E-04	3.0E-04	3.8E-04	8.4E-05
a2-AR	9.5E-04	3.6E-04	1.3E-03	7.4E-04	1.5E-04	1.1E-04	3.5E-04	2.1E-04
UCP1	4.4E+01	8.5E+00	6.1E+01	1.3E+01	5.7E+00	9.5E-01	9.1E+00	1.3E+00
PRARg	3.9E-01	5.7E-02	3.3E-01	4.7E-02	1.2E-01	6.7E-03	1.9E-01	1.8E-02

Figure 4.20: Absolute Expression of analyzed genes in Brown Adipose Fat

	Liver							
	wt Ctrl		wt Stress		βless Ctrl		βless Stress	
	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)	Mean	Mean±(SD)
IRS1	2.5E-03	4.4E-04	3.2E-03	1.3E-03	1.6E-02	8.6E-03	5.2E-03	1.4E-03
IRS2	5.8E-03	1.6E-03	7.7E-03	4.1E-03	1.9E-02	1.1E-02	9.7E-03	2.2E-03

Figure 4.21: Absolute Expression of analyzed genes in Liver

Part III

General Conclusion

The goal of this section is to summarize concepts and outcomes discussed in the preceding chapters. The association between stress, disrupted energy balance and obesity has been extensively documented, but the exact physiological and biochemical mechanism underling this relationship still remains unclear [Elzinga et al., 2003,Brunner et al., 2007,Chandola et al., 2006]. Using our chronic psychosocial stress model in mice, an ethological and naturalist experimental model, that has been shown to induce depressive like symptoms together with an obese phenotype and metabolic consequences [Bartolomucci et al., 2001, Bartolomucci et al., 2009, Bartolomucci et al., 2010], I tried to give some insight on the molecular changes taking place within metabolically active tissues under standard and high fat diet conditions.

Chronic Psychological Stress by itself alters key metabolic markers in a tissue specific manner.

In chapter 1 we observed that chronic social defeat induced a complex but regulated molecular modifications in different adipose tissues, depending on their sympathetic innervation and metabolic function, to adequately respond to the development of stress induce obesity. This conclusion is based the observations of: A) an up-regulation of the pro-adipogenic and diabetogenic gene PPARγ mostly in adipose tissues that can expand in size such as the epidydimal and perirenal visceral depots and the subcutaneous inguinal fat indicating more fat accretion, but normal sensitivity to insulin [Auwerx, 1999]; B) and contemporary an up-regulation of genes involved in energy breakdown and theromogenis such as UCP1 in highly innervated adipose depots like the visceral perirenal and the subcutaneous inguinal white adipose depots to have enough energy required for the stress response. Together the data gathered from our first set of experiments define the adipose tissue as an extremely malleable tissue that can adapt to the adverse environmental condition such as stress by regulating pro-adipogenic/lipolytic and sympathetic markers' expression in a specific manner in different fat pads.

Molecular changes detected in CD1 Dominant vs CD1 Subordinate mice are in agreement with their stress induced phenotype.

We have previously shown that chronic social stress along with a hypercaloric diet induced a healthy metabolic phenotype in dominant mice while caused obesity and metabolic syndrome subordinate [Bartolomucci et al., 2009, Sanghez et al., 2013]. In chapter 2, our molecolar data showed that domninats presented a significant up-regulation of key catabolic markers such as UCP1 in subcutaneous inguinal and isnuiln signaling markers in liver and

muscle, together responsible for anti-obesity and anti-diabetic effects observed in these high rank animals. On the other spectrum, the obese and diabetic subordinates displayed a higher expression of pro-adipogenic factors such as PPARγ and maintained adipose sensitivity to insulin which allowed for lipid accumulation and cell expansion drawing them to become them glucose intolerant as well. As a compensatory mechanism within the subordinate individuals we detected a significant increase in brown adipose tissue's UCP1.

Genetic background does influence expression of key metabolic markers following stress.

Genetics play an important role in body fat accumulation and distribution [Bouchard et al., 2007, Barsh and Schwartz, 2002, Friedman, 2004] and it is current thought that 45 to 75% of obesity is due to genetics [Speliotes et al., 2010] although it is not the only determent. Extensive studies performed in our laboratory have shown that chronic social stress induced obesity and metabolic syndrome like phenotype in CD1, C57BL/6J as well as 129EvSv subordinate mice [Bartolomucci et al., 2009, Sanghez et al., 2013, Bartolomucci et al., 2010, Dadomo et al., 2011]. In chapter 3 we detected that stress had the greatest impact on the 129EvSv strain of mice. Overall in the different adipose tissues analyzed, the NPY system gene had a very low expression in the CD1 and C57BL/6J stressed mice. We found that stress induced a remarkable increase in NPY genes of interest only in 129SvEv strain and only within the subcutaneous inguinal fat. These data were very similar to results published by Kuo et al., [Kuo et al., 2007] in stressed 129X1/SvJ mice strongly suggesting that NPY potentiation of stress effects are strain and tissue specific. Moreover, the 129SvEv subordinate mice maintained better functional insulin signaling system compared to the other strains, in agreement with our physiological data. In conclusion, our data found that the 129SvEv mice were the most responsive to stress induce molecular changes to adapt to the environmental challenge.

Chronic social stress restores sympatric markers' expression in βless mice

Global β-adrenergic receptors, β_1-AR, β_2-AR and β_3-AR, knockout mice (βless) have dysfunctional sympathetic activity, impaired adaptive thermogenesis and diet induced thermogenesis, therefore not vital at cold temperatures and become massively become massively obese under high fat diet [Bachman et al., 2002, Lowell and Bachman, 2003, Asensio et al., 2005]. In chapter 4 we observed that contrarily to all other strains investigated stress induced

weight loss and reduced fat mass paradigm did normalize the otherwise obese phenotype in βless mice as well as restored the expression of UCP1 in brown and white adipose depots indicating stress induced sympathetic innervation and lipolysis. Moreover within white depots, stress induced low NPYergic activity, inhibited anti-lipolysis and pro-adipogeneis, while in brown adipose we detected a significant decrease of NPY and increase in Y1R that we believe is involved in the up-regulation of UCP1 and transdifferention of a white into a brown phenotype. Up to now, this is the only "obese resistant strain" found following our stress procedure. A major interest now is to undercover the mechanism through which stress restored a healthy phenotype in βless mice.

To conclude, many molecular changes within the adipose tissue were discussed in this thesis. My hope is to have enlightened some important and unknown molecular changes that stress produced on metabolically active tissues. My next challenge will be to understand some of the hypothesized mechanisms that I have laid out throughout my thesis on how an environmental challenge such as psychosocial stress can modify the abc of life.

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