The role of chronic variable stress and dietary fat on insulin sensitivity in a mouse model for posttraumatic stress disorder

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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Düsseldorf, Juli 2015

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des Deutschen Diabetes-Zentrums,

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Gedruckt mit der Genehmigung der

Mathematisch-Naturwissenschaftlichen Fakultät der

Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Hadi Al-Hasani

Koreferent: Prof. Dr. Eckhard Lammert

Tag der mündlichen Prüfung: 13.07.2015

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und ohne fremde Hilfe verfasst habe. Es wurden nur die angegeben Quellen und Hilfsmittel benutzt. Wörtlich und inhaltlich übernommene Gedanken wurden als solche kenntlich gemacht.

Düsseldorf, den 13.07.2015 Sabrina Müller-Lühlhoff

„Das schönste Glück des denkenden Menschen ist,
das Erforschliche erforscht zu haben und das Unerforschliche zu verehren.“

Johann Wolfgang von Goethe

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1 Introduction

1.1 Stress

Stress is a threat to the homeostasis of an organism induced by “intrinsic or extrinsic adverse forces, called stressors” [1]. More in detail, stress can be defined as “the behavioral and physiological responses generated in the face of or in anticipation of a perceived threat” [2] in order to re-establish the homeostatic equilibrium of the organism. The stress challenge to the organism’s homeostasis implies the mobilization of energy sources to be used by target tissues involved in the fight-or-flight response like the skeletal muscle, the cardiac muscle and the brain. In an acute way, the mobilization of energy stores (glucose and fatty acids) is required for the survival of an organism and therefore considered beneficial whereas in contrast, the prolonged exposure to stressors like in chronic stress situations can contribute to metabolic impairment [3, 4].

Actually, war veterans, police officers, people from impoverished urban population or victims of natural disasters suffering from post-traumatic stress disorder (PTSD), a severe consequence of chronic stress, show a high prevalence of the metabolic syndrome, obesity and cardiovascular diseases [5-10].

Therefore, along with decreased physical activity and increased consumption of high caloric food, stress could be considered also a metabolic regulator, contributing to visceral obesity and type 2 diabetes [11].

1.2 The hypothalamus-pituitary-adrenal (HPA) axis

The HPA axis is one of the main pathways involved in the organism’s stress response [12]. It consists of a cascade of endocrine actions located in the hypothalamus, the pituitary gland and the adrenal cortex (Figure 1). Stress sets off the release of corticotropin-releasing hormone (CRH) from neurons in the paraventricular nucleus (PVN) of the hypothalamus into the portal blood adjacent to the pituitary gland. CRH secretion in turn triggers the synthesis and secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland into the general circulation which results finally in the stimulation of glucocorticoids (GCs) release from the adrenal cortex.

GCs are synthesized originally from cholesterol that undergoes several steps of transformation in the *zona fasciculata* of the adrenal cortex. First, cholesterol is converted by cholesterol desmolase to pregnenolone that can be further processed to 17-OH pregnenolone or progesterone. Subsequently, 17-OH pregnenolone can be converted to cortisol and progesterone can be converted to corticosterone through further enzymatic steps. The main type of glucocorticoid secreted in humans is cortisol whereas in rodents it is corticosterone. Although corticosterone is also secreted by humans, it circulates at 10-20 fold lower than cortisol [13-16] and is mainly considered an intermediate in the synthesis of the mineralocorticoid aldosterone [17]. In relation to structure those two hormones differ in just one additional OH-group in corticosterone.

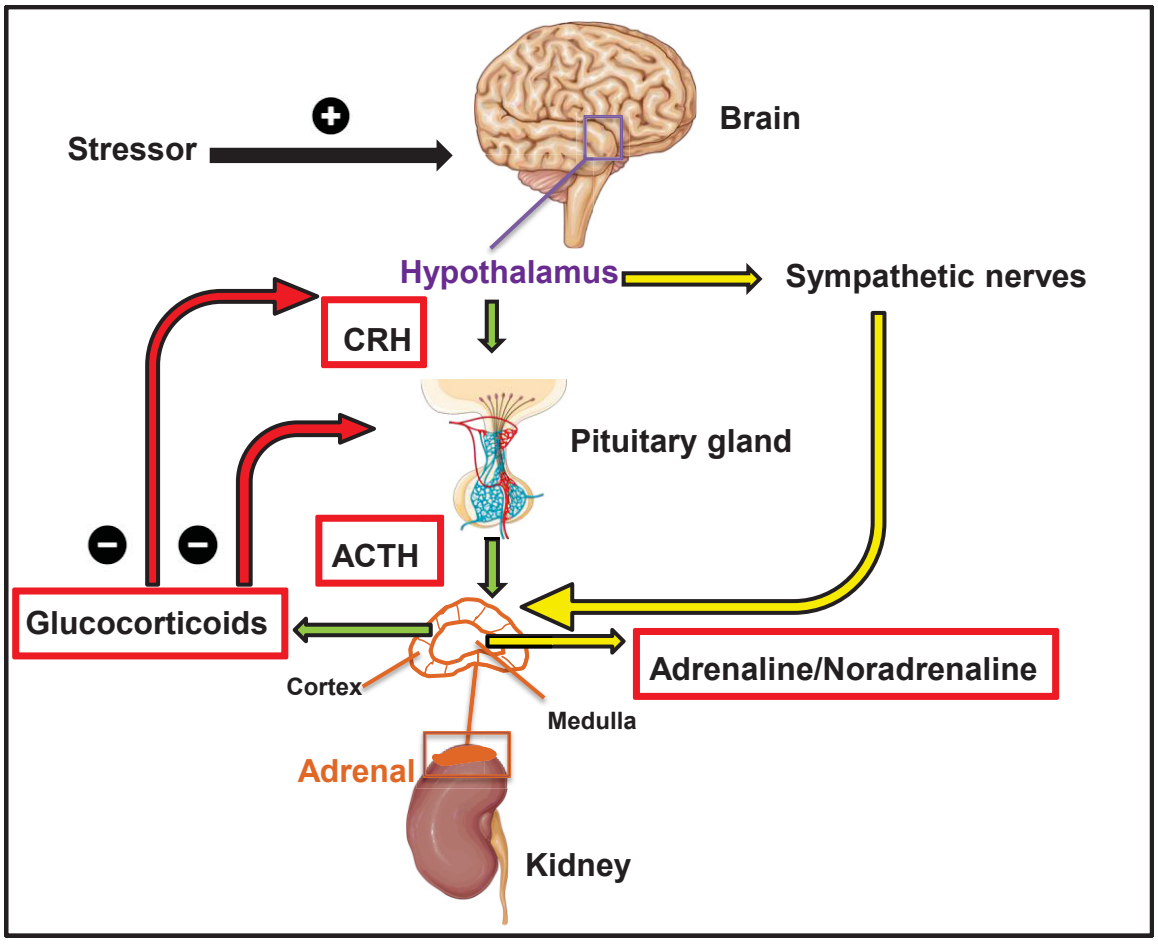


Figure 1: The stress response system. The hypothalamus-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) as the two branches of the body's stress response are activated from the hypothalamus of the brain. The HPA axis includes the release of CRH from the hypothalamus which triggers the release of ACTH from the pituitary gland. In response, glucocorticoids (GCs) are secreted from the adrenal cortex. GCs limit the actions of the hypothalamus and the pituitary gland in a negative feedback loop (red arrows). The actions of the SNS include the activation of sympathetic nerves by the hypothalamus which triggers the release of adrenaline and noradrenaline from the adrenal medulla (yellow arrows).

GCs have long-term effects [18] through the binding to the intracellular glucocorticoid receptor (GR), translocation to the cell nucleus and subsequent alteration of gene transcription.

More in detail, GCs act via a signaling pathway that is based on binding to the GR that belongs to the nuclear superfamily of ligand-activated transcription factors [19] and exists in two isoforms [20]. In the basal state, the GR is maintained in the cytoplasm and bound to an inactive multiprotein complex that includes a heat shock protein (HSP), a chaperone protein that controls and limits the GRs interaction with transcriptional regulatory sites [21]. Upon stress, released GCs transit the plasma membrane and bind to the ligand binding domain of the GR (19), conversely the GR is released from the inactive chaperone complex and translocated into the cell nucleus. Inside the nucleus, the GR can regulate and modulate gene transcription either through binding to glucocorticoid responsive elements (GREs) or interaction with other transcription factors [22, 23]. Those effects can last beyond the return of glucocorticoid levels to basal state (Figure 2).

To limit the effects of GCs, the secretion is tightly regulated through a negative feedback loop to the ACTH secretory response in the pituitary on the one hand [1] and to the CRH secretory response in the PVN on the other hand [24, 25]. Also as part of this negative feedback loop, the back-travel of increased circulating GCs includes various areas of the brain, such as the hypothalamus [26], the hippocampus [27], the medial prefrontal cortex [28] and the pituitary gland [29].

It is well-known that both the deficiency and the excess of endogenous or exogenous GCs are associated with severe disturbances in systemic energy metabolism [20, 30]. For instance patients with Addison's disease as a chronic endocrine disease characterized by a loss of GC actions due to autoimmunity against the adrenal cortex, inherited GC synthesis dysfunction or pituitary disease show impaired stress resistance, hypoglycemia, lymphoid tissue hypertrophy and weight loss [20, 30, 31]. In contrast, Cushing's patients with sustained and pronounced hypersecretion of GCs display central obesity, increased breakdown of skeletal muscle mass, hyperglycemia, fatty liver development, hypertension, elevated cholesterol, immunodeficiency and insulin resistance [20, 32]. Therefore, although the HPA axis is part of the beneficial acute stress response, prolonged activation like under chronic stress conditions may result in severe metabolic dysfunction.

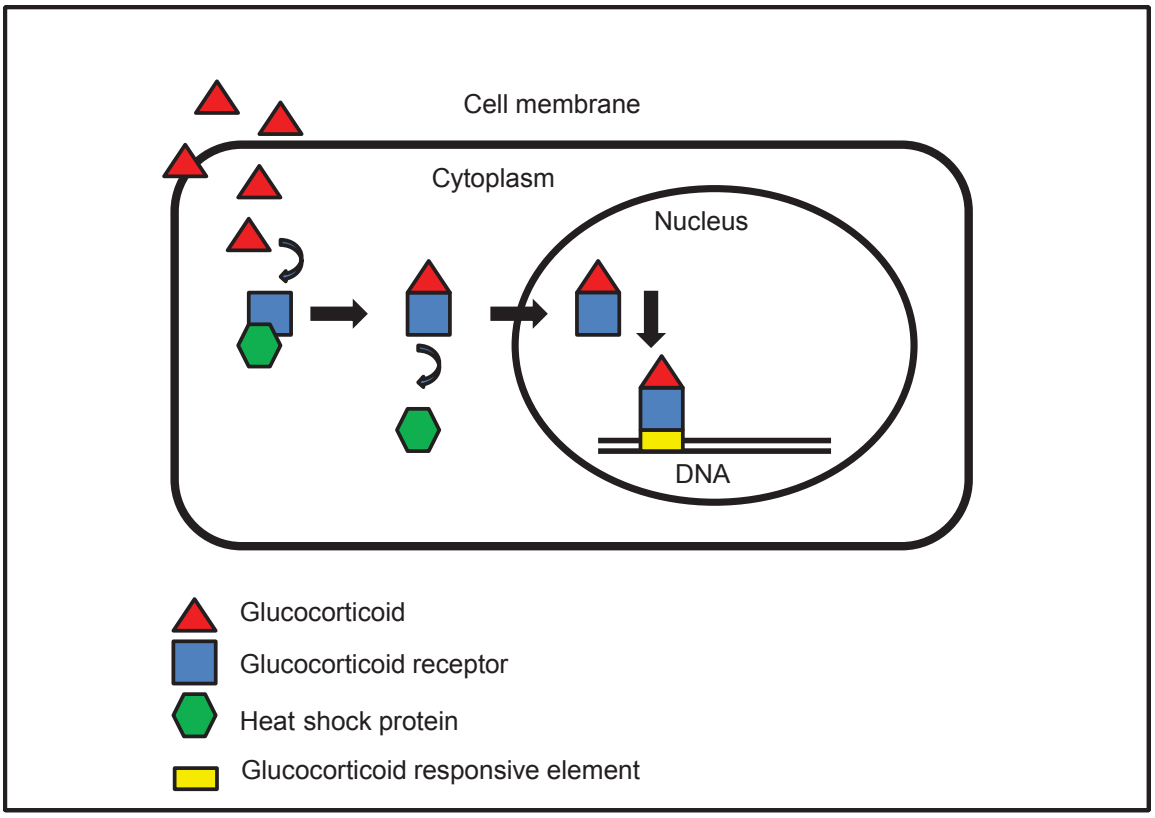


Figure 2: The intracellular mechanism of glucocorticoid action. After entering the cell, glucocorticoids (GCs) bind to the glucocorticoid receptor (GR) which is associated with a heat shock protein complex under basal conditions. Binding of GCs to their receptor leads to the dissociation of the heat shock protein complex and to a translocation of the GR into the nucleus. Inside the nucleus, the GR can regulate and modulate gene transcription either through binding to glucocorticoid responsive elements (GREs) or interaction with other transcription factors.

1.3 The sympathetic nervous system

The sympathetic nervous system (SNS) is together with the HPA axis the other branch in the stress response [2] (Figure 1).

The SNS main function is to mediate fast responses in relation to the body needs to be highly alerted and to be supplied with a burst of energy to cope with a threatening situation (fight-or-flight response) [2]. The hormones involved in that process are the catecholamines adrenaline and noradrenaline, which are released from the adrenal medulla as a consequence of sympathetic signals sent by the hypothalamus through sympathetic nerves that innervate peripheral organs. The innervation is derived from efferent preganglionic fibers in the spinal cord and the nerves synapse in the bilateral chains of sympathetic ganglia with postganglionic sympathetic neurons that project to organs like heart, skeletal muscle, kidney, gut, fat and others [1].

Catecholamines are synthesized in the chromaffin cells of the adrenal medulla [33] from the amino acid tyrosine, which is converted into L-Dopa by tyrosine-hydroxylase, an enzyme that is activated by the sympathetic nerves and serves as the rate limiting enzyme in the whole process [34]. In turn, L-Dopa is transformed into dopamine. Using vitamin C as a cofactor, a beta-hydroxylase can convert dopamine to noradrenaline and the final addition of a methyl group to noradrenaline by a methyltransferase finalizes the conversion to adrenaline.

In contrast to GCs that regulate gene expression, adrenaline and noradrenaline exert their metabolic effects through a class of G-protein coupled receptors called adrenergic receptors that consist of two main groups, α-and β-receptors which can be further divided into several subtypes [35]. These receptors can be found in different target tissues illustrating the variety of effects induced by adrenaline and noradrenaline to cope with a perceived danger.

An over-activation of beta adrenergic activity can be part of pathological conditions [36] and may result in insulin resistance, altered glucose and lipid metabolism, mitochondrial dysfunction [36] and heart failure [20].

1.4 The role of the hypothalamus in stress/metabolism

As both responses from the SNS and the HPA axis originate from the hypothalamus, this brain area is of particular interest in the physiology of stress. Furthermore, the hypothalamus is highly involved in the regulation of energy homeostasis and food intake (factors that are known to be altered in the response to stress) as a site of action for both central and peripheral metabolic signals [37]. Especially the arcuate nucleus in the mediobasal hypothalamus is highly relevant with regard to metabolism as it contains two antagonistic centrally-projecting neuron populations [37]: one group of neurons co-expressing the orexigenic (appetite-stimulating) neuropeptides Y (NPY) and agouti-related protein (AgRP) and the other group containing the anorectic (appetite suppressors) neuropeptides pro-opiomelanocortin (POMC) and cocaine-amphetamine related transcript (CART).

Those two opposing populations of neurons, also called “first order” neurons, project to “second order” neurons in other areas of the hypothalamus, for instance to the PVN (which also contains autonomic control neurons activating the SNS [38]) and to the ventromedial and dorsomedial nuclei [37]. From these brain areas, neurons project to the nucleus of the solitary tract in the brainstem and to the dorsomotor nucleus of the vagus for the integration of hypothalamic signals with peripheral ones from liver and gastrointestinal tract in order to control and regulate energy homeostasis [37]. Besides, circulating peripheral hormones play an important role in the energy balance control of AgRP/NPY and POMC/CART neurons by activation or inhibition of those two opposing neuron populations.

For instance leptin, a satiety hormone secreted by the adipose tissue, can activate POMC neurons and decrease food intake through the alpha-melanocyte stimulating hormone (α-MSH)-melanocortin 4 receptor (MC4R) axis [37]. On the other hand, the orexigenic NYP/AgRP neurons are inhibited by leptin as well as by insulin [39] whereas they are activated by ghrelin [40]. While the two antagonistic neuron groups can get activated or inhibited by circulating hormones they also control the activity of each other [2, 41].

Actually, these peptides expressed in the hypothalamic neuron network have been described to be associated with stress. For instance NPY is considered the most abundant neuropeptide within the brain [42] and plays a pivotal role not only in the regulation of food intake and energy homeostasis but also in anxiety and stress-coping [42, 43] acting through several isoforms of its receptor, Y1-Y5 [42]. Genetic variations in NPY can lead to low expression of this protein in the human brain resulting in impaired stress coping and higher prevalence of anxiety disorders [44, 45]. Furthermore, plasma levels of NPY are reduced in PTSD patients and increased in people recovered from PTSD [46]. However, it has been shown that GCs stimulate the expression of NPY in an adenosine-monophosphate-activated protein kinase (AMPK)-dependent manner in the hypothalamus of rats [47] and chickens [48] in order to regulate energy homeostasis in response to stress. In the periphery, it has been shown in mice that an NPY-NPY receptor 2 (Y2R) related pathway induces adiposity in response to 2 weeks of chronic stress (cold exposure and aggression) and parallel high fat high sugar diet feeding as indicated by higher gene expression of both NPY and Y2R [49, 50]. Also in dementia caregiving women as a model for chronic stress abdominal adiposity was increased due to high levels of NPY [51].

The apparent contradiction of NPY levels in subjects with PTSD and the GCs action could be explained in relation to the time when those measurements were taken, the tissue and endogenous versus synthetic GCs.

Same as for NPY, the expression of AgRP has been reported to be increased upon GCs administration in an AMPK-dependent manner in the hypothalamus of rats [47]. In a more physiological approach, NPY and AgRP expression were shown to be enhanced upon cold exposure stress in the hypothalamus of chickens [52].

Although POMC contrary to NPY and AgRP is anorectic, POMC is a precursor of ACTH [53]. Therefore POMC is considered an important regulator of the HPA axis and patients suffering from ACTH-mediated Cushing's syndrome show increased expression of *Pomc* [54]. *Pomc* expression in the pituitary gland is also enhanced upon acute immobilization stress [55, 56] and early-life stress in mice [53]. DNA methylation in the *Pomc* gene has been described in relation to its enhanced expression in stress [53].

In summary, hypothalamic peptides with an important role in energy homeostasis may also have relevant effects with regard to stress and could be potential candidates in a major mechanism behind stressed-induced metabolic alterations.

1.5 GCs and glucose metabolism

The release of glucose as a rapid source of energy to be used by skeletal muscle plays a key role under stress conditions. GCs, as the name already reveals, have a main impact on glucose metabolism [57] (Figure 3).

Specifically, in response to a stressful event, GCs enable a high release of glucose from the liver [20] through gluconeogenesis, in order to keep blood glucose levels stable and to provide energy to the brain, renal medulla and erythrocytes [20, 58]. The up-regulation of gluconeogenesis is induced mainly at the transcriptional level by increased induction of the gluconeogenic enzymes pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK) and finally glucose-6-phosphatase (G6Pase) that hydrolyzes glucose-6-phosphate into free glucose and inorganic phosphate [59, 60] and also controls hepatic glucose release from glycogenolysis [61].

PEPCK is the rate-limiting enzyme in gluconeogenesis [62, 63] and is encoded by the *Pck1* gene. Its expression in response to fasting is induced on the one hand by glucagon acting through cyclic adenosine monophosphate (cAMP) and on the other hand, as just mentioned, by GCs and the GR. The *Pck1* gene contains a glucocorticoid response unit (GRU) that was characterized initially in 1990 by Imai et al. and includes binding sites for the GR [30, 62]. Binding of the GR to those sites increases the expression of *Pck1*, an effect that is potentiated by peroxisome proliferator-activated receptor co-activator 1α (PGC-1α), a factor that has been identified as a co-activator for nuclear receptors like the GR besides its classical function in mitochondrial biogenesis and thermogenesis [64, 65].

Besides the expression of *Pck1* also the expression of *G6pc*, specifically the catalytic subunit, has been described to be regulated by the GC/GR-axis [30]. *In vitro* studies show that the treatment of isolated hepatocytes with dexamethasone increases the expression of *G6pc* [66, 67] and on the contrary, *in vivo* studies show that adrenolectomized mice lack *G6pc* expression [68]. Similar to the GRU in the *Pck1* gene, three glucocorticoid responsive elements (GRE) have been identified in the promoter of the *G6pc* gene [69, 70].

Finally also the PC gene expression is up-regulated by GCs as shown *in vitro* with human hepatocytes treated with dexamethasone in combination with cAMP [71].

In view of these findings, GCs increase gluconeogenesis in the liver through binding of the GR to GREs and GRUs in gluconeogenic genes, opposing the inhibiting action of insulin on gluconeogenesis. Indeed, an impairment of the suppression of gluconeogenesis by insulin

following hyperglycemia is a main characteristic of insulin resistance and type 2 diabetes [30, 72]. Therefore, GCs contribute to hepatic insulin resistance by their property to favor gluconeogenesis. Indeed, an impairment of the suppression of gluconeogenesis by insulin following hyperglycemia is a main characteristic of insulin resistance and type 2 diabetes [30, 72]. A key role for the GC/GR-axis triggering gluconeogenesis under insulin resistant/diabetic conditions has been described in mice lacking the GR in liver. Those animals showed hypoglycemia under fasting conditions, were protected from streptozotocin- induced hyperglycemia and displayed a down-regulation of gluconeogenic gene expression [72]. In line with this study, antisense oligonucleotides targeted against the GR [73] or a liver-selective GR antagonist (GR antagonist is made liver-selective through bile acid conjugation) [74, 75] reduces hepatic glucose production and ameliorates hyperglycemia in diabetic mouse models. Also in humans, a decrease of hepatic insulin sensitivity by GC action has been reported, specifically in patients suffering from Cushing's syndrome [76, 77].

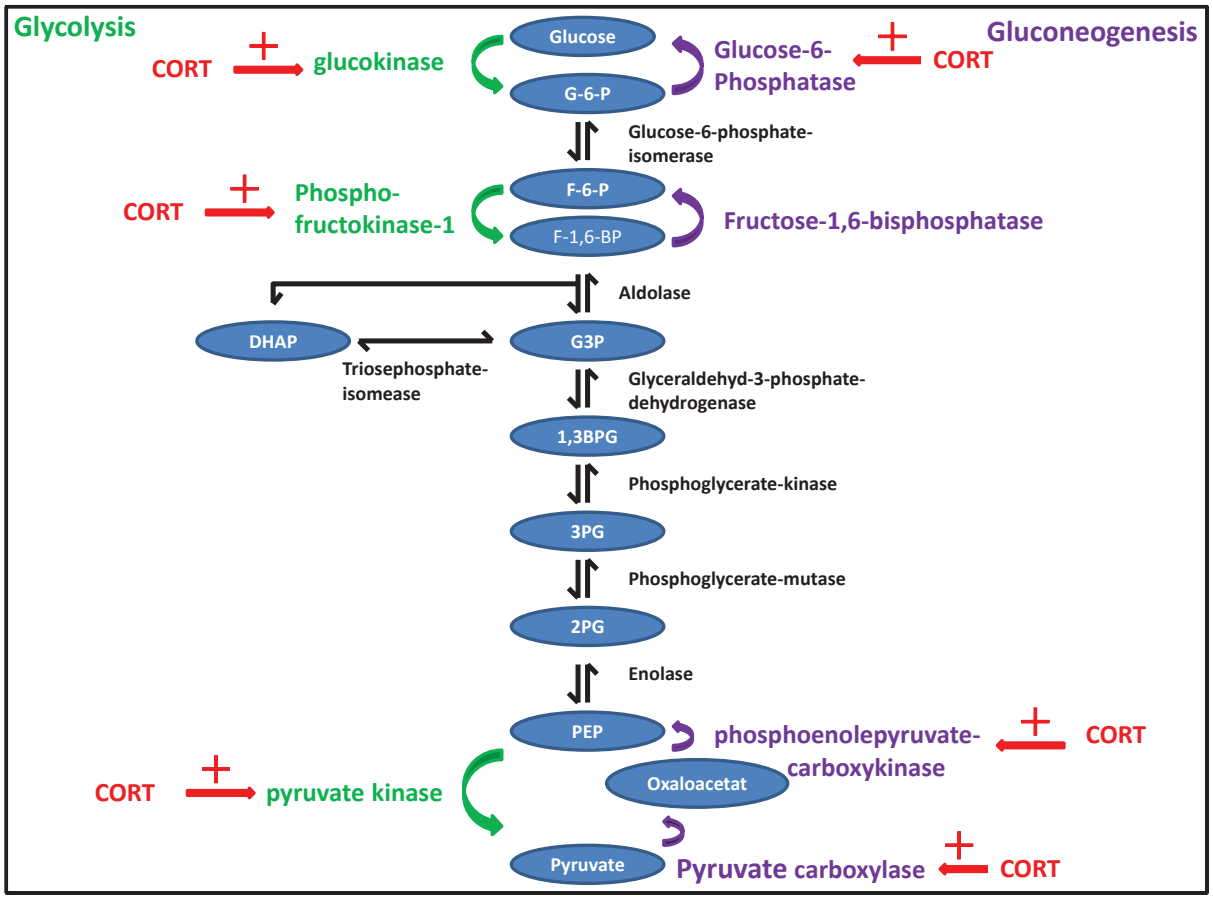


Figure 3: Effects of GCs (Corticosterone = CORT) on hepatic glucose metabolism. Key enzymes of both gluconeogenesis (purple) and glycolysis (green) are affected by GC actions. G-6-P: glucose-6-phosphate; F-6-P: fructose-6-phosphate; F-1,6-BP: fructose-1,6-bisphosphatase;DHAP: dihydroxyacetone phosphate; G3P: glyceraldehyde-3-phosphate; 1,3BGP: 1,3-bisphospho-glycerate; 3PG: 3-phosphoglyceric acid; 2PG: 2-phosphoglyceric acid; PEP: phosphoenolpyruvate.

Besides the effects on glucose metabolism at the level of the liver, GCs also directly affect glucose metabolism in the skeletal muscle [30]. Rats treated with cortisone acetate (100 mg/kg s.c., 5 days) or dexamethasone (1 mg/kg per day for 5 days, application route not indicated) showed decreased phosphorylation of the insulin receptor and phosphoinositide 3-kinase (PI3K) activity in muscle [78, 79]. Furthermore, dexamethasone treatment inhibited insulin-mediated proteinkinase B (AKT) phosphorylation in rat muscle [80] and *in vitro* in C2C12 cells [81].

Changes in insulin sensitivity are as well linked to changes in glucose uptake through glucose transporters (GLUTs) such as GLUT1 and GLUT4 [82].

GLUT1 is a transporter with a wide tissue distribution that is not markedly translocated by insulin [83], therefore it is also known as the basal glucose transporter, mediating glucose uptake under insulin non-stimulated conditions [83-85]. GLUT1 is highly expressed in skeletal muscle where it ensures sufficient transport of glucose under basal conditions as suggested by studies in cultured skeletal muscle myotubes [86-89].

In contrast to GLUT1, GLUT4 is considered an insulin-dependent glucose transporter and thus the main isoform in insulin-sensitive tissues like skeletal muscle and the adipose tissue [83]. In consequence, GLUT4 plays an essential role in whole-body glucose homeostasis [90] and multiple insulin signaling pathways (AMPK, AKT etc.) control the trafficking of GLUT4 from intracellular vesicles to the plasma membrane linking insulin signaling and vesicle trafficking pathways [90].

Although the complex signaling cascade of GLUT4 trafficking suggests a main role of GLUT4 in the restriction of glucose uptake under low-insulin states, a small amount of GLUT4 was found at the cell surface in the non-stimulated state (1% in brown adipocytes and 5-10 % in T3T-L1 adipocytes, [83]). Therefore the targeting of GLUT4 under basal conditions and the mobilization in the transition from the basal to the insulin-stimulated state is as well a question of debate [83].

A suppression of glucose uptake due to impaired translocation of GLUT4 to the cell surface has been described as well as a key mechanism in GC-induced insulin resistance of skeletal muscle. Specifically, rats injected with dexamethasone for 5 days (0.5 mg/day, i.p.) showed a decreased translocation of GLUT4 in skeletal muscle in response to *ex vivo* insulin stimulation [91]. Likewise, treatment with 0.9 mg/kg/day of dexamethasone reduced insulin-stimulated 2-[3H]deoxyglucose (2-[3H]DG) uptake and cell-surface (ATB-[2-3H]BMPA-photolabeled) GLUT4 although total GLUT4 content was increased in skeletal muscle of rats [92].

GCs also affect glucose storage as glycogen in liver and muscle although the results are controversial with some studies showing induction of glycogen synthase by GCs in liver of animals models [93] whereas in others glycogen synthase activity in skeletal muscle was

impaired either in humans after corticosteroid therapy [94] or in rats after administration of dexamethasone (0.4 mg/kg/day for 14 days) [95]. Therefore, the effects of GCs on glycogen synthase may be tissue dependent.

In summary, it has been shown that a prolonged activation of the GC/GR-axis aggravates the already present pathological state of insulin resistance and may contribute to dysfunction of glucose metabolism in skeletal muscle [20] and indeed correlations have been found between GR mRNA levels in skeletal muscle and the extent of insulin resistance in diabetic patients whereas GR mRNA levels normalized upon treatment of insulin resistance [96, 97].

Regarding the effects of GCs on glucose metabolism in the adipose tissue, numerous studies were carried out in the nineteen sixties and seventies in rats with dexamethasone and demonstrated that the ability of adipocytes to take up and metabolize glucose was decreased in response to dexamethasone administration *in vitro* and *ex vivo*. However, the results were inconsistent with regard to the question whether GCs decrease uptake and oxidation of glucose just under basal or also under insulin-stimulated conditions. Some studies showed that the diminished glucose metabolism in response to GC administration was restricted to basal conditions [98-100] whereas others observed a decrease of glucose metabolism in response to GCs also under insulin-stimulated conditions when transport of glucose into fat cells was rate-limiting [101].

Therefore, GCs may alter glucose metabolism of the fat cell, similar as in muscle, by an inhibition of GLUT trafficking. In line with this suggestion, dexamethasone has been shown to decrease the number of GLUTs in the plasma membrane of 3T3-L1 adipocytes [102] under both basal and insulin-stimulated conditions. Impaired insulin-mediated GLUT4 translocation in T3T-L1 adipocytes cultured with dexamethasone was associated with a reduction of both the protein levels and the tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) in T3T-L1 adipocytes [102]. Similarly, isolated rat adipocytes stimulated with 0.3 µmol/l dexamethasone for 24 h showed a decrease in IRS-1 expression, in PI3K and AKT content and in insulin-mediated AKT phosphorylation [103]. Total protein content of GLUT4 was not altered by dexamethasone in those studies whereas interestingly, there was an increase in insulin receptor substrate 2 (IRS-2) expression and/or phosphorylation in spite of the decrease in IRS-1.

In summary, a variety of acute *in vitro* and *ex vivo* studies present evidence that the administration of synthetic GCs promotes insulin resistance at the level of the three main tissues implicated in glucose homeostasis. Furthermore, patients suffering from Cushing's syndrome show hyperglycemia and insulin resistance.

However, it remains elusive whether the phenomena observed with exogenous synthetic GCs are also found in a physiological approach of chronic stress conditions and whether

those effects persist in time (resembling the Cushing's syndrome) and are regulated by diet composition as a new aspect that has not been addressed before.

1.6 GCs and lipid metabolism

Besides glucose, lipids are a relevant source of energy that is mobilized upon stressful events. The actions of GCs on lipid metabolism are not trivial and go, same as the effects on glucose metabolism in opposing directions as GCs promote both lipolysis and lipogenesis depending on time, concentration and research model [104].

Lipolysis is referred as the breakdown of triglycerides (TAGs) stored in adipocytes. TAGs are composed of three fatty acids bound to glycerol. Lipolytic breakdown is catalyzed by the subsequent action of the lipases adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) that results in the cleavage of the three fatty acids from the glycerol backbone [18]. Subsequently, fatty acids can be used by the organism as a fuel source through mitochondrial β-oxidation [105], especially by skeletal muscle [106]. The process of mitochondrial β-oxidation is preceded by the transport of fatty acids into the mitochondria and AMPK, acetyl-CoA carboxylase (ACC) and carnitinepalmitoyl-transferase 1 beta (CPT1β) are involved in its regulation [107].

Hypercortisolemia and GC treatment lead to high levels of circulating free fatty acids in humans [108, 109] and rodents [110, 111]. It has been observed in relation to GC-induced lipolysis an enhanced expression of the lipases ATGL and HSL in adipose tissue [104, 112, 113]. However, non-genomic effects (effects that are not related to transcriptional action) of GCs on lipolysis cannot be excluded [104] like binding of the GR complex to intracellular proteins which are relevant with regard to lipolysis like ATGL or HSL.

On the other hand, the activity of lipases is also controlled by catecholamines as upstream regulators acting through cAMP- and protein kinase A (PKA)-dependent mechanisms [112]. Notably, lipolytic effects of catecholamines on the organism are enhanced through GCs, a phenomenon referred to as the permissive effect [114] and presenting a link between the two stress response systems of the organism. However, whether the lipolytic actions of GCs are a short-or long-term effect is not completely understood although limited previous studies done in humans failed to find long-term effects of GCs on lipolysis [109, 115-117]. However, the effects may also be dependent on the model, the concentration of GCs and the duration of treatment.

Besides their lipolytic effects, GCs also display adipogenic actions [18, 30]. In rodents, those adipogenic actions have been linked in part to the enzyme 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) that converts inactive GCs (cortisone in humans and 11-dehydrocortisone in rodents) from the circulation to the active form inside tissues like adipose

tissue, liver, muscle and brain [18]. *In vitro* in T3T-L1 adipocytes, stimulation with 1 µM of dexamethasone in combination with 10 µg/ml of insulin increased the mRNA and protein levels of 11βHSD1 and induced the expression of adipogenesis marker genes, while silencing of the 11βHSD1 gene abolished dexamethasone adipogenic action suggesting that dexamethasone exerted its actions through 11βHSD1 [118]. In addition, transgenic mice over-expressing 11βHSD1 in adipocytes displayed increased abdominal adiposity enhanced by HFD consumption [119]. In contrast, knockout mice for 11βHSD1 showed lower gluconeogenic gene expression in liver in response to fasting, lower levels of plasma TAGs and higher fatty acid oxidation [30]. In line with those observations, 11βHSD1 expression has been shown to be elevated in obese animals and humans [120, 121]. Therefore, an up-regulation of 11βHSD1 may be the first step for increased adiposity by GC action.

Increased visceral adiposity by GCs is well described in patients suffering from Cushing's syndrome [122] and is characterized by a pronounced redistribution of subcutaneous peripheral fat to central depots like the abdomen, the nape of the neck, the cheeks and subclavicular regions [20, 109, 123], also an accumulation of TAGs in the liver could be observed [124] in accordance with other reports stating that GCs promote hepatic fatty acid synthesis and storage [30, 125, 126].

In conclusion, GCs promote adiposity on several levels:

1. Besides up-regulation of 11βHSD1, GCs may induce hypertrophy of adipocytes through the enzyme lipoprotein lipase (LPL). LPL has a dual function and is related to hydrolysis of triglycerides as well as to re-esterification and storage of fatty acids [127] and has been demonstrated to be highly expressed in the presence of GCs [127-129].
2. Furthermore, GCs can promote hyperplasia (increase in adipocyte cell number) promoting the differentiation of pre-adipocytes to mature adipocytes [18].
3. Finally, GCs can also increase lipogenesis by increasing the expression of fatty acid synthase (FAS), an enzyme that catalyzes the synthesis of palmitate from Acetyl-CoA and malonyl-CoA [130, 131].

Taken these studies together, GCs have a great impact on fatty acid metabolism on different levels (lipolysis, lipogenesis) depending on model, tissue and duration of GC exposure. However, there is still a lack of understanding for the mechanism orchestrating the short-and long-term actions of both GCs and catecholamines in response to a stressful period with regard to lipid metabolism. Therefore it is of high relevance to investigate *in vivo* under a physiological approach to which extent changes in lipid metabolism are relevant for long-term pathophysiological conditions caused by stress.

1.7 GCs and inflammation

GCs exert potent anti-inflammatory effects, which are essential for the orchestration of metabolic and immune responses [132]. Those anti-inflammatory effects are mediated by the down-regulation of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) [133], interleukin-4 (IL-4) and interleukin-5 (IL-5) [134], and the up-regulation of anti-inflammatory factors such as lipocortin-1 and interleukin-10 (IL-10) [135]. These effects take place through the binding of the GC/GR complex to the respective GREs [136] as already described above or on the inhibitory interaction between activated GRs and the pro-inflammatory transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and activator protein-1 (AP-1) [135].

However, there are also reports stating that GCs can promote inflammation. Studies in parents of cancer patients, as a model for chronic stress, showed an impaired suppression of pro-inflammatory interleukin-6 (IL-6) production in response to synthetic GC administration [137]. Similarly, IL-6 levels have been shown to be increased in splenocytes of mice exposed to chronic social disruption [138] demonstrating that chronic stress impairs the immune's system response to anti-inflammatory signals. Furthermore, humans suffering from long-term stressful events were more prone to develop a cold in response to a rhinovirus-challenge due to an inadequate regulation of inflammation, reflected in higher levels of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, as a consequence of GR resistance or impaired sensitivity of immune cells to GC hormones which usually block the inflammatory response [139].

Therefore, in contrast to the acute stress response that is associated with a decrease in inflammation, chronic stress may be associated with a pro-inflammatory state and the development of GR resistance. It has been proposed that acute stress has anti-inflammatory effects increasing immunity whereas chronic stress can overcome those short-term effects [140, 141]. However, there is still a lack of studies investigating in a physiological model the long-term effects of stress in combination with dietary fat on inflammation and its potential relation with insulin resistance.

1.8 GCs/ stress and hormones

Although GCs and catecholamines are considered the main hormones in the stress response of the organism, other centrally and peripherally active hormones could also be involved in the stress-related metabolic alterations. Few studies have focused on the gastrointestinal hormone ghrelin as well as on the adipokines leptin, adiponectin, resistin but with inconsistent findings depending on the kind of stress and model. In the present thesis

the role of those hormones as potential mediators in the regulation of insulin sensitivity in response to CVS also regarding time and diet was analyzed. The analysis was as well extended to other hormones that may have a key role in stress-induced metabolic regulation.

1.8.1 Ghrelin

One candidate that could be of fundamental interest is the hormone ghrelin. Ghrelin is a 28 amino acid peptide synthesized mainly by the X/A-like cells of the oxyntic mucosa of the stomach [142] and the intestine that promotes a positive metabolic state by increasing food intake and adiposity. These effects occur on the one hand via central activation of the melanocortin system of the hypothalamus and a subsequent SNS-mediated up-regulation of lipogenic enzymes in WAT [143]. On the other hand, ghrelin decreases energy expenditure as well as locomotor activity [144]. Ghrelin exerts its actions through the growth hormone secretagogue receptor (GHSR) which is to date the only known ghrelin receptor [145]. The GHSR has a broad tissue distribution with the highest expression found in the arcuate nucleus of the hypothalamus, a brain center that is highly associated with the control of energy homeostasis and contains NPY/AgRP expressing neurons that can be activated by ghrelin action in order to stimulate food intake [40]. Therefore, taken together the relevance of the hypothalamus regarding energy homeostasis and the proximity of ghrelin action there is increasing interest in the role of ghrelin with regard to stress metabolic regulation.

Indeed the administration of exogenous ghrelin, either central or peripheral, can activate the HPA axis in rodents leading to anxiety [146]. On the one hand, this phenomenon is caused by the stimulation of hypothalamic CRH neurons and increased CRH gene expression by ghrelin [147], conversely, ghrelin-induced anxiogenic effects can be inhibited by the administration of a CRH receptor antagonist [148]. On the other hand, ghrelin directly stimulates ACTH and cortisol secretion [149]. Also *vice versa*, ghrelin and GHSR expression levels are increased in the PVN neuronal cell line 4 B in response to dexamethasone stimulation and that increase is blocked by the administration of the GR antagonist RU-486 [150]. In physiological *in vivo* approaches, ghrelin plasma and mRNA levels are elevated with acute and chronic stress in rodents [151-154]. Besides the HPA axis, the SNS can also stimulate ghrelin secretion [155].

Various studies *in vitro* as well as *in vivo* in animal models and humans demonstrated that ghrelin also has effects on glucose tolerance, insulin secretion and insulin sensitivity although the findings are contradictory. *In vitro*, ghrelin acts directly on β-cells by an inhibition of glucose-stimulated insulin secretion in a dose-dependent manner shown in cultured pancreata [156], isolated pancreatic islets [157, 158] and immortalized β-cell lines [157, 159]. Also in humans, the administration of exogenous ghrelin has been linked to an

inhibition of insulin secretion in some studies [160, 161] while others did not confirm that finding [162], therefore it is possible that pharmacologic doses of ghrelin affect insulin secretion while endogenous do not.

Besides direct insulin secretion, ghrelin was observed to affect insulin sensitivity *in vitro* in hepatoma cells by inhibiting AKT kinase activity and up-regulating key enzymes of gluconeogenesis [163] as well as *in vivo* in mice where ghrelin inhibited insulin-mediated suppression of gluconeogenesis [164]. In line with those findings, ghrelin and GHSR knockout (KO) mice show improved hepatic and peripheral insulin sensitivity measured with an insulin tolerance test (ITT) and hyperinsulinemic-euglycemic clamps [165, 166] whereas another study did not confirm that finding [167]. In humans, two independent studies in healthy men and post-gastrectomy patients showed that exogenous ghrelin decreased insulin-stimulated glucose disposal leading to insulin resistance, measured by hyperinsulinemic-euglycemic clamps [168, 169] or decreased insulin secretion without affecting insulin sensitivity [170]. Since ghrelin seems to be a modulator of insulin sensitivity and is secreted under stress conditions, it is possible that ghrelin plays a role in insulin sensitivity alterations with stress.

On the other hand, ghrelin may be able to regulate stress-induced metabolic alterations at the level of the immune system. Both ghrelin and its receptor the GHSR are expressed in immune cells and ghrelin and GHSR agonist administration display anti-inflammatory properties [171, 172].

These results suggest that ghrelin may play a unique role in meeting the metabolic and psychological challenges of stress potentially through its properties to increase food intake, adiposity and anxiety and its immune-regulatory effects [173, 174].

1.8.2 Leptin

Leptin is a 167 amino acid protein that is secreted mainly by the adipocytes [175]. Once secreted by the adipose tissue, leptin is transported through the blood brain barrier via a saturable transporter system and acts on the arcuate nucleus of the hypothalamus to reduce food intake and to increase energy expenditure, therefore promoting a negative metabolic state [176]. The arcuate neuron populations express leptin receptors [177], however, the leptin actions are opposite inhibiting NPY/AgRP neurons and activating POMC/CART neurons [178, 179]. Furthermore, leptin may also exert its anorectic properties through stimulation of the adrenergic system and down-regulation of the somatostatinergic system [180].

Leptin may be involved in the metabolic alterations caused by stress as shown *in vitro* where the administration of dexamethasone increased both the expression of the *ob* gene

(encoding leptin) and leptin secretion in adipocytes from rodents and obese human subjects [181, 182]. Interestingly, the *ob* gene expression and leptin secretion were further enhanced through the additional administration of insulin suggesting that leptin and insulin exert overlapping effects by converging upon the same signal transduction pathway, namely the insulin-receptor-substrate phosphatidylisositol 3-kinase pathway [183]. Also *in vivo*, prenatal exposure in rats to dexamethasone in drinking water led to higher leptin levels and decreased body weight in the offspring [184]. Leptin levels were also higher in rats exposed to chronic stress (restraint and tail suspension) for 5 weeks and simultaneously fed a HFD [185].

These findings suggest that leptin may play a linking role between stress and metabolic regulation. However, the majority of experiments investigating the effects of GCs on leptin expression and secretion are done *in vitro* and *in vivo* with the administration of synthetic GCs.

1.8.3 Adiponectin

Adiponectin is the most abundant adipocyte-secreted hormone in blood [186]. Adiponectin exerts its effects through the ubiquitously expressed receptors adiponectin receptor 1 (ADIPOR1) and adiponectin receptor 2 (ADIPOR2) which possess 7 transmembrane domains similar to G-protein-coupled receptors [187, 188]. As well, the glycosyl phosphatidylinositol-anchored cell surface glycoprotein T-cadherin interacts with adiponectin in mice in order to protect from cardiac stress [189].

Adiponectin enhances insulin sensitivity, mainly through elevated glucose utilization by skeletal muscle, decreased gluconeogenesis in liver, increased fatty acid oxidation in liver and muscle due to the activation of AMPK [187, 188, 190] and suppressed inflammation antagonizing TNF-α and its expression [187, 191]. Therefore low levels of adiponectin are linked to insulin resistance [192] and actually, adiponectin levels are reduced in obesity and type 2 diabetes in animal models [193, 194] as well as in humans [195-197]. Specifically, in humans, a strong negative correlation between body mass index (BMI) and levels of adiponectin has been reported [198].

GCs and adiponectin display contrary effects on energy metabolism with GCs primary involved in the activation of catabolic processes and insulin resistance in liver, muscle and WAT [30] whereas adiponectin exerts insulin-sensitizing actions [199].

Studies *in vitro*, in animal models and in humans investigating the effect of GCs on adiponectin expression and secretion have produced conflicting results. Thus, stimulation of 3T3-L1 pre-adipocytes as well as isolated adipocytes from human visceral or subcutaneous adipose tissue with dexamethasone decreases adiponectin expression and secretion [200].

Also *in vivo*, a down-regulation of adiponectin has been described in rats treated with hydrocortisone (5 or 15 mg/kg/day i.p. for 20 days) [201] or dexamethasone (2 mg/kg s.c. twice per day for 3 days) [202, 203]. In contrast, other studies in neonatal rats revealed an up-regulation of plasma adiponectin levels in response to dexamethasone (subcutaneous tapering dosing regimen from 0.5 mg/kg to 0.05 mg/kg) [204]. Furthermore, dexamethasone in T3T-L1 adipocytes, human bone marrow adipocytes and canine adipocytes did not affect adiponectin expression or secretion [202, 205-207].

The contradictory findings regarding the effects of GCs on adiponectin are also extended to studies in human subjects. Specifically some studies show a trend to lower adiponectin levels in women suffering from Cushing's syndrome compared to age-, gender- and BMI-matched healthy subjects [208] whereas others did not find that difference [209]. Hydrocortisone administration (3 h infusion of 1.5 µg/kg/min) in normal weight and obese women showed that although obese women in general had lower adiponectin levels than normal weight women, no significant changes in response to hydrocortisone across the study could be found in either group [210]. In contrast, there are studies reporting that the infusion of synthetic GCs in healthy volunteers with regular exercise training (60 mg prednisolone/day for one week) as well as in polymyalgia rheumatic patients (25 mg prednisolone/day for one month) led to an increase in blood/plasma adiponectin concentrations [211, 212].

In relation to the SNS there is also a lack of homogenous results. Adrenalectomy led to a significant increase of adiponectin expression in WAT and serum levels in ob/ob mice while in control wild type mice serum levels were lower and gene expression was not affected [169] meanwhile in rats, adrenalectomy did not alter serum adiponectin [163, 164].

Reports on the effect of stress on adiponectin levels in animal models are highly limited with studies showing a decrease in adiponectin levels in C57BL/6 mice with acute immobilization stress for 6 h [213] or with CVS and subsequent HFD consumption [214].

In conclusion, the effects of GCs on adiponectin expression and secretion have been widely studied although no consensus has been achieved. A potential reason for that may be that numerous mechanisms regulate adiponectin expression and furthermore, those studies were mainly pharmacological in different species with different doses and administration routes [188].

1.8.4 Resistin

Resistin, an adipokine discovered in 2001 and named after its action of promoting insulin resistance [215], is mainly synthesized by the adipose tissue in rodents [215, 216] and immune cells in humans [217, 218]. Although the source of expression and secretion is different in humans and rodents, resistin is linked to obesity and insulin resistance in both

species [219, 220]. The receptor through which resistin exerts its actions has been an open question for long, however, it has been shown that resistin binds to the endotoxin receptor toll-like receptor 4 [221] and furthermore to ΔDCN, an isoform of decorin on the surface of adipose progenitor cells [222]. Furthermore, just recently, adenylyl cyclase-associated protein1 (CAP-1) has been described as a receptor for resistin in humans [223].

As previously mentioned, resistin promotes insulin resistance centrally [224] and peripherally [225-227] at the level of the muscle, liver and adipose tissue. In muscle, resistin induces insulin resistance mainly through an inhibition of insulin-mediated glucose uptake via down-regulation of the expression of GLUT4 [228] and a decrease in AMPK activation [227, 229]. In the liver, it has been shown in rodents that the administration of exogenous resistin or transgenic overexpression increases hepatic glucose output [216, 227, 230] due to increased expression of gluconeogenic enzymes and an impairment of glycogen storage [231]. Likewise in muscle, those effects are related to a decrease in AMPK activation [215, 231-233]). In adipose tissue, elevated resistin leads to impaired insulin signaling including decreased phosphorylation of IRS-1 and -2 and decreased activation of AKT, with the down-regulation of AMPK playing again a role in resistin-induced insulin resistance in the adipose tissue [227]. In spite of the uniform findings in rodents of resistin promoting insulin resistance, the role of resistin in humans is still a conflicting matter. While some studies support a role of resistin for insulin resistance in humans [234, 235] or at least in human hepatocytes (HepG2 cells) [236], other studies failed to find a link between resistin and insulin resistance [237-239]. The differences between rodents and humans regarding resistin and regulation of insulin sensitivity could be explained by the different sources of resistin.

As GCs can induce insulin resistance like resistin, resistin may play a role in changes in insulin sensitivity in response to stress. Indeed T3T-L1 adipocytes cultured with dexamethasone showed 2.5 to 3.5 fold higher expression and protein levels of resistin [240]. Also mice receiving intramuscular dexamethasone showed up-regulation of resistin in WAT [240]. In relation to stress, higher plasma resistin levels have been observed with CVS and subsequent HFD consumption [214].

In spite of the scientific evidence linking GCs with resistin, the way resistin may act as a mediator of stress-induced metabolic alterations needs to be further elucidated. It could be possible that resistin acts through a common receptor to rodents and humans under stress conditions to overcome the difference in synthesis but more studies are needed to verify this option.

Observation/Outcome	Synthetic GCs / GR agonists	Stress	Clinical models
Gluconeogenesis	↑ **Dexamethasone *in vitro*** (1-5x10^{-6} M, cells) [66, 67]	↑ **Chronic stress (rats)** Restraint and swim for 2,4 or 24 weeks [241]	↑ **Patients of Cushing's syndrome** [242]
Post-receptor insulin signaling (IRS, AKT,PI3K) in muscle and adipose tissue (*in vitro* and *ex vivo*)	↓ **Dexamethasone** 1 mg/kg/d for 5 d, rats [79]; 10 nM-1 µM for 24 h, T3T-L1 adipocytes [102]; 0.3 µM/L for 24 h, isolated rat adipocytes [103]; **Cortisone** 100 mg/kg for 5 d, rat skeletal muscle [78]	**No data**	**No data**
Glucose tolerance/Insulin sensitivity	**Dexamethasone** 200 µM/L, 3 µL/min for 90 min into ARC and PVN of rat hypothalamus ↓ **Hyperinsulinemic-euglycemic clamp** Suppression of EGP (%) ↑ Rate of disappearance (µmol/kg/min) [243] **Dexamethasone** Humans, 4 mg/d **Hyperinsulinemic-euglycemic clamp** ↓ Glucose disposal rate (mg/kg lean mass/min) [244]	**Chronic stress** (CVS + HFD, mice) ↓ Glucose tolerance (**GTT 3 months after CVS intervention)** [214] Chronic social defeat stress (mice) ↑ HOMA-IR [245]	**Patients of Cushing's syndrome** ↓ HOMA-β, ↓ Oral disposition index [246]
Insulin-mediated GLUT translocation in muscle and adipose tissue (*in vitro* and *ex vivo*)	↓ **Dexamethasone** 0.5 mg/d for 5 d, rat skeletal muscle *ex vivo* [91] 0.9 mg/kg for 2 d, rat skeletal muscle *ex vivo* [92] 10 nM-1 µM for 24 h, 3T3-L1 adipocytes [102] **Cortisol** 30 µg/ml medium, isolated rat adipocytes [98]	**No data**	**No data**
Glycogen synthase activity (muscle)	↑↓ **Dexamethasone** 0.4 mg/kg/d for 14 d, rats [95] Methylprednisolone 7 mg every 2nd day, humans [94]	↓ **Chronic stress** Chronic restraint stress (21 d), mice [247]	↓ **Patients of Cushing's syndrome [248]**

Table 1: Effects of synthetic GCs and stress on glucose metabolism. Results from previous studies are presented. d = day, EGP = endogenous glucose production. ↑ up-regulation, ↓ down-regulation

Observation/Outcome	Synthetic GCs / GR agonists	Stress	Clinical models
Circulating FFA	↑ **Dexamethasone** rats [111] 2 mg/kg BW, adrenalectomized rats[249] **hydrocortisone** 80-200 µg/kg/h, overnight [250]	↑ **Acute stress** Electric shock stress, rats [251] Cold exposure (4°C, 120 min), dogs [252]	↑↓ **Patients of Cushing's syndrome [253], [254]**
Stimulation of lipases (f.i. ATGL and HSL) (*in vitro* and *ex vivo*)	↑ **corticosterone** 1-100 µM, 3T3-L1 adipocytes [104] **dexamethasone** 0.1 mg/kg/ d for 7 weeks, rats [112] 10-1000 nM, 4 and 24 h, isolated rat adipocytes [113]	**No data**	**No data**
Expression of LPL/hypertrophy of adipocytes	↑ **Dexamethasone** 30 nM, 7 d, human adipose tissue fragments [129] 0.1-100 nM, 7 d, human adipose tissue [127]	↑↓ **Chronic stress** 2 h restraint stress per d, for 2 periods of 5 and 4 consecutive days, rats [255] Isolation/defeat stress for 1.5 m, mice [256]	↑↓ **Patients of Cushing syndrome [257], [248]**
Fatty liver/hepatic steatosis	↑ **Dexamethasone** 0.5 µM for 5 h, rat primary hepatocytes [126]	↑ **Chronic stress (+HFD)** Electric stimulation stress, twice per d for 8 weeks, rats [258] Chronic social defeat stress, 10 d, mice [259]	↑ **Patients of Cushing's syndrome [260]**
Fatty acid oxidation/ Mitochondrial function	**Dexamethasone** 6mg/kg/d, stimulation of mitochondrial biogenesis in skeletal muscle of rats [261] **Dexamethasone** Stimulatied transcription of genes endoded on mitochondrial DNA, hepatoma cell lines [262, 263]	**Chronic stress** Crowding stress for 15 days, rats, disturbed mitochondrial activity in intestine [264] Restraint for 6 h/day for 3 weeks, rats, decrease in myocardial mitochondrial lipid metabolism enzymes (CPT2, ACOT1) [265]	**Patients of Cushing's syndrome** Pleomorphism of mitochondria in adrenals [266]

Table 2: Effects of synthetic GCs and stress on lipid metabolism. Results from previous studies are presented. d=day
↑up-regulation, ↓down-regulation

Observation/Outcome	Synthetic GCs / GR agonists	Stress	Clinical models
TNF-α	↓ **Dexamethasone** 1 µM for 30 min, THP-1 cells (human promonocytic cells) [133]	↑ **Chronic stress** Humans suffering from long-term stressful experience [139]	↔ **Patients of Cushing's syndrome [267]**
IL-4 and -5	↓ **Dexamethasone** 10^{-6}-10^{-9} M, RBL-2H3 cells (analogue of mucosal mast cells) [134]	**No data**	↑ **Patients of rheumatoid arthritis with acute stress [268]**
IL-6	↓ **Hydrocortisone**	↑ **Chronic stress** 1 week of social disruption, mice [138] Humans from long-term stressful events [139]	↑↓ **Patients of Cushing's syndrome [267]** ↑ **Patients with cortical adenomas [267]**
IL-1β	↓ **Dexamethasone**	↑ **Acute and chronic stress** Acute (20 min), chronic once per , 20 min, 21 consecutive days Forced swimming and high-light open field stress, rats [269]	↑ **Patients of Cushing's syndrome [270]**
Cellular antioxidant machinery	↓ **Dexamethasone** 100 nM, primary cultured rat hepatcytes [271]	↓ **Chronic stress** 21 days of social isolation stress with subsequent acute immobilization challenge in rats, decrease in liver antioxidative enzymes [272] Repeated short-term stress (restraint), mice, decrease in antioxidative enzymes in epidermal tissues [273]	↓ **Patients of Cushing's syndrome [274]**

Table 3: Effects of synthetic GCs and stress on inflammation. Results from previous studies are presented. d= day.
↑up-regulation, ↓down-regulation, ↔no effect

Observation/Outcome	Synthetic GCs / GR agonists	Stress	Clinical models
Ghrelin	↑ **Dexamethasone** 10 nM-1µM , PVN hypothalamic 4B cells [150]	↑ **Acute and chronic stress (rodents)** 60 min water avoidance stress, rats [275] 5 d water avoidance stress, rats [152] 10 d of chronic social defeat stress, mice [153] 5 d of chronic restraint stress, rats [154]	↑↓ **Patients of Cushing's syndrome** [209, 276, 277]
Leptin	↑ **Dexamethasone** 0.078-5 nM, isolated rat adipocytes [181] 25 nM, isolated human adipose tissue [182]	↓ **Chronic stress** 5 weeks of chronic social defeat stress, rats [278]	↑↔ **Patients of Cushing's syndrome** [279-282] [208]
Adiponectin	↑↓↔ **Dexamethasone** 50 nM, human visceral adipose tissue [200] 0.05-0.5 mg/kg, neonatal rats [204] **prednisolone** 25 mg/d for one month, polymyalgia rheumatic patients [212] **hydrocortisone** 5-15 mg/kg/d for 20 d, obese rats [201]	↓ **Chronic stress** CVS + HFD, mice Measured 1 month after CVS intervention [214] **Acute stress** Immobilization stress, 6 h, mice [213]	↓↔ **Patients of Cushing's syndrome** [283] (in non-obese patients) [283] (obese CD vs. non-obese), [208, 209, 284]
Resistin	↑ **Dexamethasone** 0.01-1 µM/L, T3T-L1 adipocytes [240] 10 mg/kg for 5 d, mice [240]	↑ **Chronic stress** CVS + HFD, mice Measured 1 month after CVS intervention [214]	↑ **Female patients of Cushing's syndrome [267]** ↑ **Patients with adrenal incidentaloma [267]**
PAI-1	↑ **Dexamethasone** 10-6 mol/l for 24 h, primary human bone marrow adipocytes [205] 100 nmol-5 µmol, human proximal renal tubular epithelial cells (HPTECs) [285]	↑ **Chronic stress** Unpredictable chronic mild stress, mice, late-onset effects [286] Humans (feelings of fatigue, demoralization) [287]	↑ **Patients of Cushings's syndrome [288]**

Table 4: Effects of synthetic GCs and stress on the expression and circulating levels of hormones. Results from previous studies are presented. d= day.
↑up-regulation, ↓down-regulation, ↔ no effect

1.9 Research aims

Although GCs and catecholamines have an impact on glucose and lipid metabolism and actually stress has been suggested to contribute to insulin resistance, the mechanisms behind the physiology of stress are still poorly understood. Most of the studies were performed with synthetic GCs like dexamethasone, often *in vitro* or *in vivo* just in an organ, with the observations limited to acute effects and some contradictory findings. Epidemiological studies in humans link chronic stress and PTSD with late diabetes onset; however, those studies do not allow the analysis of the overall mechanisms behind the observed effects. Therefore, further research is needed in order to elucidate whether chronic stress may affect overall insulin sensitivity beyond one tissue level, what factors and mediators drive and orchestrate those metabolic alterations and as the new and main aspect in the present doctoral thesis, whether those effects last in time and how they can be regulated by dietary fat.

It has been shown that early exposure to chronic variable stress (CVS) in combination with subsequent HFD consumption leads to late impaired glucose tolerance associated with an altered adiponectin:resistin ratio in male C57BL/6 mice [214]. Furthermore, white adipose tissue showed an increased expression of genes associated to adipogenesis, adipocyte differentiation, hypoxia and inflammation [214]. These findings suggest the adipose tissue to play an important role in stress-induced metabolic disorders; however, the contribution by other key tissues in glucose homeostasis like the liver and skeletal muscle cannot be excluded and may be of high relevance in the analysis of whole-body insulin sensitivity.

Therefore, the aims of the present research project were to investigate in a mouse model for PTSD whether:

1. CVS impairs whole body insulin sensitivity *in vivo*.
2. Those effects last in time after the CVS intervention is over.
3. Those effects are regulated by dietary fat.

Insulin sensitivity was analyzed with the gold-standard technique of hyperinsulinemic-euglycemic clamps

1. After CVS in 3 months and a half old male mice.
2. Three months after the CVS intervention in combination with dietary fat (6 months and a half of age).

These analyses were complemented by measurements of lipid metabolites, hormones and cytokines in plasma and gene expression and protein levels in hypothalamus, muscle, liver and adipose tissue.

2 Material and methods

2.1 Material

2.1.1 Animals

C57BL/6J male mice (12 weeks old, Janvier, Saint Berthevin Cedex, France) were group housed under a 12:12 hour light-dark cycle (6 a.m. lights on) with *ad libitum* access to low-fat CHOW (58 % carbohydrates, 9 % fat, 33 % protein, R/M-H Extrudat, ssniff Spezialdiäten GmbH, Soest, Germany) or a high-fat western type diet (HFD) for up to three months (42 % carbohydrates, 43% fat, 15 % protein, TD88137, ssniff Spezialdiäten GmbH, Soest, Germany) and tap water. All animal protocols were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, NRW, Germany.

2.1.2 Consumable material

Borosilicate glass tubes (6 ml), (12x75 mm)	VWR, Langenfeld, Germany
Centrifugation tubes (1 ml), thick wall, polycarbonate (11x34 mm)	Beckman Coulter, Brea, CA, USA
di-K^+-EDTA-coated microcuvettes	Sarstedt, Nümbrecht, Germany
Filter papers	Oehmen, Essen, Germany
Insulin needles	Braun, Melsungen, Germany
Microwell plates (96 well)	Roth, Karlsruhe, Germany
PCR microwell plates (96 well)	Applied Biosystems, Darmstadt, Germany
Pipette tips	Eppendorf, Wesseling-Berzdorf, Germany
Reaction tubes (1.5 ml)	Eppendorf, Wesseling-Berzdorf, Germany
Reaction tubes (2 ml)	Eppendorf, Wesseling-Berzdorf, Germany
Reaction tubes (200 µl)	Eppendorf, Wesseling-Berzdorf, Germany
Stainless steel beads	Qiagen, Hilden, Germany
Whatman Nitrocellulose membrane	Oehmen, Essen, Germany
Syringes (0.1-1 ml)	Braun, Melsungen, Germany

2.1.3 Biochemicals

Acetic acid	KMF, Lohmar, Germany
Acrylamide 30 %	AppliChem, Darmstadt, Germany
Ammonium persulfate (APS)	MP Biomedicals, Illkirch, France
Aprotinin	Sigma Aldrich, Steinheim, Germany
Bovine serum albumin (BSA) powder	AppliChem, Darmstadt, Germany
Chloroform	AppliChem, Darmstadt, Germany
Complete Protease Inhibitor Cocktail Tablets	Roche, Mannheim, Germany
Deuterated D[6,6-2H]glucose	Cambridge Isotope Laboratories, Andover, MA, USA
Diprotin A	Bachem, Bubendorf, Switzerland
Ethanol	Roth, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Roth, Karlsruhe, Germany
Ethylene glycol tetraacetic acid (EGTA)	Serva, Heidelberg, Germany
Glycerol	MP Biomedicals, Illkirch, France
Glycine	AppliChem, Darmstadt, Germany
Heparin (10 000 U/ml)	Medical Equipment Affiliates, Tahlequah, OK, USA
Insulin Huminsulin® Lilly	Lilly, Giessen, Germany
Isopropanol	AppliChem, Darmstadt, Germany
Methanol	Roth, Karlsruhe, Germany
Molecular weight marker Dual Color	BioRad Laboratories, Munich, Germany
Non-fat dry milk powder	Roth, Karlsruhe, Germany
Phosphatase Inhibitor Cocktail Tablets	Roche, Mannheim, Germany
Ponceau S	Sigma Aldrich, Steinheim, Germany
Potassium hydroxide (KOH)	Merck, Darmstadt, Germany
Protease Inhibitor Cocktail Tablets	Roche, Mannheim, Germany
RNAase-free water	Qiagen, Hilden, Germany
Saline solution	Fresenius Kabi, Bad Homburg, Germany
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	AppliChem, Darmstadt, Germany
Sucrose	Sigma Aldrich, Steinheim, Germany
Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe, Germany

Tris(hydroxymethyl)aminomethan (TRIS)	Roth, Karlsruhe, Germany
Triton-X 100	Sigma Aldrich, Steinheim, Germany
TRIzol Reagent	Ambion, Life technologies, Darmstadt, Germany
Tween-20	MP Biomedicals,

2.1.4 Biomolecular kits

Bio-Plex pro Mouse Cytokine	BioRad Laboratories, Munich, Germany
Bio-Plex pro Mouse Diabetes 8-plex assay	BioRad Laboratories, Munich, Germany
Bio-Plex pro Mouse Diabetes Adiponectin Assay	BioRad Laboratories Munich, Germany
Cholesterol liquid 1000	Erba Lachema, Brno, Czech Republic
Corticosterone Double Antibody RIA Kit	MP Biomedicals, Orangeburg, NY, USA
Enhanced Chemiluminescence Substrate, Western Lightning Pro	Perkin Elmer, Waltham, MA, USA
Enhances Chemiluminescence Substrate, Western Lightning Ultra	Perkin Elmer, Waltham, MA, USA
GoScript Reverse Transcription System	Promega, Madison, WI, USA
NEFA-HR (2) kit	Wako, Neuss, Germany
Pierce BCA Protein Assay Kit	Thermo Scientific, Schwerte, Germany
Quantifast SYBR Green PCR kit	Qiagen, Hilden, Germany
Triglyceride liquid 1000	Erba Lachema, Brno, Czech Republic

2.1.5 Buffers

Anticoagulation cocktail (50 ml recipe)	25 ml 0.5 M EDTA 92 mg aprotinin 4 ml heparin (10 000 U/ml) 21.6 mg diprotin A 21 ml saline

Laemmli Electrophoresis buffer	25 mM Tris 0.192 M glycerol 0.1 % SDS (w/v)
Laemmli sample buffer	20 % glycerol (w/v) 8 % SDS (w/v) 10 mM EDTA 250 mM Tris
Lysis buffer for total protein	20 mM Tris-Hcl 150 mM NaCl 1 mM EDTA 1 mM EGTA 1 % Triton-X 100
Lysis buffer A for tissues (protein, microsomal fraction)	20 mM Tris-Hcl 1 mM EDTA 0.25 mM EGTA 250 mM sucrose 1 tablet Protease inhibitor 1 tablet Phosphatase inhibitor
Lysis buffer B for tissues (protein, plasma membrane fraction)	250 mM Tris-Hcl 1 mM EDTA 0.25 mM EGTA 2 mM Triton-X 100 1 tablet Protease inhibitor 1 tablet Phosphatase inhibitor
Ponceau staining solution	0.25 % Ponceau S 40 % methanol (v/v) 15 % acetic acid (v/v)
Separation gel buffer (pH 8.8)	1.5 M Tris 0.4 % SDS (w/v)

Stacking gel buffer (pH 6.8)	0.5 M Tris 0.4 % SDS (w/v)
Transfer buffer	25 mM Tris 0.192 M glycerol 20 % methanol (v/v)
Wash buffer for membranes	10 mM Tris 0.15 M NaCl 0.05 % Tween-20 (v/v)

2.1.6 Antibodies for Western Blots

phospho-Acetyl-CoA Carboxylase (p-ACC) (Ser79)	Cell Signaling Technology, Danvers, MA, USA
Acetyl-CoA Carboxylase (ACC)	Cell Signaling Technology, Danvers, MA, USA
phospho-AKT (Ser473)	Cell Signaling Technology, Danvers, MA, USA
AKT	Cell Signaling Technology, Danvers, MA, USA
phospho-AMPKα (Thr172)	Cell Signaling Technology, Danvers, MA, USA
AMPKα	Cell Signaling Technology, Danvers, MA, USA
GAPDH	Cell Signaling Technology, Danvers, MA, USA
GLUT1	custom-made, German Institute for Nutrition, Potsdam, Germany
GLUT 4	custom-made
1α Na^+Ka^+ ATPase	Cell Signaling Technology, Danvers, MA, USA
phospho-p70S6K1 (Thr389)	Cell Signaling Technology, Danvers, MA, USA
p70S6K1	Cell Signaling Technology, Danvers, MA, USA

2.1.7 Devices/ instruments

Bioplex 200 suspension array system	Biorad, Hercules, CA, USA
Centrifuge 5424 R	Eppendorf, Wesseling-Berzdorf, Germany
Chemidoc™ XRS^+ System	Biorad Laboratories, Munich, Germany
Cryofuge Heraeus 8500i	Thermo Scientific, Peqlab, Wilmington, USA
Cooling Unit	
ARCTIC A28 Refrigerated Circulator	Thermo Scientific, Schwerte, Germany
Combs for SDS gels (15 well)	Biorad Laboratories, Munich, Germany

Electrophoresis Chamber	
Mini-PROTEAN ® Tetra Cell	BioRad Laboratories, Munich, Germany
ELISA-Reader Synergy2	BioTek Instruments, Winooski, VT, USA
FeedTime system	TSE Systems GmbH, Bad Homburg, Germany
Fridge Sanyo Labcool MPR-1410R	Sanyo, Moriguchi, Japan
Gamma Counter LB2111	Berthold, Bad Wildbad, Germany
iMark™ Microplate Reader	BioRad Laboratories, Munich, Germany
Nanodrop 2000	Thermo Scientific, Peqlab, Wilmington, MA, USA
NMR Whole Body Composition Analyzer	Echo MRI™, Houston, Texas, USA
Overhead shaker	Heidolph, Schwabach, Germany
PCR Mastercycler	Eppendorf, Wesseling-Berzdorf, Germany
Pipettes	Eppendorf, Wesseling-Berzdorf, Germany
Short plates for SDS gels	BioRad Laboratories, Munich, Germany
Spacer plates (1.5 mm) for SDS gels	BioRad Laboratories, Munich, Germany
Standard Analog Shaker	VWR, Langenfeld, Germany
StepOnePlus™ Real-time PCR system	Applied Biosystem, Life Technologies, Darmstadt, Germany
Tankblot Eco Mini	Biometra, Göttingen, Germany
Tecan Micro plate reader	Tecan, Männedorf, Switzerland
Thermomix	Eppendorf, Wesseling-Berzdorf, Germany
TissueLyser II	Qiagen, Hilden, Germany
Ultracentrifuge Optima™ MAX-XP	Beckman Coulter, Brea, CA, USA

2.1.8 Software

Bio-Plex Manager Software	BioRad Laboratories, Hercules, CA, USA
Gen 5	BioTek Instruments, Winooski, VT, USA
Graph Pad Prism 5	San Diego, CA, USA
ImageLab	BioRad Laboratories, Hercules, CA, USA
Nanodrop 2000/2000c software	Thermo Scientific, Peqlab, Wilmington, MA, USA
Quantity One	BioRad Laboratories, Hercules, CA, USA
StepOne	Applied Biosystems, Life Technologies
Tecan i-Control™	Tecan, Männedorf, Switzerland

2.2 Methods

2.2.1 Body composition

Body composition measurements were taken using magnetic nuclear resonance (Whole Body Composition Analyzer; Echo MRI™, Houston, Texas, USA) after 15 day of CVS intervention and after subsequent three months of CHOW or HFD consumption without further stress intervention. This method is used to measure fat and lean mass from the whole body of a living mouse.

The advantages of this measuring system are that the measurements require a short time (~ 1 min per measurement), sedation or anesthesia is not necessary and it is easy to use and free of radiation and body invasion [289]. Therefore it cannot be considered a severe stressor with regard to stress research and allows to measure body composition of control animals without keeping the HPA axis active for a prolonged time.

The principle of the measurement is that a scan produces a record of nuclear magnetic resonance responses (NMR echoes) to a sequence of radio pulses. The sequence is composed of several periodic Carr-Purcell-Meiboom-Gill parts (CPMG segments) [289] separated by pauses of different durations. The lengths of the periodic parts and the durations of the pauses are considered to catch all the crucial characteristic (relaxation) time scales of the NMR responses (transverse, “T2” and longitudinal, “T1”, relaxation) that are distinctive for fat, lean mass and free water. The calculation of the amounts of fat, lean mass and free water is achieved through linear regressions taking into account in the signal from the body the linear combination of those three components and the different relaxation rates of them [289].

2.2.2 Chronic variable stress (CVS)

Young-adult male C57BL/6J mice matched by age (12 weeks) and body weight (BW) underwent a 15 day-regimen of CVS according to a protocol that resembles PTSD in rodents [214, 290, 291] consisting of five different stressors:

1. Swimming in warm water (physical + psychogenic stressor)
   A big plastic recipient was filled with 30 °C warm water to a level that the mice did not touch the bottom with the tail. Mice were placed inside (one cage at a time; 4-6 mice) and were forced to swim for 20 min.

2. Shaking (psychogenic stressor)
   Mice were placed (per group; 4-6 mice) in cages without bedding, subsequently the cages were placed on a rocker. Shaking was performed for 1 h at 100 rpm.

3. Cold exposure (4°C, physical stressor)
   Mice were individually placed in a mouse cage without bedding in a cold box for 1 h at 4 °C.

4. Restraint (psychogenic stressor)
   Each mouse was placed inside a 50 ml tube equipped with a hole to enable breathing to restricted movement for 30 min.

5. Overnight isolation in an oversize cage (psychogenic stressor)
   In the afternoon (~ 5 p.m.) each mouse was individually placed in an oversize cage until the following morning (~9 a.m.). During that time mice had *ad libitum* access to food and water.

The animals were exposed to two stressors per day in a randomly alternating manner whereas the first stressor was applied ~ 9 a.m. and the second stressor ~ 2 p.m. Immobilization stress (restraint) was exclusively applied in the afternoon at ~ 4 p.m. considering the close dark phase or active phase. The alternating stressor paradigm is designed to avoid adaptation of the animals to any individual stressor. Individual timing of the stressors was selected on the basis of the stress duration required to provoke an adrenocortical response, without endangering the animals' well-being. The mice subjected to the stressors were kept separately from the non-stressed control mice (CTRL) during the 15 day-procedure. In order to study the short-term effects of CVS, one set of mice (CTRL and CVS) immediately underwent analyses of insulin sensitivity, whereas another set of animals was divided into four subgroups consuming either the regular low-fat CHOW diet from the animal facilities (58 % carbohydrates, 9 % fat, 33 % protein, R/M-H Extrudat, ssniff Spezialdiäten GmbH, Soest, Germany) (CTRL CHOW and CVS CHOW) or a high fat diet (HFD) (42 % carbohydrates, 43 % fat, 15 % protein, Western Type diet TD88137, ssniff Spezialdiäten GmbH, Soest, Germany) (CTRL HFD and CVS HFD) for 3 months after the CVS paradigm was over followed by the same experiments as performed in the short-term study. No more stress intervention was applied during those three months (Figure 4).

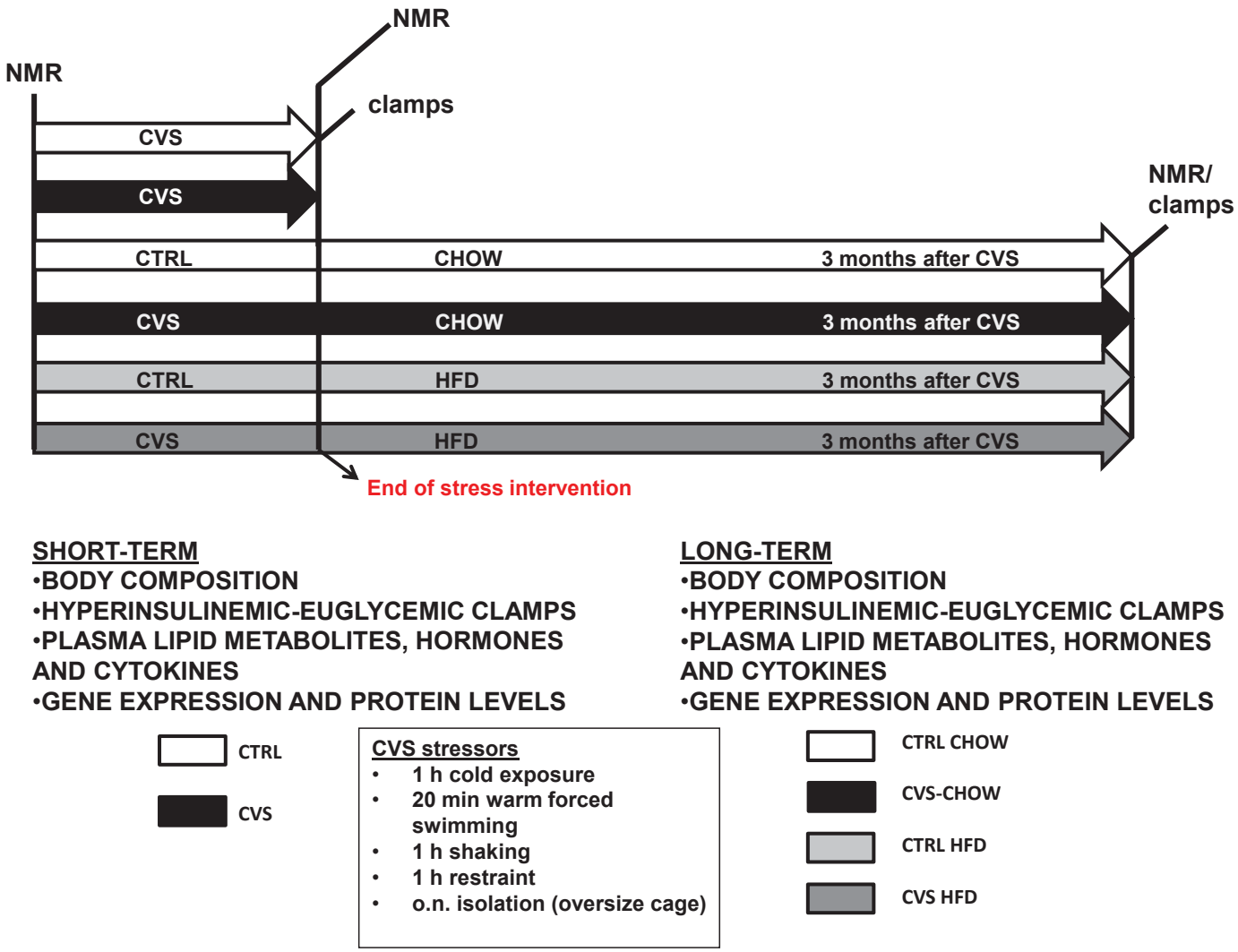


Figure 4: Experimental protocol to analyze short-and long-term effects of chronic variable stress (CVS) in relation to dietary fat. Male C57BL/6 mice underwent a 15 day chronic variable stress paradigm. Insulin sensitivity as well as plasma lipid metabolites, hormone and cytokine levels and gene expression and protein levels were measured immediately after CVS or three months later after CHOW or HFD consumption. o.n. = overnight.

2.2.3 Measurement of corticosterone levels in plasma collected after CVS

Mice were fasted for 4 h after the last stressor (overnight isolation) with an automatic feeder system (FeedTime, TSE Systems, Bad Homburg, Germany) and tail blood was collected (8 a.m. to noon) to measure plasma corticosterone. A small cut was carried out in the skin of the tail and blood was collected from the tail vein in di-K^+-EDTA-coated microcuvettes (Sarstedt, Nümbrecht, Germany). Bleeding was restricted to 2 min to avoid a rapid rise in blood corticosterone levels especially in non-stressed CTRL mice. After bleeding, microcuvettes were immediately chilled on ice. Plasma was obtained by centrifugation at 3000 *x g*, 4°C for 15-20 min and was stored at -80°C.

Corticosterone concentrations in the plasma of mice were measured by radioimmunoassay (RIA) using the Corticosterone Double Antibody ^{125}I RIA kit for rats and mice (MP Biomedicals, Orangeburg, NY, USA). With this method, the amount of an antigen (corticosterone) in the sample is quantitated via competing with a known amount of labeled antigen (Corticosterone ^{125}I, tracer) for binding to antibody binding sites. A known amount of corticosterone is labeled with gamma-radioactive isotopes of iodine (^{125} I). This labeled antigen is mixed with an antibody directed against corticosterone and subsequently binds specifically to the antibody. When the mouse plasma sample containing an unknown amount of corticosterone is added, it competes with the radiolabeled corticosterone for antibody binding sites. Conversely, increasing amounts of antigen in the sample bind an increasing amount of antibody, displacing the radiolabeled corticosterone variant. Therefore, the amount of detected counts (from radioactively-labeled corticosterone) of the sample is inversely proportional to the amount of corticosterone.

Plasma samples were diluted 1:200 with steroid diluent (phosphosaline gelatin buffer, pH7, containing rabbit gamma globulins) by taking 5 or 10 µl of plasma to 95 or 1990 µl, respectively, of steroid buffer. One hundred µl of diluted samples, standards (50, 100, 250, 500, 1000 ng/ml, diluted with steroid diluent) as well as one low- and one high-corticosterone control provided in the kit were pipetted into appropriate borosilicate glass tubes in duplicate or single. For the “zero tube” (100 % binding tube), 100 µl steroid diluent was pipetted and for the non-specific-binding (NSB, blank tube, no binding) 300 µl was pipetted into respective separate tubes. Two hundred µl of tracer solution (containing ^{125}I corticosterone) were added to all tubes. Subsequently, 200 µl of anti-serum (containing the antibody, tittered to bind 50-60 % of the ^{125}I corticosterone derivative in the absence of nonradioactive corticosterone) was added to all tubes with the exception of the NSB tubes. After mixing, tubes were incubated for 2 h at room temperature (RT). Antibody-bound antigen was precipitated with 500 µl precipitant solution (containing polyethylene glycol (PEG) and goat anti-rabbit gamma globulins in TRIS buffer) per tube. After thorough mixing, tubes were centrifuged at 3000 *x g*, RT, for 15 min. The supernatant was decanted and counts in the precipitate were measured in a gamma counter (Berthold, Bad Wildbad, Germany).

The determination of concentration was done via comparison of the measured values with the data of the standard curve (prepared with above-mentioned concentration) as follows:

$$\%B/Bo = \frac{\text{average counts (sample)} - \text{average counts NSB}}{\text{average counts zero tube} - \text{average counts NSB}} \times 100$$

2.2.4 Hyperinsulinemic-euglycemic clamp

The results were obtained in collaboration with Prof. Roden and Dr. Jelenik, Institute for Clinical Diabetology, German Diabetes Center, Düsseldorf, Germany.

The hyperinsulinemic-euglycemic clamp is the gold standard technique to study insulin sensitivity *in vivo*. It refers to the principle of stable maintenance of blood glucose (=euglycemia) when the glucose production of the organism (mainly by the liver, endogenous glucose production, **EGP**) equals glucose disposal (uptake mainly by the skeletal muscle and the adipose tissue).

In that technique, insulin is perfused through the jugular vein at a high physiological dose. In order to avoid hypoglycemia in response to insulin, varying amounts of glucose are co-perfused with insulin to keep blood glucose around its original level (euglycemia). The rate of glucose infused that is needed to maintain the euglycemic state is called the glucose infusion rate (**GIR**). The higher the GIR that is needed to maintain euglycemia, the higher is the whole-body insulin sensitivity of the organism and *vice versa*. At the end of the clamp, when both insulin and blood glucose levels are stable, the EGP plus the GIR are equal to the glucose disposal in peripheral tissues (**stimulation Rd, Rd %**). However, under hyperinsulinemia, EGP should be suppressed close to 100 % in insulin-sensitive subjects and therefore, impaired suppression of EGP indicates an insulin resistant state at the level of the liver.

A silicon catheter was placed into the right jugular vein under isoflurane anesthesia. CTRL and CVS mice were allowed to recover for 5±1 days. At the day of experiment, mice were fasted for 6 h (3 a.m. - 9 a.m.). To assess basal whole-body glucose turnover, deuterated D-[6,6-2H2] glucose (Cambridge Isotope Laboratories, Andover, MA, USA) was infused at a rate of 0.7 mg/kg/min for 120 min. Hyperinsulinemic-euglycemic clamp was initiated with insulin (Huminsulin®, Lilly, Giessen, Germany) priming dose (40 mU/kg) and continued with insulin infusion at a constant rate of 4 mU/kg/min. Euglycemia was maintained by periodically adjusting a variable infusion of 20% glucose. D-[6,6-2H2]glucose was infused in both insulin and 20% glucose solution to obtain stable tracer concentrations during varying glucose infusion rates. Blood samples for D-[6,6-2H2] glucose enrichment analysis were taken every 10 min during the last 30 min of the basal as well as hyperinsulinemic-euglycemic clamp period when blood glucose was at steady-state of 120±10 mg/dl. Blood samples were mixed with 40 µl of KF-EDTA-NaCl.

In parallel to the clamps, I processed the samples for D-[6,6-2H2] glucose enrichment analysis. Fifty µl of $ZnSO_4$ were added to each sample and mixed. Subsequently, 100 µl of $Ba(OH)_2$ were added and after another mixing step, samples were shaken for 20 min at 1600 rpm and centrifuged for 20 min, -2 °C at 21 000 *x g*. One hundred twenty-five µl from the

supernatant were transferred into glass vials with glass stoppers, shut and frozen at -20 °C before analysis in the Technical Laboratory of the German Diabetes Center.

2.2.5 Plasma biochemistry analysis

Before and after clamp just before sacrifice, a small amount of plasma was collected from the tail vein in di-K^+-EDTA-coated-microcuvettes (Sarstedt, Nümbrecht, Germany) in order to measure plasma biochemistry parameters (cholesterol, triglycerides (TGs) and non-esterified free fatty acids (NEFA) and immediately chilled on ice. Mice were killed by decapitation following clamps at the end of the study and blood was collected in 1.5 ml reaction tubes (Eppendorf, Hamburg, Germany) provided with anticoagulation cocktail and immediately chilled on ice. All blood samples were centrifuged at 3000 *x g*, 4°C and plasma was stored at -80°C.

The concentration of cholesterol was measured in undiluted plasma with the cholesterol liquid 1000 kit (Erba Lachema, Brno, Czech Republic). The principle of this colorimetric test is based in the multi-step-hydrolysis of cholesterol ester into cholesterol and free fatty acids and a chromophore that can be detected at a specific wavelength. The absorbance of this chromophore is proportional to the concentration of cholesterol present in the sample.

Three different reactions are included in that procedure:

1. Cholesterol esterase hydrolyses cholesterol esters enzymatically to cholesterol and free fatty acids.
2. Cholesterol oxidase then oxidizes free cholesterol to cholest-4-en-3-one and hydrogen peroxide.
3. The hydrogen peroxide along with 4-aminoantipyrine forms a chromophore (quinoneimine dye) which may be quantitated at 505 nm.

Three µl of undiluted plasma sample or standard were pipetted into appropriate wells on a 96well-plate in duplicates. Three hundred µl of working reagent (containing 50 mmol/l Good's buffer, 5 mmol/l phenol, 0.3 mmol/l 4-aminoantipyrine, ≥200 U/l cholesterol esterase, ≥50 U/l cholesterol oxidase, ≥3 kU/l peroxidase) were added. Subsequently, the plate was incubated at 37 °C for 10 min and absorption was detected at 500 nm.

Results were calculated with the formula:

$$\text{Cholesterol (mmol/L)} = \frac{\Delta A_{sample}}{\Delta A_{sample}} \text{ x } C_{standard}$$

being A_{sample} the absorption of the unknown sample, $A_{standard}$ the absorption of the standard und $C_{standard}$ the concentration of the standard.

The concentration of triglycerides (TGs) was measured in undiluted plasma with the triglyceride liquid 1000 kit (Erba Lachema, Brno, Czech Republic). The principle of this colorimetric test is based in the multi-step-hydrolysis of TGs into free fatty acids, glycerol and a dye that can be detected at a specific wavelength.

Four different reactions are included in that procedure:

1. TGs are enzymatically hydrolyzed by lipase to free fatty acids and glycerol.
2. The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-3-phosphate and adenosine diphosphate (ADP).
3. Glycerol-3-phosphate is oxidized to dihydroxy-acetone phosphate (ADP) by glycerol phosphate oxidase producing hydrogen peroxide (H_2O_2).
4. In a Trinder type colour reaction catalyzed by peroxidase, the H_2O_2 reacts with 4-aminoantipyrine (4AAP) and 4-chlorophenol to produce a red colored dye. The absorbance of this dye is proportional to the concentration present in the sample.

Three µl of undiluted plasma sample or standard were pipetted into appropriate wells on a 96 well-plate in duplicates. Three hundred ul of working reagent were added. Subsequently, the plate was incubated at 37 °C for 10 min and absorption was detected at 500 nm (reference wavelength 546 nm).

Results were calculated with the formula:

$$\text{TGs (mg/dL)} = \frac{\Delta\, A_{sample}}{\Delta\, A_{sample}} \text{ x } C_{standard}$$

being A_{sample} the absorption of the unknown sample, $A_{standard}$ the absorption of the standard and $C_{standard}$ the concentration of the standard.

The concentration of non-esterified fatty acids (NEFA) was measured using the NEFA-HR(2) kit from Wako (Wako Chemicals GmbH, Neuss, Germany) as a colorimetric test based on the ACS-ACOD method including following reactions: Along with Coenzyme A (CoA) and adenosine 5-triphosphate disodium salt (ATP) the enzyme Acyl-CoA synthetase (ACS) converts NEFAs in the sample to Acyl-CoA, AMP and pyrophosphoric acid (PPi). Acyl-CoA is further processed to 2,3-*trans*-Enoyl-CoA and hydrogen peroxide by the enzyme Acyl-CoA oxidase (ACOD). In the presence of peroxidase (POD), a blue purple pigment is built up from hydrogen peroxide along with 3-methyl-N-ethyl-N-(beta-hydroxyethyl)-aniline (MEHA) and 4-

amino-antipyrine (a-AA) via quantitative oxidation condensation. The absorption of the blue purple pigment can be measured at a specific wavelength and is proportional to the initial NEFA concentration in the sample.

Seven µl of the sample, standard and blank were pipetted into appropriate wells of a 96-well plate. Three hundred µl of reagent 1 (R1) (containing 0.53 U/ml ACS, 0.31 mmol/l CoA, 4.3 mmol/l ATP, 1.5 mmol/l 4-AA, 2.6 U/ml AOD, 0.062 % sodium azide and 0.8 % lyophilized color A dissolved in 50 mmol/l phosphate buffer pH 7.0 with 0.05 % sodium azide) was added to each well and the plate was incubated for 3 min at 37 °C. Subsequently, 150 µl of reagent 2 (R2) (containing 12 U/ml ACOD, 14 U/ml POD dissolved in 2.4 mmol/l MEA) was added to each well. After another incubation of 4.5 min at 37 °C, the absorption was measured at 546 nm (reference wavelength 660 nm) and the determination of concentration was done via comparison of the measured values with the data of the standard:

$$\text{NEFA (mmol/L)} = \frac{\Delta\, \mathrm{A}_{sample}}{\Delta\, A_{sample}} \text{ x } \mathrm{C}_{standard}$$

being A_{sample} the absorption of the unknown sample, $A_{standard}$ the absorption of the standard and $C_{standard}$ the concentration of the standard.

2.2.6 Measurement of cytokines and hormones in final plasma

Three commercially available multiplex bead based immunoassays (Bio-Plex Pro™ Mouse Diabetes 8-plex, Diabetes adiponectin, Cytokine Group 1 23-plex; Biorad Laboratories, Hercules, CA, USA) were used to determine plasma levels of adiponectin, leptin, resistin, glucagon, PAI-1, glucagon-like-peptide 1 (GLP-1), gastric inhibitory peptide (GIP), ghrelin, interleukin (IL)1a, IL1b, IL2, IL3, IL4, IL5, IL6, IL9, IL10, IL12(p40), IL12(p70), IL13, IL17, eotaxin (CCL11), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), platelet-derived growth factor-inducible protein KC, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein alpha (MIP-1α), macrophage inflammatory protein 1 beta (MIP-1β, CCL4), RANTES (CCL5) and tumor necrosis factor alpha (TNF-α). Analysis was performed using a Bioplex 200 suspension array system (Biorad, Hercules, CA, USA) according to the manufacturer's instructions. Protein concentrations were calculated from the appropriate optimized standard curves using Bio-Plex Manger software version 6.0 (Biorad Laboratories, Hercules, CA, USA).

2.2.7 Isolation of hypothalamus

For the analysis of the gene expression profile of the hypothalamus that part of the brain was isolated out of the whole brain from the mouse. In order to accomplish that working step, the base of the brain with the hypothalamus in the center was exposed. The hypothalamus was removed with a spring-type micro scissors (see black circle, Figure 5) and snap frozen in liquid nitrogen.

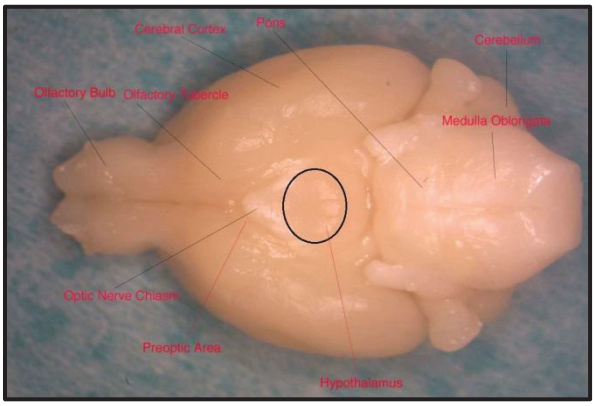


Figure 5: Ventral view of a mouse brain. The hypothalamus as isolated is marked by a black circle. Modified after "Neurogenetics at UT Health Science Center, Mouse Brain Gross Anatomy Atlas, Nervenet.org MBL.org"

2.2.8 Real-time polymerase chain reaction

Liver, gastrocnemius muscle, epididymal white adipose tissue (,WAT), brown adipose tissue (BAT) and the hypothalamus were collected from mice after euthanasia and snap frozen in liquid nitrogen. Samples were subsequently stored in -80°C until the analysis of gene expression for agouti-related protein (*Agrp*), angiopoietin-like protein 4 (*Angptl4*), chemokine (C-C motif) ligand 2 (*Ccl2*), carnitinepalmitoyl-transferase 1 alpha (*Cpt1a*), carnitinepalmitoyl-transferase 1 beta (*Cpt1b*), fatty acid synthase (*Fasn*), glucokinase (*Gck*), glucose-6-phosphatase (*G6pc*), hexokinase2 (*Hk2*), hormone sensitive lipase (*Lipe*), lipoprotein lipase (*Lpl*), neuropeptide Y (*Npy*), Phosphoenolpyruvate carboxykinase 1 (*Pck1*), proopiomelanocortin (*Pomc*), uncoupling protein 1 (*Ucp1*) and uncoupling protein 3 (*Ucp3*).

For RT-PCR primers design the FASTA sequence (http://www.ncbi.nlm.nih.gov/nucleotide/) is introduced in the software Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) that automatically generates primers based on the information provided by the user such as length of the product and species. This tool can be also used to check the primer/s specificity or whether the primer/s could recognize sequences of other genes besides the intended target one.

Mouse gene	Accession n°	Forward sequence	Reverse sequence	Product length (bp)
Actb	NM_007393	5'CCACCATGTACCC AGGCATT3'	5'AGGGTGTAAAACG CAGCTCA3	253
AgRP	NM_001271806.1	5'TGTGTAAGGCTGCA CGAGTC3'	5'GGCAGTAGCAAAAGGCAT TG3'	100
Angptl4	NM_020581.2	5'CGCTACTATCCTC TGCAGGC3'	5'AGTCTCTCCAGTT ACGGGCT3'	215
Ccl2	NM_011333	5'GTGCTGACCCCAA GAAGGAA3'	5'GTGCTGAAGACCT TAGGGCA3'	117
Cpt1a	NM_013495	5'CTCAGTGGGAGCG ACTCTTCA3'	5'GGCCTCTGTGGTAC ACGACAA3'	105
Cpt1b	NM_009948	5'CAGCGCTTTGGGA ACCACAT3'	5'CACTGCCTCAAGAGC TGTTCTC3'	204
Fasn	NM_007988	5'TTGCTGGCACTAC AGAATGC3'	5'AACAGCCTCAGAG CGACAAT3'	192
Gapdh	NM_001289726.1 NM_008084.3	5'-CCAGGTTGTCTCC TGCGACT-3'	5'ATACCAGGAAATGAGCTT GACAAAGT3'	106
Gck	NM_010292	5'CTTTCCAGGCCAC AAACATT3'	5'TGAGTGTTGAAGC TGCCATC3'	187
G6pc	NM_008061	5'CCTCCTCAGCCTA TGTCTGC3'	5'AACATCGGAGTGA CCTTTGG3'	186
Hk2	NM_013820	5'ACCCGGGATGTTA GGCAAAG3'	5'TGTACAAACACCC CGAGACG3'	218
Lipe	NM_001039507.2	5'GGAGCTCCAGTCG GAAGAGG3'	5'GTCTTCTGCGAGTGTCAC CA3'	98
Lpl	NM_008509	5'CAGCTGGGCCTAA CTTTGAG3'	5'AATCACACGGATG GCTTCTC3'	206
Npy	NM_023456.2	5'TGTTTGGGCATTC TGGCTGA3'	5'CTCAGGGCTGGAT CTCTTGC3'	153
Pck1	NM_011044	5'TCAACACCGACCT CCCTTAC3'	5'CCCTAGCCTGTTC TCTGTGC3'	235
Pomc	NM_001278584.1 NM_008895.4 NM_001278583.1 NM_001278582.1 NM_001278581.1	5'GAGTTCAAGAGGG AGCTGGA3'	5' GGTCATGAAGCCA CCGTAAC 3'	150
Rps18	NM_011296	5'ATCATGCAGAACC CACGACA3'	5'GCAAAGGCCCAGAG ACTCATT3'	268
Ucp1	NM_009463	5'GGGCCCTTGTAAA CAACAAA3'	5'GTCGGTCCTTCCT TGGTGTA3'	196
Ucp3	NM_009464	5'GTCTGCCTCATCA GGGTGTT3'	5'CCTGGTCCTTACC ATGCAGT3'	204

Table 5: Primer sequences for RT-PCR.

Three main steps were followed in the RT-PCR analysis:

1. RNA extraction: Total RNA was extracted from liver, gastrocnemius muscle, EPI WAT, BAT and hypothalamus samples using the TRIzol-phenol-chloroform-method. Samples were homogenized in TRIzol, a solution containing phenol (dissolvent for RNA) and guanidine isothiocyanate (for the inhibition of RNAses and other enzymes)

(Ambion; Life technologies, Darmstadt, Germany) using the TissueLyser II (Qiagen, Hilden, Germany). After the addition of 0.2 ml chloroform per 1 ml of TRIzol the samples were centrifuged at 4 °C, 12 000 *x g*, 15 min (Eppendorf centrifuge 5424 R, Wesseling-Berzdorf, Germany). That process forms a three-phase-mixture with the RNA in the upper aqueous phase, the DNA in the interphase and finally the proteins in the lower hydrophobic chloroform/phenol phase. From the aqueous phase, RNA was precipitated with 100% isopropanol. The pellet obtained after centrifugation at 4°C was washed in 75% ethanol, air- dried and resuspended in 15 µl RNase-free water. Finally the concentration and quality of the RNA were determined with the Nanodrop 2000 device (Thermo Scientific, peqLab, Wilmington, MA, USA) and software (Nanodrop 2000/2000c software, Thermo Scientific, peqLab, Wilmington, MA, USA).

2. RNA reverse transcription:
   a) Liver, muscle, WAT and BAT: cDNA samples for qPCR were synthesized using 1-5 µg of total RNA by the GoScript Reverse Transcription System (Promega, Madison, WI, USA). After RNA dilution the oligo (dT)-primer was added and the RNA was preheated at 70°C for 5 min and subsequently chilled on ice. For the final reaction volume (20 µl per tube) GoScript 5x reaction buffer, $MgCl_2$, PCR nucleotide mix and GoScript reverse transcriptase were added**.** cDNA synthesis was started following the protocol on table 6 using a Mastercycler (Eppendorf, Wesseling-Berzdorf, Germany).
   b) Hypothalamus: cDNA samples for qPC were synthesizd using 1 µg of total RNA filled up to 10 µl with H_2O, 1 µl of dNTPs and 2 µl of hexanucleotide primers (diluted 1:10). The RNA mix was preheated at 65 °C for 5 min, chilled on ice for 1 min and centrifuged for 10 sec. For the final reaction volume (20 µl per tube) GoScript 5x reaction buffer, $MgCl_2$, PCR nucleotide mix and GoScript reverse transcriptase were added**.** cDNA synthesis was started following the protocol on table 6 using a Mastercycler (Eppendorf, Wesseling-Berzdorf, Germany).

3. RT-PCR: qPCR was performed using Quantifast SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to the standard protocol. cDNA samples were diluted 1 to 15. Subsequently, 5 µl of sample together with 15 µl of mastermix were pipetted in a 96 well plate (Applied Biosystems, Life Technologies, Darmstadt, Germany). The mastermix consists of Quantifast SYBR Green Kit (HotStarTaq Plus DNA Polymerase, NH^{4+}/K^{+}-buffer with unique Q-Bond additive, SYBR Green I dye, ROX dye, dNTP mix) nucleotide free water, and the gene specific primers, that were used

at a final concentration of 0.33 µM. Finally all samples were run in triplicates in a real-time PCR cycler (StepOnePlus™, Applied Biosystems, Darmstadt, Germany) following the standard protocol (Table 7).

Process	Time	Temperature
Annealing	5 min	25 °C
cDNA synthesis	60 min	42 °C
Inactivation of enzyme	15 min	70 °C

Table 6: Protocol of cDNA synthesis.

Step	Time	Temperature	Cycle
HotStarTaq Plus DNA Polymerase is activated	20 sec	95 °C	hold
Denature Anneal/Extend	3 sec 30 sec	95°C 60-62°C	40
Melting curve state	15 sec 60 sec 15 sec	95 °C 60-62 °C 95 °C	hold

Table 7: RT-PCR protocol.

In a PCR a specific region of a gene is amplified through the use of complementary primers that bind to the cDNA. In RT-PCR the amount of PCR product is detected in every PCR cycle by the fluorescence emission from the SYBR green dye, which binds to the double stranded cDNA. The detected fluorescence signal is presented as the delta of the normalized reporter signal (ΔRn = (Rn+)-(Rn)), whereas the Rn is the value of an unreacted sample and R+ the value of a reaction containing all components including the target gene. In the amplification plot the fluorescence signal correlates with the initial number of target gene during the exponential phase of the PCR [378]. The Ct (cycle threshold) is defined as the cycle number required for the fluorescent signal cross the specific threshold. Therefore the Ct values are inversely proportional to the amount of target cDNA in the sample.

The Ct values of the analyzed genes were normalized to Ct values of the housekeeping genes beta actin (*Actb*), glyceralydehyde 3-phosphate-dehydrogenase (*Gapdh*) or ribosomal protein S18 (*Rsp18*) in each sample. Housekeeping genes are genes with relatively constant expression values across the experimental groups and therefore with an expression independent of external variables [378] such as in our case CVS or HFD consumption.

For the analysis of the gene expression the Ct values of the housekeeping gene were subtracted from the Ct values of the target gene. So the ΔCt is equal to the difference in Ct values for target and reference ($\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$). For the representation of the results, the common logarithm of the mean $2^{(-\Delta Ct)}$ values per group was calculated. That way of representation allows to converge the data as much as possible and therefore to ensure a normal distribution for subsequent statistical analysis.

To avoid and to figure out mismatches or primer hybridization melting curve analysis was performed. Every double stranded DNA has its specific melting point (T_m), depending on DNA length, sequence order, G:C content and Watson-Crick pairing. When the T_m is reached, 50% of the replicated fluorescence dye binding DNA becomes single stranded resulting in a sudden decrease of fluorescence signal, due to double stranded DNA dissociation and release of the dye. Finally this point is determined and translated in a melting curve [378]. Therefore, every additionally peak is indicative for mismatches or primer hybridizations, whereas one clear peak indicates a specific primer annealing and therefore DNA replication of the target gene.

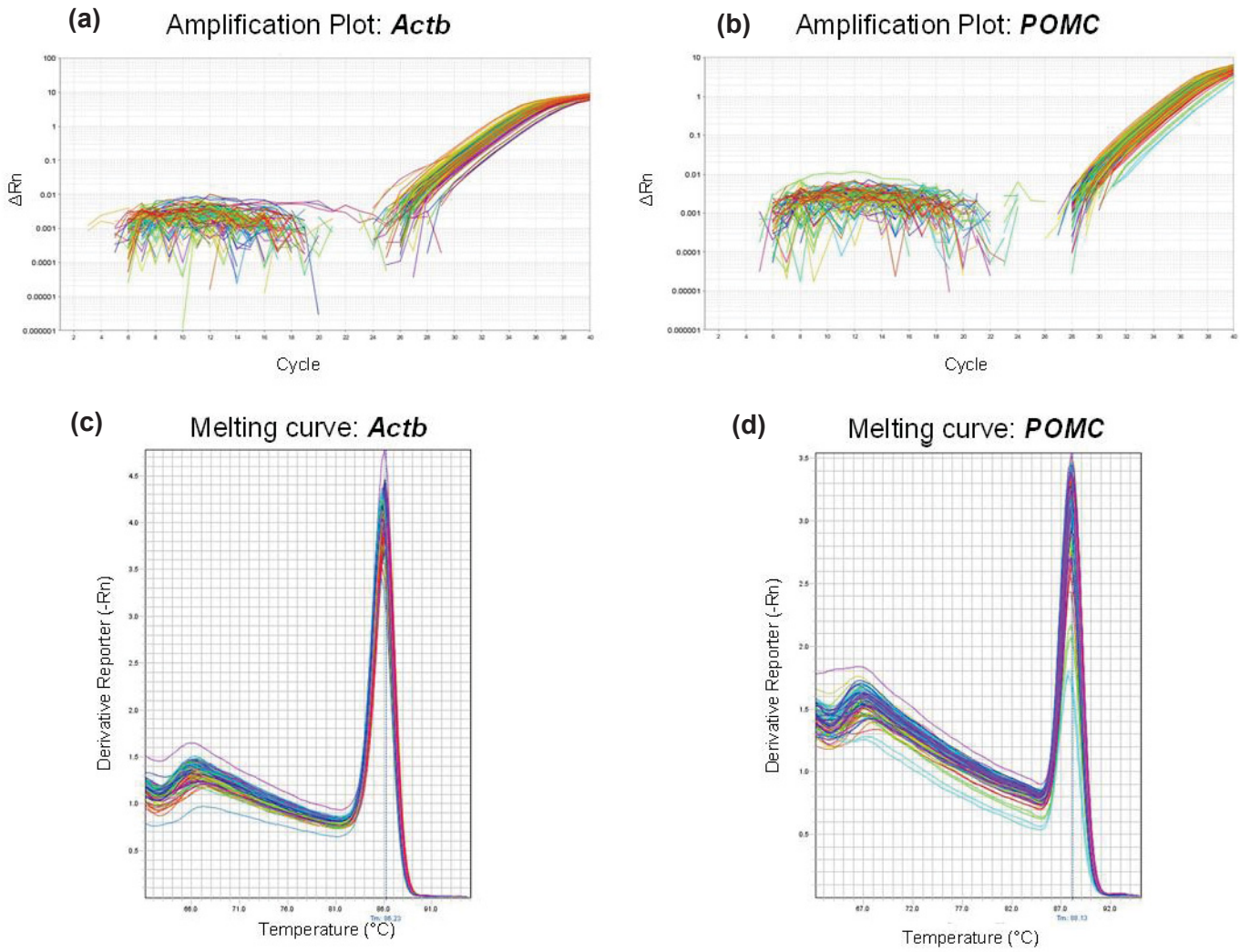


Figure 6: Amplification plot and melting curve of representative real time PCR results. In the amplification plot the cycle number, which correlates with the initial amount of cDNA during the exponential phase of PCR, versus fluorescence signal is presented. **a** amplification plot of housekeeping gene *Actb*, **b** amplification plot of gene of interest (*Pomc*). The melting curves of *Actib* (**c**) and *Pomc* (**d**) show a clear peak what indicates that the DNA synthesis and the primer annealing is specific only for the target gene.

2.2.9 Protein extraction from gastrocnemius muscle separated in microsomal and plasma membrane fraction

Separation of microsomal and plasma membrane fraction of muscle cells was performed as reported in [292]. Pieces of ~ 100 mg of frozen *gastrocnemius* muscle were pestled in liquid nitrogen, transferred to borosilicate vials (VWR, Langenfeld, Germany) provided with 600 µl of lysis buffer A (20 mM Tris-HCl, 1 mM EDTA, 0.25 mM EGTA, 250 nM sucrose, protease and phosphatase inhibitor) and subsequently homogenized on ice with a manual homogenizer at 17 500 rpm. Homogenates were transferred to centrifugation tubes

(Beckman Coulter, Brea, CA, USA) with a Pasteur pipette and centrifuged at 100 000 *x g*, 1 h, 4°C in an ultracentrifuge (Beckman Coulter, Brea, CA, USA). The supernatant (=microsomal fraction) was transferred to a 1.5 ml reaction tube (Eppendorf, Wesseling-Berzdorf, Germany) with an insulin syringe (Braun, Melsungen, Germany) and frozen at -80 °C. The pellet was re-homogenized with 200 µl of lysis buffer B (250 mM Tris-HCl, 1 mM EDTA, 0.25 mM EGTA, 2 mM Triton-X 100, protease and phosphatase inhibitor) and another centrifugation step at 100 000 *x g*, 1 h, 4°C in the ultracentrifuge was performed. The supernatant (=plasma membrane fraction) was transferred to a 1.5 ml reaction tube (Eppendorf, Wesseling-Berzdorf, Germany) with an insulin syringe and frozen at -80 °C. Protein content of all microsomal and plasma membrane fraction samples was determined using bicinchoninic protein assay (BCA assay) (Pierce BCA assay kit, Thermo Scientific, Schwerte, Germany).

2.2.10 Protein extraction and quantification by BCA protein assay

Pieces of ~ 50 mg of frozen liver or muscle were put in 2 ml reaction tubes (Eppendorf, Wesseling-Berzdorf, Germany) provided with lysis buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X 100 and one stainless steel bead (Qiagen, Hilden, Germany) per tube. After homogenization in an automatic tissue lyser (TissueLyser II, Qiagen, Hilden, Germany) for 4 min with 25 Hz, samples were incubated for 1 h at 4°C in an overhead shaker (Heidolph, Schwabach, Germany) and subsequently centrifuged (14 000 *x g*, 4°C, 10 min). Protein content of the supernatants was determined using bicinchoninic protein assay (BCA assay) (Pierce BCA assay kit, Thermo Scientific, Schwerte, Germany).

This biochemical assay is based on the property of peptide bonds in proteins to reduce Cu^{2+} ions to Cu^{+} ions in alkaline medium, a reaction that is proportional to the amount of protein present in a solution. Subsequently, the Cu^{+} ions are bound by two molecules of bicinchoninic acid to form a chelate complex that strongly absorbs light at a wavelength of 562 nm and is nearly linear with increasing protein concentrations over a broad working range (20-20000 µg/ml). Protein samples were diluted 1:20-1:50 with distilled water and pipetted into respective wells on the microwell plate same as eight standards with defined concentrations of bovine serum albumin (BSA) (0.025, 0.125, 0.25, 0.5, 0.75, 1, 1.5, 2 µg/µl) as well as the blank. Subsequently, the working reagent was prepared by mixing 50 parts of BCA reagent A (containing bicinchoninic acid) with 1 part of BCA reagent B (containing 4 % cupric sulfate) provided by the Pierce BCA Protein Assay Kit (Therm Scientific, Schwerte, Germany). After a brief mixing, the plate was incubated at 37 °C for 30 min, then cooled down to RT and the absorption of each sample was measured at a wavelength of 562 nm with a micro plate reader (Tecan, Männedorf, Switzerland). The determination of

concentration was done via comparison of the measured values with the data of the standard curve (prepared with above-mentioned concentration). Based on the calculation, samples were diluted to a final concentration of 15 µg/25 µl with 4x Laemmli sample buffer (containing 20 % glycerol (w/v), 8 % SDS (w/v), 10 mM EDTA and 250 mM Tris) and boiled at 95 °C for 5 min.

2.2.11 SDS-PAGE and Western Blot

2.2.11.1 SDS-PAGE

A separation gel of 8-12% acrylamide concentration depending on the molecular weight of the protein of interest (table 8) was prepared by mixing a respective amount of acrylamide solution with a respective amount of separation gel buffer (containing 1.5 M Tris and 0.4 % SDS (w/v), pH 8.8), distilled water, APS (MP Biomedicals, Illkirch, France) and TEMED (Roth, Karlsruhe, Germany). The gel was covered with a thin layer of 0.2 % SDS solution and allowed to polymerize within ~20 min. After polymerization of the separation gel, the SDS solution on top was poured to the sink and the stacking gel of 5 % acrylamide concentration was prepared by mixing a respective amount of acrylamide solution with a respective amount of stacking gel buffer (containing 0.5 M Tris and 0.4 % SDS (w/v), pH 6.8), distilled water, APS and TEMED. The stacking gel was cast on top of the separation gel, a comb with 15 slots was placed and the gel was left to polymerize for ~30 min.
The gels were placed into a Mini-PROTEAN ® Tetra Cell electrophoresis chamber (BioRad Laboratories, Munich, Germany) and the combs were removed. The chamber and the space within two gels each was filled with Laemmli electrophoresis buffer (containing 25 mM Tris, 0.192 M glycerol and 0.1 % SDS (w/v)). Twenty five µl of protein sample (containing 15 µg of total protein) were loaded per well as well as 1x 10 µl of a molecular weight marker (Dual Color, BioRad Laboraties, Munich, Germany) per gel. Samples migrated through the stacking gel with a constant voltage of 50 volt (V) and through the separation gel with a constant voltage of 150 V.

2.2.11.2 Blotting

After completed electrophoresis, protein in the gel was transferred onto a nitrocellulose membrane (Whatman, Oehmen, Essen, Germany) by clamping the gel and the blotting membrane in a frame between four filter papers (Oehmen, Essen, Germany) and two fiber pads (Biometra, Göttingen, Germany). The cassettes containing one membrane and gel

each surrounded by filter papers and the fiber pads were put into a vertical buffer tank (Tankblot Eco Mini, Biometra, Göttingen, Germany) filled with transfer buffer (containing 25 mM Tris, 0.192 M glycerol, 20 % methanol (v/v)) and connected to a cooling unit (ARCTIC A28 Refrigerated Circulator, Thermo Scientific, Schwerte, Germany) to allow blotting at 4°C. Transfer was performed at constant current (200 mA), time was chosen based on the molecular weight of the protein of interest (table 8).

After completed transfer, membranes were washed briefly with wash buffer (containing 10 mM Tris, 0.15 M NaCl, 0.05 % Tween-20 (v/v) and the efficient transfer was verified by reversible staining of the membrane with Ponceau S staining solution (containing 0.25 % Ponceau S, 40 % methanol (v/v), 15 % acetic acid (v/v)). After blocking for 1 h at RT with wash buffer containing 5 % non-fat dry milk (Roth, Karlsruhe, Germany), the membrane was either washed briefly (when primary antibody was diluted in blocking solution, see table 8) or for 30 min (when primary antibody was diluted in wash buffer containing 5 % BSA, see table 8) before probing with primary antibody. Incubation with primary antibody was either overnight (o.n.) or for 2 h at room temperature (see table 8). Subsequently, the membrane was washed 3 times briefly and once for 30 min before incubation with HRP-coupled secondary antibody (anti-rabbit or anti-mouse IgG, see table 8) diluted 1:10 000-1:20 000 (see table 8) in 5 % non-fat dry milk (NFDM) for 1 h at RT. After incubation with the secondary antibody, membrane was washed again 3 times briefly and once for 30 min.

Based on the expected amount of the protein of interest in the sample (sensitivity), either Western Lightning Pro or Ultra (both Perkin Elmer, Waltham, MA, USA) was used to visualize specific bands through the Chemidoc^{TM} XRS^{+} System (BioRad Laboratories, Munich, Germany). Protein bands were quantified using Quantity One software (BioRad Laboratories, Hercules, CA, USA).

Antibody	Gel %	Blot time	Incubation time and dilution	Type of 2ary and dilution	ECL system
p-ACC	10%	3 h	o.n. 4°C, 1:1000, 5 % BSA	anti-rabbit, 1:10 000	Western Lightning Ultra
ACC	10%	3 h	o.n. 4°C, 1:1000, 5 % BSA	anti-rabbit, 1:10 000	Western Lightning Ultra
p-AKT	12%	2 h	o.n. 4°C, 1:1000, 5 % BSA	anti-rabbit, 1:10 000	Western Lightning Ultra
AKT	12%	2 h	o.n. 4°C, 1:1000, 5 % BSA	anti-rabbit, 1:10 000	Western Lightning Ultra
p-AMPK	12%	2 h	o.n. 4°C, 1:1000, 5 % BSA	anti-rabbit, 1:10 000	Western Lightning Ultra
AMPK	12%	2 h	o.n. 4°C, 1:1000, 5 % BSA	anti-rabbit, 1:10 000	Western Lightning Ultra
GAPDH	depending	depending	o.n. 4°C or 2 h RT	anti-rabbit, 1:10 000	Western Lightning Pro
	on POI	on POI	1:5000, 5 % BSA		
GLUT 1	12%	o.n.	2 h RT, 1:1000, 5 % NFDM	anti-rabbit, 1:20 000	Western Lightning Pro
GLUT 4	12%	o.n.	2 h RT, 1:1000, 5 % NFDM	anti-rabbit, 1:20 000	Western Lightning Pro
1αNa+K+ ATPase	depending	depending	o.n. 4°C, 1:1000, 5 % BSA	anti-rabbit, 1:10 000	Western Lightning Ultra
	on POI	on POI			
p-p70S6K1	10%	2 h	o.n. 4°C, 1:1000, 5 % NFDM	anti-mouse, 1:10 000	Western Lightning Ultra
p70S6K1	10%	2 h	o.n. 4°C, 1:1000, 5 % BSA	anti-rabbit, 1:10 000	Western Lightning Ultra

Table 8: Antibodies used for Western Blot. POI = protein of interest, o.n. = overnight

2.2.12 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). For calculation of statistical differences between two data points, two-tailed unpaired student's t-test was used. Differences between two data points were considered statistically significant with $p<0.05$. All statistical analyses were performed with the software Graphpad Prism 5.04 (GraphPad Software, San Diego, CA, USA).

3 Results

In the present thesis, 3 months old male C57BL/6J mice were exposed to CVS, changes in insulin sensitivity were determined by hyperinsulinemic-euglycemic clamps either directly after the stress regimen or three months later, during which the mice were consuming either a low-fat (CHOW) or a high-fat diet (HFD). The obtained data on insulin sensitivity were complemented by further investigations on plasma cytokines and hormones, gene expression and tissue protein levels analysis.

3.1 Exposure to CVS increases plasma corticosterone and decreases lean mass

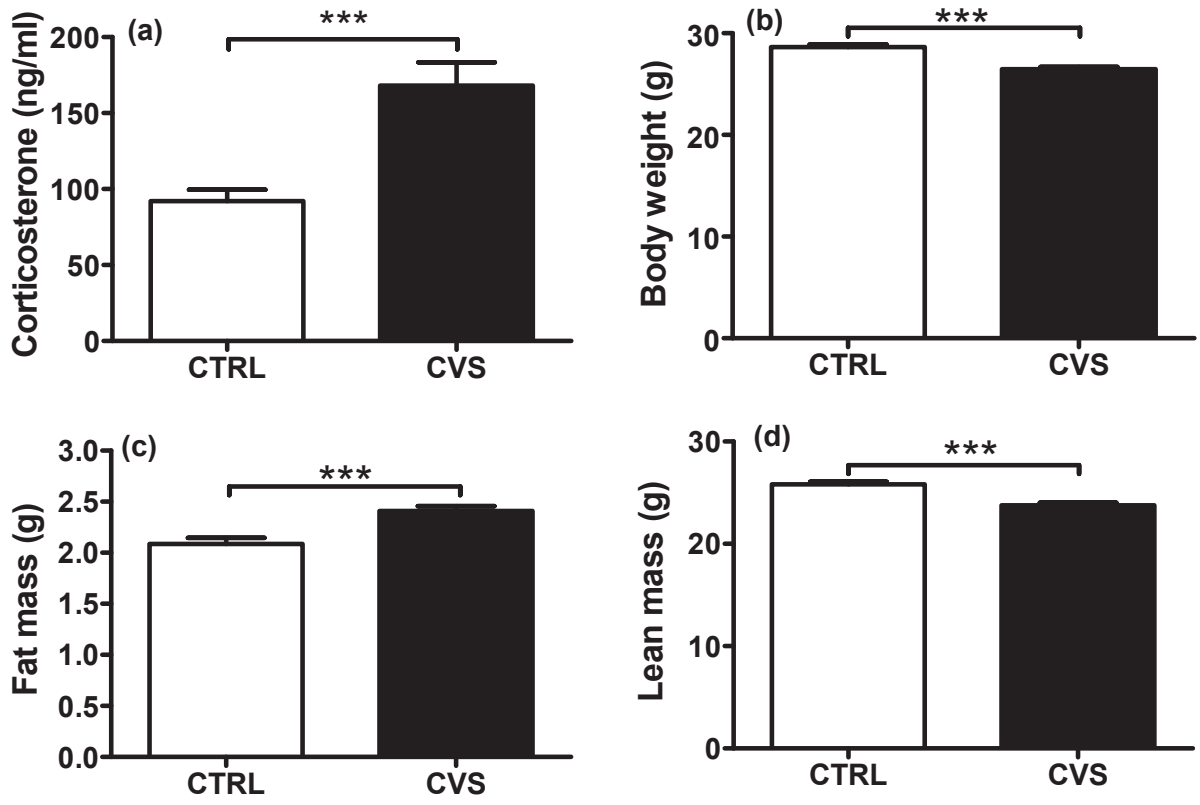


Figure 7: Body composition changes in response to CVS. CVS decreases body weight (**b**) and lean mass (**d**) and increases plasma corticosterone (**a**) and fat mass (**c**). Results are presented as mean ± SEM, two-tailed unpaired t-test, ***p<0.001 (n=65-69).

Three months old male C57BL/6J mice were divided into CTRL and CVS groups matched by BW. Body composition and plasma corticosterone were measured after the CVS intervention in both groups. CVS mice showed higher plasma levels of corticosterone (168.10±15.33 vs. 92.02 ± 7.61 ng/ml, n=65-67; $p<0.001$; Figure 7a) and lower lean mass (23.76±0.24 vs. 25.81±0.26 g, n=69; $p<0.001$; Figure 7d). The fat mass was increased with CVS (2.41±0.05 vs. 2.09±0.06 g, n=69; $p<0.001$; Figure 7c) whereas the body weight was decreased (26.46±0.23 vs. 28.64 ±0.25 g, n=69; $p<0.001$; Figure 7b).

3.2 Effects of CVS and diet on body composition

Plasma levels of corticosterone and body composition were determined again 3 months after CVS and diet consumption. With CHOW consumption following the CVS intervention, plasma corticosterone did not differ between CVS CHOW and CTRL CHOW (n=12; Figure 8a), as well no differences were observed in lean mass (n=23-24; Figure 8b), fat mass (n=23-24; Figure 8c) and body weight (n=23-24; Figure 8d).

When the animals were consuming HFD subsequent to the CVS intervention, plasma corticosterone was increased compared to CTRL HFD ($p<0.01$, n=10-12; Figure 8e) with no differences in lean mass (n=22; Figure 8f), fat mass (n=22; Figure 8g) and body weight (n=22; Figure 8h).

Therefore CVS did not cause long-term effects on BW and lean mass independently of the diet, however, plasma corticosterone was increased with CVS and HFD.

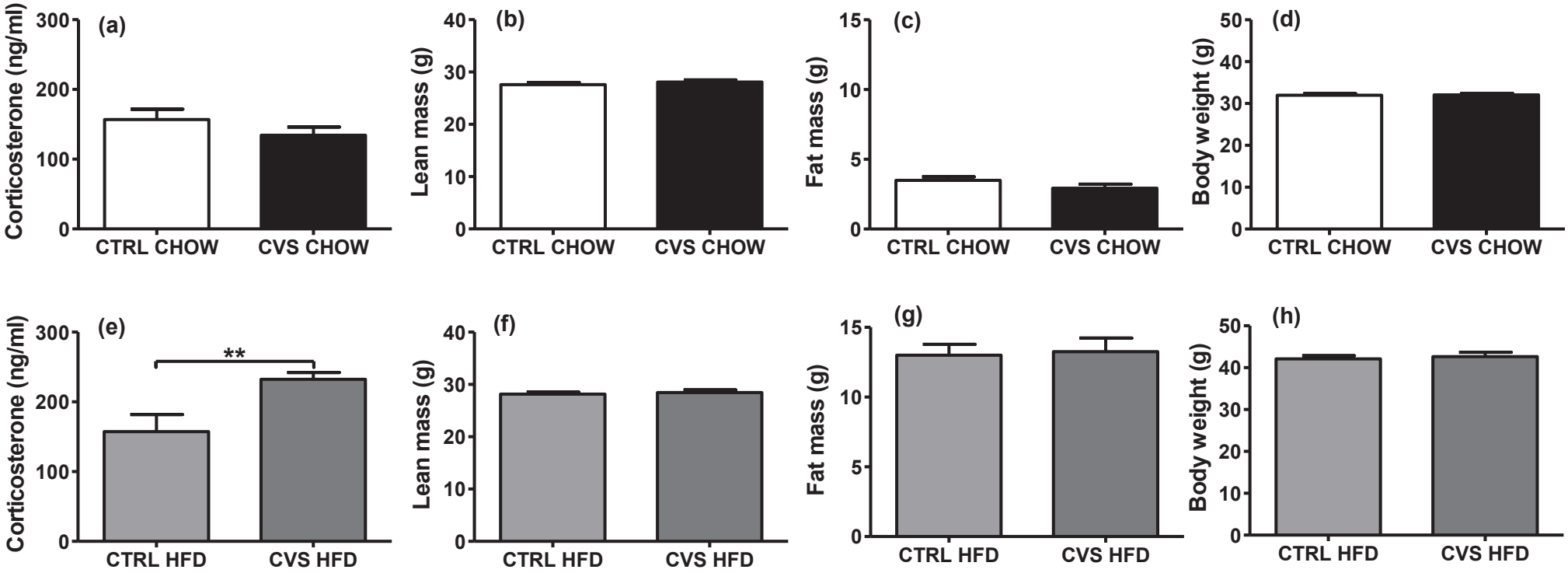


Figure 8: Body composition changes in response to CVS and diet. Three month after the CVS intervention with CHOW consumption, no differences were found between CVS CHOW and CTRL CHOW in plasma levels of corticosterone, lean mass, fat mass and body weight (**a** n=CTRL:CVS=12:12, **b** n= 23:24, **c** n=23:24, **d** n=23:24). With HFD consumption for 3 months after the CVS intervention, plasma levels of corticosterone were increased whereas lean mass, fat mass and body weight did not show differences between CVS HFD and CTRL HFD. (**e** n=CTRL:CVS=10:12, **f** n=22:22, **g** n=22:22, **h** n=22:22). Results are presented as mean ± SEM, two-tailed unpaired t-test, **p<0.01.

3.3 CVS effects on insulin sensitivity are tissue - and diet-dependent

After the chronic stress intervention, basal endogenous glucose production (EGP) by the liver was decreased in the CVS mice compared with the CTRL mice (19.16±0.91 vs. 23.02±1.00 mg/kg/min, n=10-11; $p<0.05$; Figure 9a). CVS did not affect the whole-body insulin sensitivity expressed by the glucose infusion rate (GIR) (65.38±2.33 vs. 64.31±3.72 mg/kg/min, n=10; Figure 9b). This could be a consequence of the opposite effects of the suppression of EGP by insulin that was decreased in CVS mice compared with CTRL mice (91.66±6.46 vs. 117.10±6.65 %, n=9; Figure 9c) while insulin stimulation of glucose disposal in peripheral tissues was increased as indicated by the stimulation of the rate of glucose disposal (Rd) (356.20±20.49 vs. 275.80±19.54%, n=10; $p<0.05$; Figure 9d).

CVS mice consuming CHOW for 3 months showed a trend to lower basal EGP compared with the CTRL CHOW mice (20.01±1.12 vs. 23.86±1.48 mg/kg/min, n=10-11; $p=0.055$; Figure 9e). CVS CHOW mice also showed increased whole-body insulin sensitivity (GIR) (72.05±3.72 vs. 62.16±2.18 mg/kg/min, n=10-11; $p<0.05$; Figure 9f). CVS in combination with CHOW feeding did not affect the suppression of EGP (101.80±6.14 vs. 91.54±8.85 %, n=10-11; Figure 9g), however, insulin stimulation of glucose disposal in peripheral tissues was increased compared to the CTRL mice (363.30±28.71 vs. 275.80±16.71 %, n=10-11; $p<0.05$; Figure 9h).

When CVS mice were fed a HFD for 3 months, no differences in basal EGP compared to the CTRL mice were observed (23.71±1.89 vs. 23.66±2.75 mg/kg/min, n=6-8; Figure 9i). GIR was decreased in CVS HFD mice compared the CTRL HFD mice indicating lower whole-body insulin sensitivity (26.72±2.39 vs. 35.50±3.39 mg/kg/min, n=8-9; $p<0.05$; Figure 9j). Suppression of EGP by insulin was not affected by CVS and HFD (72.50±8.48 vs. 65.06±8.37%, n=6-8; Figure 9k) while insulin stimulation of glucose disposal in peripheral tissues was decreased compared to the CTRL HFD mice (134.90±11.53 vs. 191.80±13.65%, n=6-8; $p<0.01$; Figure 9l).

Therefore, exposure to CVS affects insulin sensitivity in a tissue-dependent manner impairing hepatic glucose production and improving glucose disposal in peripheral tissues. Furthermore, when fat consumption is low, chronic stress improves long-term insulin sensitivity through high glucose disposal in peripheral tissues. On the contrary, HFD consumption exacerbates insulin resistance under these experimental conditions.

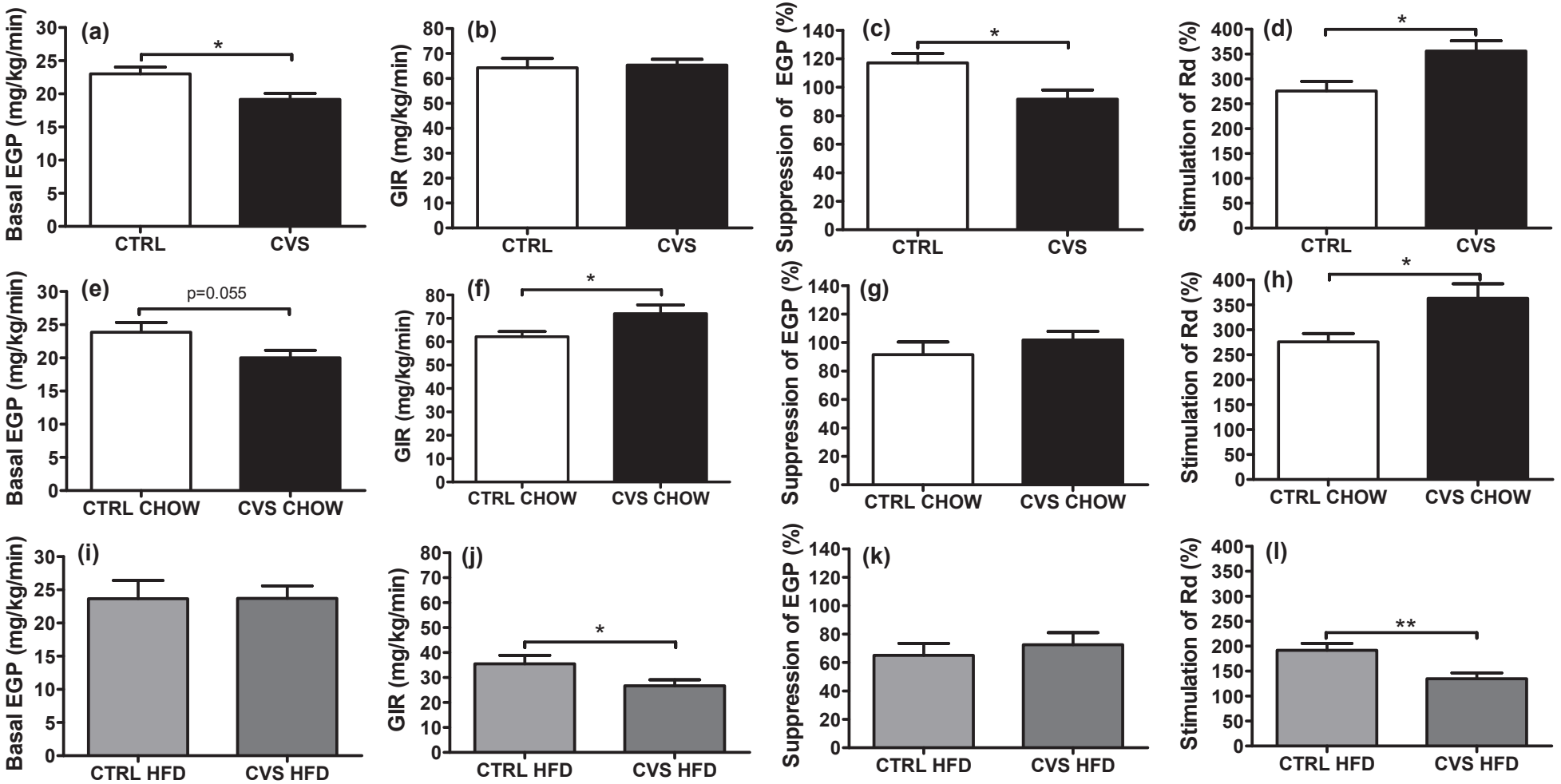


Figure 9: Short- and long-term effects of CVS and diet on insulin sensitivity. CVS decreases basal endogenous glucose production (EGP) (**a** n=CTRL: CVS=11:10). No changes were found in the glucose infusion rate (GIR) (**b** n=10:10). CVS decreases the suppression of EGP (**c** n=9:9) and increases the rate of glucose disposal in peripheral tissues (stimulation of Rd) (**d** n=10:10).CVS and CHOW consumption decrease basal EGP (**e** n=11:10), increase GIR (**f** n=11:10) and the rate of glucose disposal in peripheral tissues (stimulation of Rd) (**h** n=11:10) while suppression of EGP is not affected (**g** n=11:10). CVS and HFD consumption decrease GIR (**j** n= 8:9) and the stimulation of Rd (**l** n=6:8) and do not affect basal EGP (**i** n=6:8) and suppression of EGP (**k** n=6:8). Results are presented as mean ± SEM, two-tailed unpaired t-test, *p<0.05, **p<0.01. (*Results got in collaboration with Prof. Roden and Dr. Jelenik, Institute for Clinical Diabetology, German Diabetes Center, Düsseldorf, Germany*).

	CTRL		CVS	
	Pre-clamp	Post-clamp	Pre-clamp	Post-clamp
n	10	11	10	11
Cholesterol (mmol/l)	1.94±0.11	1.10±0.04$^{###}$	1.78±0.08	1.10±0.03$^{###}$
TG (mg/dl)	81±6	74±13	58±3**	94±11$^{\#}$
NEFA (mmol/l)	0.64±0.08	0.19±0.02$^{###}$	0.61±0.12	0.17±0.02$^{###}$

Table 9: Plasma lipid metabolites pre- (6-h-fast) and post-hyperinsulinemic-euglycemic clamp in response to CVS. Data presented as mean±SEM, two-tailed unpaired t-test, **p<0.01 vs. CTRL; #p<0.05, ###p<0.001 vs. pre-clamp.

Given that GCs, the SNS and insulin affect lipid metabolism, plasma lipid species in response to CVS and diets before and after the hyperinsulinemic-euglycemic clamps were analyzed.

Pre-clamp plasma cholesterol and NEFA did not differ between the CVS and the CTRL group (Table 9). Cholesterol and NEFA levels were lower in the CTRL and the CVS group after clamps, an effect probably related to the inhibitory effect of insulin on lipolysis. Interestingly, plasma TG were lower pre-clamp in the CVS mice compared with the CTRL mice. Furthermore, TG levels were higher after clamps in the CVS mice with no changes in the CTRL mice (Table 9).

Three months after the chronic stress intervention, this profile was similar between the CTRL and the CVS mice kept on CHOW. Like in the short-term group, plasma TG were lower pre-clamp in the CVS mice compared to the CTRL mice. Likewise NEFA and TGs, cholesterol was decreased after clamps in both CTRL and CVS mice (Table 10).

In mice consuming a HFD for 3 months, no differences were observed in the pre-/post-clamps metabolite profile of the HFD CTRL group compared with the acute and CHOW CTRL groups. However, cholesterol and NEFA levels pre- and post-clamps were not different in CVS HFD mice. Likewise, cholesterol as well as TG and NEFA levels were not different pre-clamp between the CVS HFD and CTRL HFD mice (Table 10).

	CTRL CHOW		CVS CHOW		CTRL HFD		CVS HFD	
	Pre-clamp	Post-clamp	Pre-clamp	Post-clamp	Pre-clamp	Post-clamp	Pre-clamp	Post-clamp
n	11	11	10	10	8	8	9	9
Cholesterol (mmol/l)	2.03±0.06	1.62±0.08$^{###}$	2.10±0.09	1.55±0.08$^{###}$	4.29±0.32	3.29±0.18$^{\#}$	3.95±0.39	3.12±0.29
TG (mg/dl)	95±7	38±2$^{###}$	77±2*	32±2$^{###}$	83±5	65±4$^{\#}$	87±7	61±4$^{\#\#}$
NEFA (mmol/l)	1.34±0.13	0.60±0.04$^{###}$	1.38±0.06	0.51±0.07$^{###}$	0.98±0.07	0.75±0.08$^{\#}$	0.93±0.06	0.83±0.09

Table 10: Plasma lipid metabolites pre- (6-h-fast) and post-hyperinsulinemic-euglycemic clamp in response to CVS and diet. Data presented as mean±SEM, two-tailed unpaired t-test, *p<0.05 *vs.* diet-matched CTRL; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{###}$p<0.001 *vs.* diet-matched pre-clamp.

3.4 Inflammation as a mechanism of the regulation of insulin sensitivity by CVS and diet

Although classically anti-inflammatory properties are attributed to GCs, they can also promote inflammation under chronic stress conditions through an increase of pro-inflammatory factors like IL-6 and TNF-α due to GC or GR resistance [137-139]. Furthermore, it has been well described that HFD consumption affects inflammation [293]. Therefore, it is possible that subsequent consumption of HFD after CVS exposure has a synergistic effect on inflammation in the contribution to insulin resistance. In order to investigate that possibility, 23 plasma cytokines were analyzed (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 [p40], IL-12 [p70], IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β (CCL4), RANTES (CCL5), TNF-α).

Compared with CTRL mice, CVS mice showed a systemic anti-inflammatory state as indicated by the higher levels of anti-inflammatory IL-10 and lower levels of pro-inflammatory RANTES/CCL5 and Eotaxin/CCL11 as well as a trend to lower levels of pro-inflammatory IL-1α and MIP-1β/CCL4. No differences were observed between the groups in the other analyzed cytokines (Table 11).

When mice were fed CHOW for 3 months, CVS mice showed higher levels of G-CSF compared to the CTRL mice (Table 12) with no differences in the other analyzed cytokines between the groups. When mice were fed HFD for three months, no differences were observed between the groups in any of the analyzed cytokines (Table 13).

Therefore, on the short-term, CVS mice display a systemic anti-inflammatory state that is in accordance with the well-described anti-inflammatory properties of GCs. However, since changes in plasma cytokine levels were not observed 3 months after the CVS intervention,

systemic inflammation does not seem to be the key mechanism to explain changes in insulin sensitivity with time and diet consumption.

Name of cytokine	CTRL (pg/ml) (n= 8-10)	CVS (pg/ml) (n= 8-10)	*P*-value
IL-1α	118.60±22.96	75.14±11.06	**p=0.097**
IL-1β	557.00±56.83	532.00±73.16	p=0.790
IL-2	58.05±14.26	59.90±13.19	p=0.925
IL-3	23.53±5.08	24.32±7.15	p=0.928
IL-4	---	---	OOR
IL-5	46.19±8.01	40.08±7.92	p=0.595
IL-6	118.90±24.81	127.80±30.04	p=0.820
IL-9	---	---	OOR
IL-10	212.20±19.12	300.40±37.67	**p=0.047***
IL-12 (p40)	1057.00±242.50	718.70±154.30	p=0.245
IL-12 (p70)	173.60±32.39	189.10±49.11	p=0.791
IL-13	623.30±131.30	631.50±151.70	p=0.967
IL-17	77.29±12.45	72.25±12.74	p=0.780
Eotaxin (CCL11)	656.20±12.45	418.00±70.74	**p=0.044***
G-CSF	3808.00±707.70	2885.00±745.60	p=0.391
GM-CSF	346.30±43.59	298.90±48.61	p=0.477
IFN-γ	28.14± 4.09	23.40±4.70	p=0.462
KC	454.10±62.77	515.30±85.38	p=0.565
MCP-1	938.00±171.90	1014.00±167.30	p=0.754
MIP-1α	131.50±51.03	56.84±10.25	p=0.168
MIP-1β (CCL4)	204.50±18.82	144.50±21.01	**p=0.054**
RANTES (CCL5)	280.90±32.38	142.70±40.23	**p=0.020***
TNF-α	763.40±131.60	744.80±165.80	p=0.931

Table 11: Levels of cytokines in response to CVS. CVS increased anti-inflammatory IL-10 and decreased pro-inflammatory RANTES/CCL5, Eotaxin /CCL11, MIP-1β/CCL4 and IL-1α levels. Results are presented as mean ± SEM, two-tailed unpaired t-test.,*p<0.05. OOR = out of range.

Name of cytokine	CTRL CHOW (pg/ml) (n= 8-11)	CVS CHOW (pg/ml) (n= 7-10)	*P*-value
IL-1α	73.20±9.26	63.29±11.17	p=0.499
IL-1β	319.10±22.55	336.50±39.15	p=0.697
IL-2	---	---	OOR
IL-3	---	---	OOR
IL-4	---	---	OOR
IL-5	20.04±1.79	19.93±2.82	p=0.974
IL-6	66.43±14.07	87.07±28.75	p=0.514
IL-9	---	---	OOR
IL-10	113.90±16.10	136.20±28.96	p=0.509
IL-12 (p40)	1252.00±201.80	1723.00±385.20	p=0.280
IL-12 (p70)	---	---	OOR
IL-13	157.30±16.95	163.90±28.71	p=0.840
IL-17	29.06±3.47	28.20±4.80	p=0.888
Eotaxin	---	---	OOR
G-CSF	4861.00±660.60	10231.00±2508.00	**p=0.045***
GM-CSF	---	---	OOR
IFN-γ	---	---	OOR
KC	597.60±55.10	504.90±86.59	p=0.369
MCP-1	2137.00±365.90	1868.00±473.20	p=0.655
MIP-1α	57.91±3.13	69.54±12.10	p=0.293
MIP-1β (CCL4)	131.30±18.46	155.60±42.96	p=0.597
RANTES (CCL5)	149.90±20.60	172.20±34.61	p=0.565
TNF-α	223.20±22.54	257.30±37.16	p=0.432

Table 12: Levels of cytokines in response to CVS and CHOW consumption. CVS and three month CHOW consumption increased G-CSF levels. Results are presented as mean ± SEM, two-tailed unpaired t-test. *p<0.05. OOR = out of range.

Name of cytokine	CTRL HFD (pg/ml) (n= 6-8)	CVS HFD (pg/ml) (n= 8-9)	*P*-value
IL-1α	178.70±66.08	97.60±15.31	p=0.225
IL-1β	88.36±21.74	103.60±12.62	p=0.542
IL-2	---	---	OOR
IL-3	---	---	OOR
IL-4	---	---	OOR
IL-5	14.76±1.09	15.15±1.10	p=0.811
IL-6	234.00±111.20	164.20±70.41	p=0.595
IL-9	---	---	OOR
IL-10	96.92±14.37	119.50±19.17	p=0.386
IL-12 (p40)	2097.00±996.10	2243.00±674.40	p=0.902
IL-12 (p70)	---	---	OOR
IL-13	155.80±31.19	171.10±20.13	p=0.670
IL-17	---	---	OOR
Eotaxin	---	---	OOR
G-CSF	11940.00±4124	11480.00±3033.00	p=0.928
GM-CSF	---	---	OOR
IFN-γ	---	---	OOR
KC	782.00±245.30	1073.00±340.80	p=0.542
MCP-1	5483.00±2174.00	2239.00±686.70	p=0.155
MIP-1α	69.06±19.34	70.68±10.75	p=0.938
MIP-1β (CCL4)	88.36±21.74	103.60±12.62	p=0.542
RANTES (CCL5)	328.70±103.90	298.90±48.04	p=0.790
TNF-α	236.60±59.56	225.10±28.64	p=0.854

Table 13: Levels of cytokines in response to CVS and HFD consumption. Results are presented as mean ± SEM, two-tailed unpaired t-test. OOR=out of range.

3.5 Hormones as mediators of the regulation of insulin sensitivity by CVS and diet

Besides cytokines, GCs and stress can affect the levels of adipokines [181, 182, 200, 201, 204, 212-214, 240, 278] and gastrointestinal hormones [103, 151-154, 294] which could contribute to the regulation of insulin sensitivity in combination with diet. Actually a decrease in the ratio of adiponectin:resistin in response to CVS and HFD consumption played an important role in glucose tolerance in previous studies [214].

To address the role of circulating hormones in the regulation of insulin sensitivity in response to CVS and diet, the levels of adipokines, gastrointestinal and pancreatic hormones were measured in final plasma after the clamp.

When analyzing the levels of circulating adipokines, CVS mice showed higher leptin levels compared with CTRL mice (1.438± 0.145 vs. 1.127± 0.065 ng/ml, n=9; p=0.07; Figure 10a) and also a trend to higher adiponectin levels (11522±1443 vs. 8221±874 ng/ml, n=9-10; p=0.06; Figure 10c) whereas resistin (132.8±8.1 vs. 144.3±9.6 ng/ml, n=10; Figure 10b) and PAI-1 (21.88±0.61 vs. 22.75±0.03 ng/ml, n=8; Figure 10d) levels were not affected by CVS.

When consuming CHOW for three months, CVS mice showed lower levels of adiponectin compared with CTRL (5875±430 vs. 11294±1389 ng/ml; n=10-11; $p<0.01$; Figure 10g), furthermore, CVS CHOW mice compared with CTRL CHOW mice showed lower levels of leptin (0.960±0.165 vs. 1.601± 0.235 ng/ml, n=10-11; $p<0.05$; Figure 10e), resistin (127.9± 5.2 vs. 142.7± 5.2 ng/ml, n=10; p=0.06, Figure 10f) and PAI-1 (22.17±0.30 vs. 22.97±0.23, n=9; $p<0.05$; Figure 10h) levels.

When consuming HFD, CVS mice compared to CTRL mice showed lower levels of adiponectin (7989±892 vs. 16787±3584 ng/ml; n=7-8; $p<0.05$; Figure 10k) and a trend to higher levels of resistin (166.0±18.9 vs. 112.9±16.9 ng/ml, n=7-9; p=0.06, Figure 10j). Leptin levels (21.240±3.358 vs. 17.050±5.655, n=8-9; Figure 10i) as well as PAI-1 levels (22.73±0.17 vs. 22.69±0.18 ng/ml, n=7-9; Figure 10l) did not differ between CVS HFD and CTRL HFD mice.

When analyzing the levels of pancreatic and gastrointestinal hormones, CVS mice showed lower levels of glucagon (0.242±0.010 vs. 0.310±0.023 ng/ml, n=9; $p<0.05$; Figure 11a) as well as of ghrelin (9.851±0.630 vs. 12.410±0.907 ng/ml, n=8-10; $p<0.05$; Figure 11b) compared with CVS mice. No changes between CVS mice compared with CTRL mice were found in GIP (0.217± 0.016 vs. 0.250±0.017 ng/ml, n=9-10; Figure 11c) and GLP-1 (0.045±0.002 vs. 0.051±0.004 ng/ml, n=9-10; Figure 11d).

Mice exposed to CVS following CHOW consumption did not show changes in glucagon (0.244±0.029 vs. 0.275± 0.037 ng/ml, n=9-10; Figure 11e), ghrelin (16.210±2.619 vs. 15.780±1.598 ng/ml, n=9-10; Figure 11f), GIP (0.221±0.017 vs. 0.257±0.028 ng/ml, n=9-11;

Figure 11g) and GLP-1 (0.043±0.005 vs. 0.053±0.005 ng/ml, n=9-10; Figure 11h) compared with CTRL CHOW.

Mice exposed to CVS following HFD consumption showed increased levels of glucagon (0.350±0.048 vs. 0.208±0.035 ng/ml, n=7-8; $p<0.05$; Figure 11i) and ghrelin (8.944±0.706 vs. 6.519±0.937 ng/ml, n=8; $p=0.06$; Figure 11j) but no changes in GIP (0.305± 0.036 vs. 0.276± 0.042 ng/ml, n=7-9; Figure 11k) and GLP-1 (0.066± 0.008 vs. 0.067± 0.004 ng/ml, n=7-8; Figure 11l) compared with CTRL HFD.

In summary, the hormones analyzed in the present research work may be regulators of insulin sensitivity on the short-term as well as on the long-term. On the short-term, the increase in the insulin-sensitizing adiponectin may contribute to the higher Rd. Ghrelin has been linked in previous studies to an inhibition of glucose-stimulated insulin secretion/sensitivity, therefore a decrease in ghrelin levels in CVS mice may underline the fact that those animals do not have an overall insulin resistant state. Since glucagon is an activator of gluconeogenesis, the lower levels in glucagon in CVS mice may explain lower basal EGP observed in the clamp.

In all three groups, levels of resistin reflect the state of whole-body insulin sensitivity (GIR) observed in clamps. The increase in resistin and the decrease in adiponectin in CVS HFD mice compared to CTRL HFD are in accordance with previous studies suggesting an imbalances adiponectin:resistin ratio as a key regulator of impaired glucose homeostasis [214]. As well, higher levels of ghrelin and glucagon may be mediators of impaired whole-body insulin sensitivity in that group.

However, adiponectin was also decreased in CVS CHOW mice compared to CTRL CHOW. The decrease of PAI-1 in that group could contribute to the improved whole-body insulin sensitivity since PAI-1 has been linked to obesity and insulin resistance [295, 296].

It is as well important to keep in mind that there may be other hormones beyond the specific data set analyzed in this doctoral thesis that may as well play a role in the regulation of insulin sensitivity by CVS, time and diet.

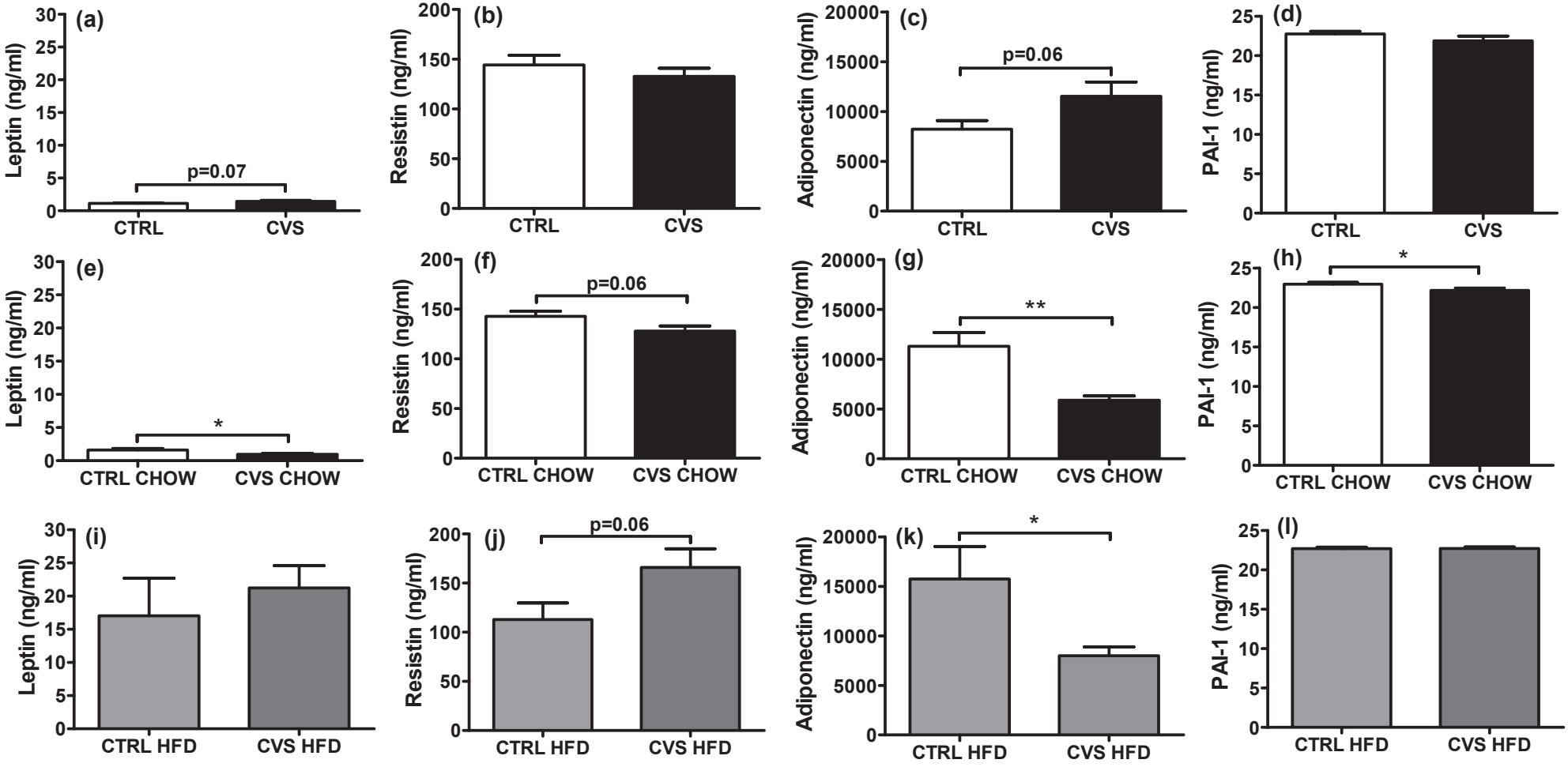


Figure 10: Plasma levels of adipokines in response to CVS and diet. CVS mice showed a trend to higher levels of leptin and adiponectin whereas resistin and PAI-1 were not affected (**a** n = CTRL:CVS= 9:9, **b** n=10:10, **c** n=10:9, **d** n=8:8). Mice exposed to CVS following CHOW consumption showed lower levels of leptin, resistin, adiponectin and PAI-1 (**e** n=CTRL:CVS=11:10, **f** n=10:10, **g** n=11:10, **h** n=9:9). Mice exposed to CVS following HFD consumption showed a trend to higher levels of resistin and lower levels of adiponectin whereas leptin and PAI-1 were not affected (**i** n=CTRL:CVS=8:9, **j** n=7:9, **k** n= 7:8, **l** n=7:9). Results are presented as mean ± SEM, two-tailed unpaired t-test, *p<0.05, **p<0.01.

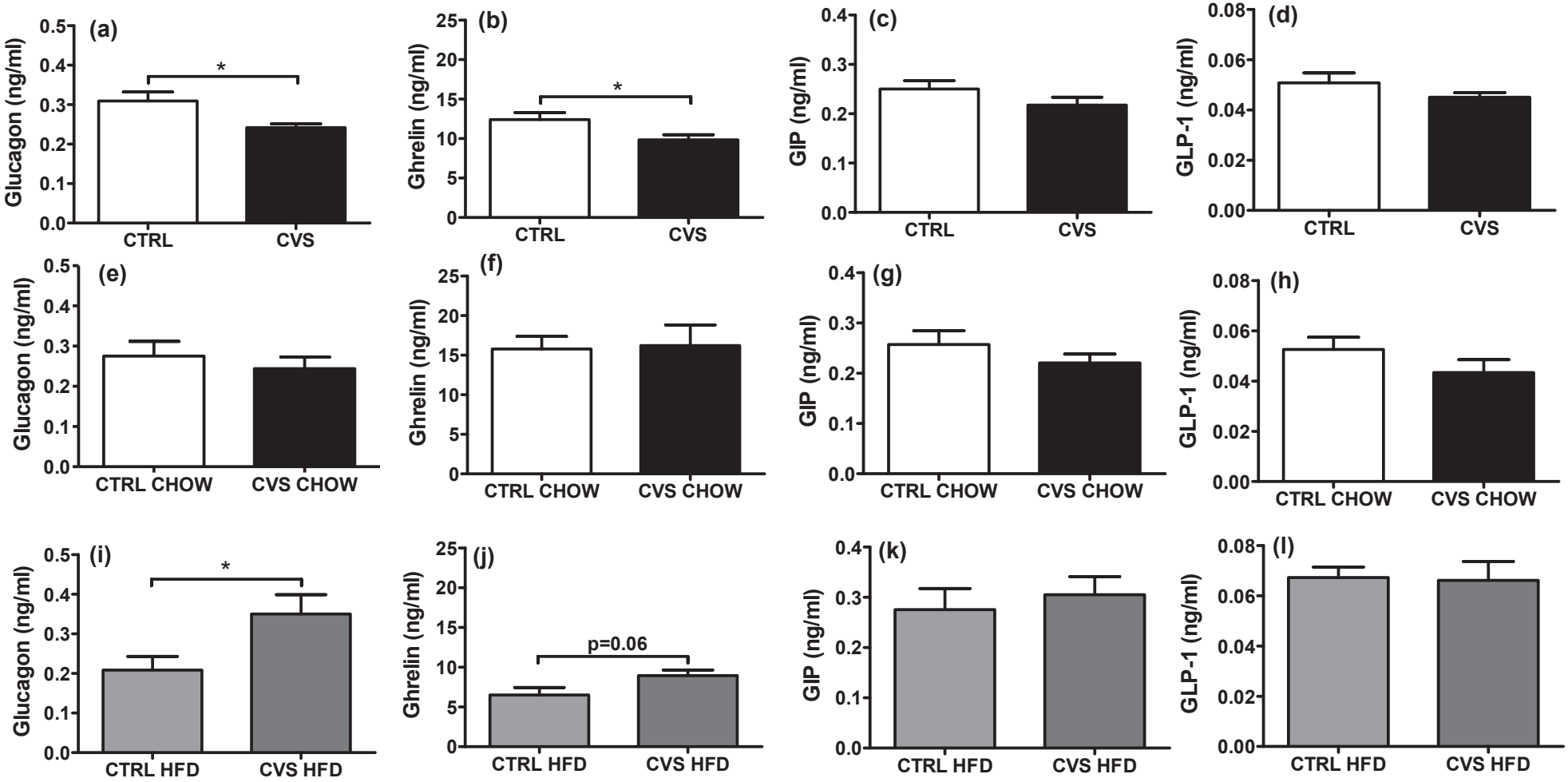


Figure 11: Plasma levels of pancreatic and gastrointestinal hormones in response to CVS and diet. CVS mice showed lower levels of glucagon and ghrelin and no changes in GIP and GLP-1 (**a** n=CTRL:CVS=9:9, **b** n= 8:10, **c** n=10:9, **d** n=10:9). No differences were found between CVS CHOW and CTRL CHOW mice in glucagon, ghrelin, GIP and GLP-1 (**e** n=10:9, **f** n=10:9, **g** n=11:9, **h** n=10:9). CVS HFD mice showed higher levels of glucagon and ghrelin and no changes in GIP and GLP-1 compared to CTRL HFD (**i** n=7:8, **j** n=8:8, **k** n=7:9, **l** n=7:8). Results are presented as mean ± SEM, two-tailed unpaired t-test.,*p<0.05.

3.6 Role of the hypothalamus and the SNS in CVS and diet effects on insulin sensitivity

In animal models, an increase in NPY expression in the hypothalamus has been observed upon GC administration [47, 48] or in response to cold stress [52] supporting a role of NPY in stress coping. In line with these findings, the prevalence for anxiety disorders is higher in humans with polymorphisms in the NPY gene leading to low expression of this neuropeptide [44, 45]. However, plasma NPY levels are lower in PTSD patients and increased in people recovered from PTSD [46]. As mentioned before, the controversy between these studies in animal and clinical models could be due to variations in the time points of measurement, the way of measurement and differences in the models.

Besides NPY, also the expression of AgRP [47, 52] and POMC [53, 55, 56] has been shown to be increased upon GC administration or stress in animal models.

However, there is still a lack of studies to analyze how stress may regulate hypothalamic neuropeptide expression on the long-term also in relation to dietary fat content. In order to address the potential role of those neuropeptides in long-term stress-induced metabolic changes, the expression of *Npy*, *Agrp* and *Pomc* was measured in the three groups.

On the short-term, CVS mice displayed higher expression of *Npy* ($p<0.01$, n=9-10), *Agrp* ($p<0.01$, n=9-11) and *Pomc* ($p<0.001$, n=8-10; Figure 12) compared with CTRL mice. Mice exposed to CVS following CHOW consumption did not show differences in the expression of *Npy*, *Agrp* and *Pomc* (n=12; Figure 12) compared with CTRL CHOW. Mice exposed to CVS following HFD consumption as well did not show differences in the expression of *Npy*, *Agrp* and *Pomc* (n=10-11; Figure 12) compared to CTRL HFD.

Therefore, a general up-regulation of hypothalamic neuropeptides was observed with CVS on the short-term. However on the long-term, no changes in hypothalamic neuropeptide expression were observed independently from the diet.

As mentioned in the introduction of this thesis stress activates the SNS besides the HPA axis. NPY and POMC have been shown to modulate sympathetic activity in the liver and adipose tissue [297] although the data regarding the kind of effect NPY has on SNS activity are not consistent. NPY has been suggested as an activator of sympathetic activity [298-300] and specifically has been shown to be up-regulated with chronic stress [49, 50] whereas there are as well studies proposing NPY as an inhibitor of SNS activity [301]. However, a differential tissue innervation of the SNS by NPY and POMC multisynaptic pathways has to be considered [302, 303].

The contribution of the SNS to CVS and diet effects on insulin sensitivity was studied by analyzing the expression of surrogate parameters for sympathetic activity.

In BAT, the expression of uncoupling protein 1 (*Ucp1*) and adrenergic receptor β3 (*Adrb3*) was analyzed. *Adrb3* expression was decreased with CVS compared to CTRL on the short-

term ($p<0.05$; n=10-11). On the long-term with CHOW consumption, *Adrb3* expression showed a trend to be increased ($p=0.08$; n=9-10). No differences in *Adrb3* expression were found between CVS HFD and CTRL HFD mice (n=8-9; Figure 13a). Same as for *Adrb3* expression, the expression of *Ucp1* was decreased with CVS on the short-term ($p<0.05$, n=9-10), but did not differ between CVS CHOW and CTRL CHOW mice (n=10) nor between CVS HFD and CTRL HFD mice (n=8-9; Figure 13a).

The lower expression of *Ucp1* and *Adrb3* with CVS would indicate a lower sympathetic activity contrary to the activation expected. However, these results would be due to the inhibitory effect of glucocorticoids on adrenergic stimulation in that tissue [304, 305].

Nevertheless, in muscle the expression of uncoupling protein 3 (*Ucp3*), also a surrogate parameter for sympathetic activity proposed to be associated with fatty acid metabolism [306, 307], was increased with CVS ($p<0.05$, n=8-10) on the short-term. On the long-term, no differences between CVS CHOW and CTRL CHOW (n=9-11) or between CVS HFD and CTRL HFD (n=7-8; Figure 13b) were found. These changes would be in line with the changes observed in hypothalamic *Npy* expression.

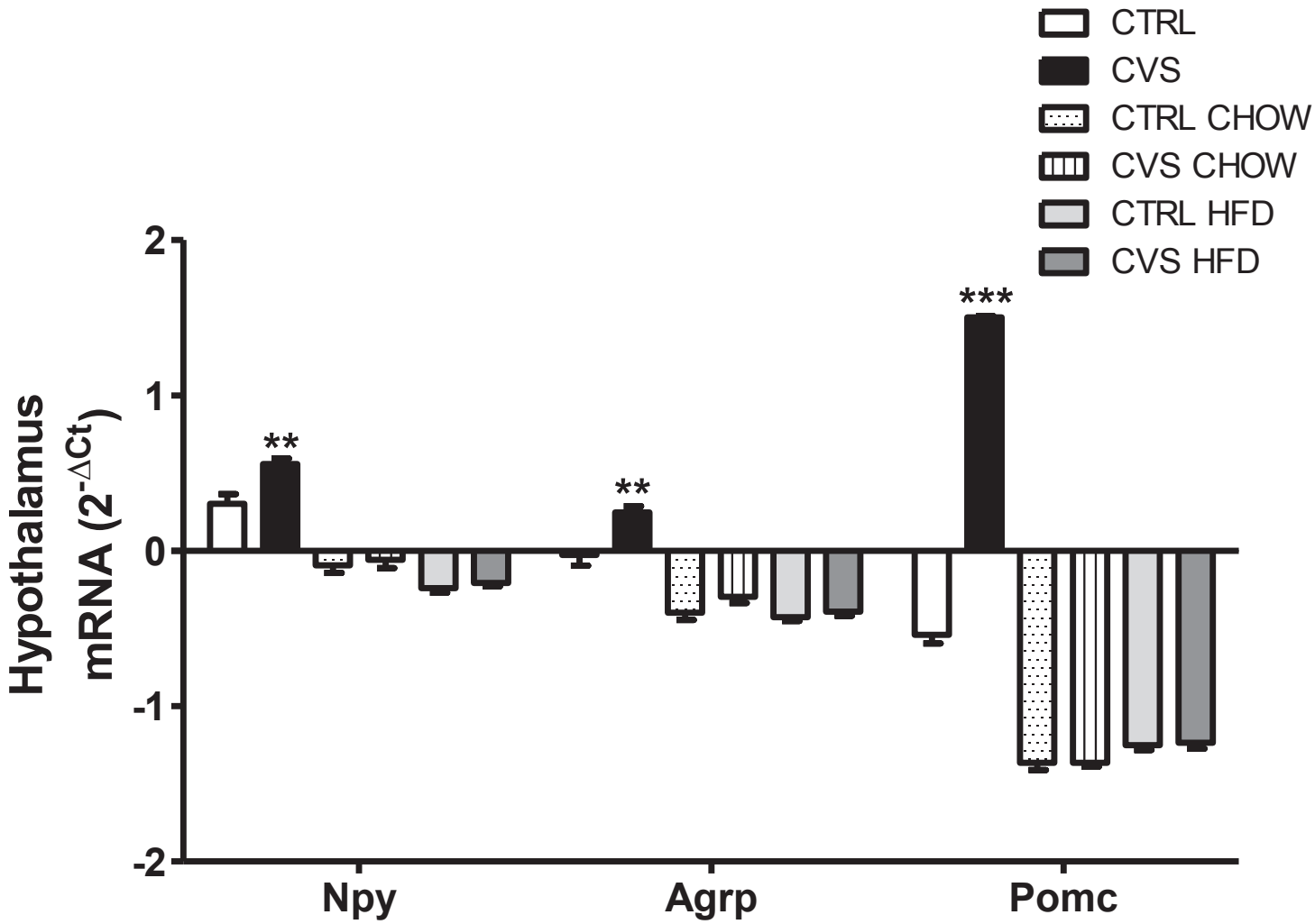


Figure 12: Gene expression of hypothalamic genes in response to CVS and diet. CVS increased the expression of *Npy* (n=CTRL:CVS=9:10), *Agrp* (n=9:11) and *Pomc* (n=8:10) compared to CTRL. CVS mice consuming CHOW did not display differences in *Npy* (n=12:12), *Agrp* (n=12:12) and *Pomc* (n=12:12) compared to CTRL CHOW. CVS mice consuming HFD did not display differences *in Npy* (n=11:11), *Agrp* (n=11:11) and *Pomc* (n=10:11). Results are presented as mean ± SEM, two-tailed unpaired t-test, **p<0.01, ***p<0.001.

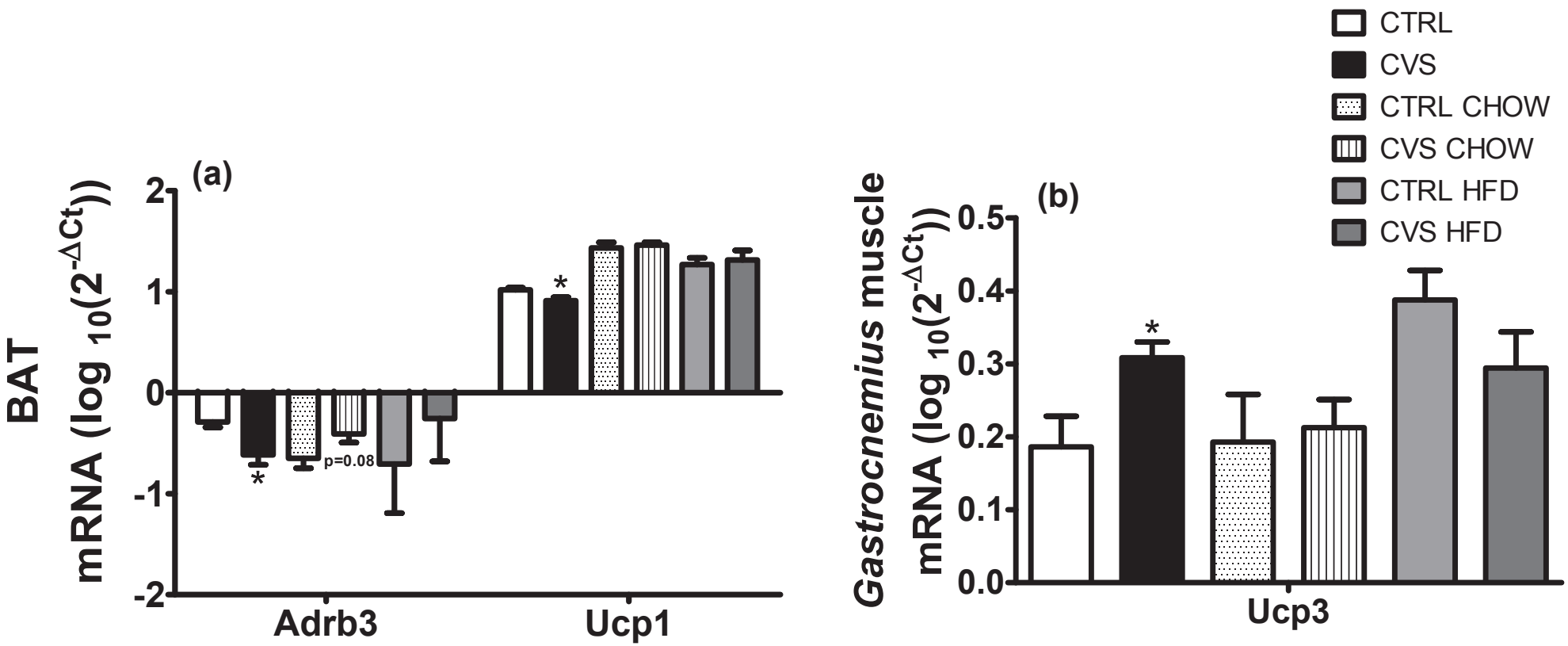


Figure 13: Gene expression of surrogate parameters for sympathetic nervous system (SNS) activity in *gastrocnemius* muscle and BAT. **a** In BAT, CVS decreased the expression of *Adrb3* (n=10:11) and *Ucp1* (n=9:10) compared to CTRL. With CHOW consumption following CVS, *Adrb3* (n=10:9) showed a trend to be increased with no changes in *Ucp1* (n=10:10). Between CVS HFD and CTRL HFD, no differences in *Adrb3* (n=8:9) as well as in *Ucp1* (n=8:9) were found. **b** In *gastrocnemius* muscle, CVS increased the expression of *Ucp3* compared to CTRL (n=CTRL:CVS=10:8). No differences in *Ucp3* expression were found between CVS CHOW and CTRL CHOW (n=CTRL: CVS=11:9) and between CVS HFD and CTRL HFD (n=CTRL:CVS=7:8). Results are presented as mean ± SEM, two-tailed unpaired t-test, *p<0.05.

3.7 Effects of CVS and diet on glucose and lipid metabolism

To further determine the effects of CVS on glucose and lipid metabolism and the regulatory role of dietary fat in time, the expression of gene markers for glucose and lipid metabolism in liver, epididymal WAT and *gastrocnemius* muscle was analyzed.

Given that skeletal muscle is the main user of glucose and lipids as an energy source and in order to elucidate its contribution to the effects of CVS/dietary fat on insulin sensitivity, the studies in this tissue were extended to protein levels analysis.

3.7.1 Liver

It has been shown that both GCs [66, 67] and chronic stress [241] increase glucose output from the liver by an induction of gluconeogenic enzymes. In order to determine under the present experimental conditions how CVS and dietary fat affect gluconeogenesis of the liver and how those observations may underline the results from the clamps, the expression of *Pck1* and *G6pc* as two control points for the regulation of gluconeogenesis was analyzed in liver. CVS mice showed increased expression of *G6pc* compared with CTRL mice ($p<0.05$; n=9-10; Figure 14). No differences in the gene expression between CVS and CTRL were found for *Pck1* (n=10; Figure 14). CVS mice consuming CHOW did not show differences in *Pck1* (n=10; Figure 14) and *G6pc* (n=10; Figure 14) compared to CTRL CHOW (n=11). CVS mice consuming HFD also did not show differences in *Pck1* (n=9) and *G6pc* (n=9) expression compared to CTRL HFD mice (n=8; Figure 14).

Furthermore, GCs and stress may also affect the regulation of glycolysis. The expression of glucokinase (*Gck*) as the first enzyme of glycolysis functioning as a glucose sensor was also analyzed in liver. No differences in *Gck* expression were found between CVS and CTRL mice (n=10) and between CVS HFD and CTRL HFD mice (n=8-9). However, *Gck* expression was increased in the liver of CVS CHOW mice compared with CTRL CHOW mice ($p<0.01$, n=9-11; Figure 14).

Since GCs actions have been linked to hepatic fatty acid synthesis and storage [30, 125, 126], the expression of fatty acid synthase (*Fasn*) was analyzed as well. However, *Fasn* expression did not differ between CVS and CTRL (n=10), neither between CVS CHOW and CTRL CHOW (n=9-10) nor CVS HFD and CTRL HFD (n=8-9; Figure 14).

In the short-term group, the increased expression of *G6pc* is in accordance with the well-described effects of glucocorticoids to increase gluconeogenesis and with the impaired suppression of EGP observed in the clamp in that group indicating liver insulin resistance.

Likewise, since changes in the suppression of EGP seem to be less relevant on the long-term, the expression of key enzymes of gluconeogenesis was not altered compared to

respective CTRL three months after the CVS intervention independent of the diet. However, CVS CHOW mice displayed increased expression of *Gck* suggesting higher glycolytic activity.

3.7.2 Epididymal WAT

Since GCs have been linked both to lipolysis and lipogenesis, the expression of key enzymes involved in those metabolic pathways was studied in epididymal WAT.

Lipoprotein lipase (LPL) is a bifunctional enzyme that is related to hydrolysis of triglycerides as well as to re-esterification and storage of fatty acids [127]. The expression of *Lpl* was increased by CVS compared with CTRL ($p<0.05$, n=9). In mice consuming CHOW following the CVS intervention, the expression of *Lpl* was decreased ($p<0.001$, n=10-11). In CVS HFD mice, the expression of *Lpl* was not altered compared with CTRL HFD (n=8; Figure 15).

The expression of *Fasn* as an enzyme involved in fatty acid synthesis did not differ neither between CVS and CTRL mice (n=8-9) nor between CVS HFD and CTRL HFD mice (n=8-9) but showed a trend to be decreased in CVS CHOW compared with CTRL CHOW mice (n=10-11; Figure 15).

In CVS CHOW mice, the expression of *Lipe* encoding the lipolytic enzyme hormone sensitive lipase (HSL) was increased compared to CTRL CHOW ($p<0.05$, n=10-11). Neither differences in *Lipe* expression were found on the short-term between CVS and CTRL (n=8) nor between CVS HFD and CTRL HFD (n=8-9; Figure 15).

Angiopoietin-like 4 is an inhibitor of LPL activity and its expression has been shown to be induced by GCs [126, 308] and inhibited by insulin [309]. Increased expression of *Angptl4* in WAT and BAT has been observed in diabetic mouse models [310]. Indeed, CVS HFD mice showed increased expression of *Angptl4* ($p<0.01$, n=8-9). On the short-term, CVS did not affect *Angptl4* expression compared with CTRL mice (n=8-9). Likewise, on the long-term, no changes in *Angptl4* expression were found between CVS CHOW and CTRL CHOW mice (n=8-11; Figure 15).

The expression of the fatty acid oxidation marker *Cpt1α* did not differ between CVS and CTRL (n=8-9). Likewise, no changes in *Cpt1α* expression were found between CVS CHOW and CTRL CHOW mice (n=10-11) and between CVS HFD and CTRL HFD mice (n=8-9; Figure 15).

The expression of Chemokine (C-C motif) ligand 2 (CCL2) was analyzed as a marker for inflammation as previous studies showed changes in the regulation of this gene in adipose tissue by CVS and diet [214]. In line with the systemic anti-inflammatory state observed by bioplex measurements, CVS mice showed a decreased expression of *Ccl2* compared with

CTRL mice ($p<0.01$, n=8-9). In CVS HFD mice, the expression of *Ccl2* was increased compared with CTRL HFD ($p<0.05$, n=8-9), whereas no changes were found between CVS CHOW and CTRL CHOW mice (n=10-11; Figure 15).

Thus, in the short-term group, the increase in *Lpl* expression is in line with the described up-regulation of that enzyme in the presence of GCs to induce hypertrophy of adipocytes [127-129] and therefore is also in accordance with the increased fat mass observed in that group. On the long-term with CHOW consumption, the changes in *Lpl, Fasn* and *Lipe* expression suggest decreased adipogenic activity in contrast to the observations on the short-term.

Lower *Ccl2* expression with CVS on the short-term goes in line with the systemic anti-inflammatory state of those animals; in contrast, *Ccl2* expression is increased with CVS an HFD in accordance with previous studies [214]. As well, higher *Aptl4* expression with CVS and HFD is in line with previous reports showing increased *Angptl4* expression in WAT of diabetic mouse models [310].

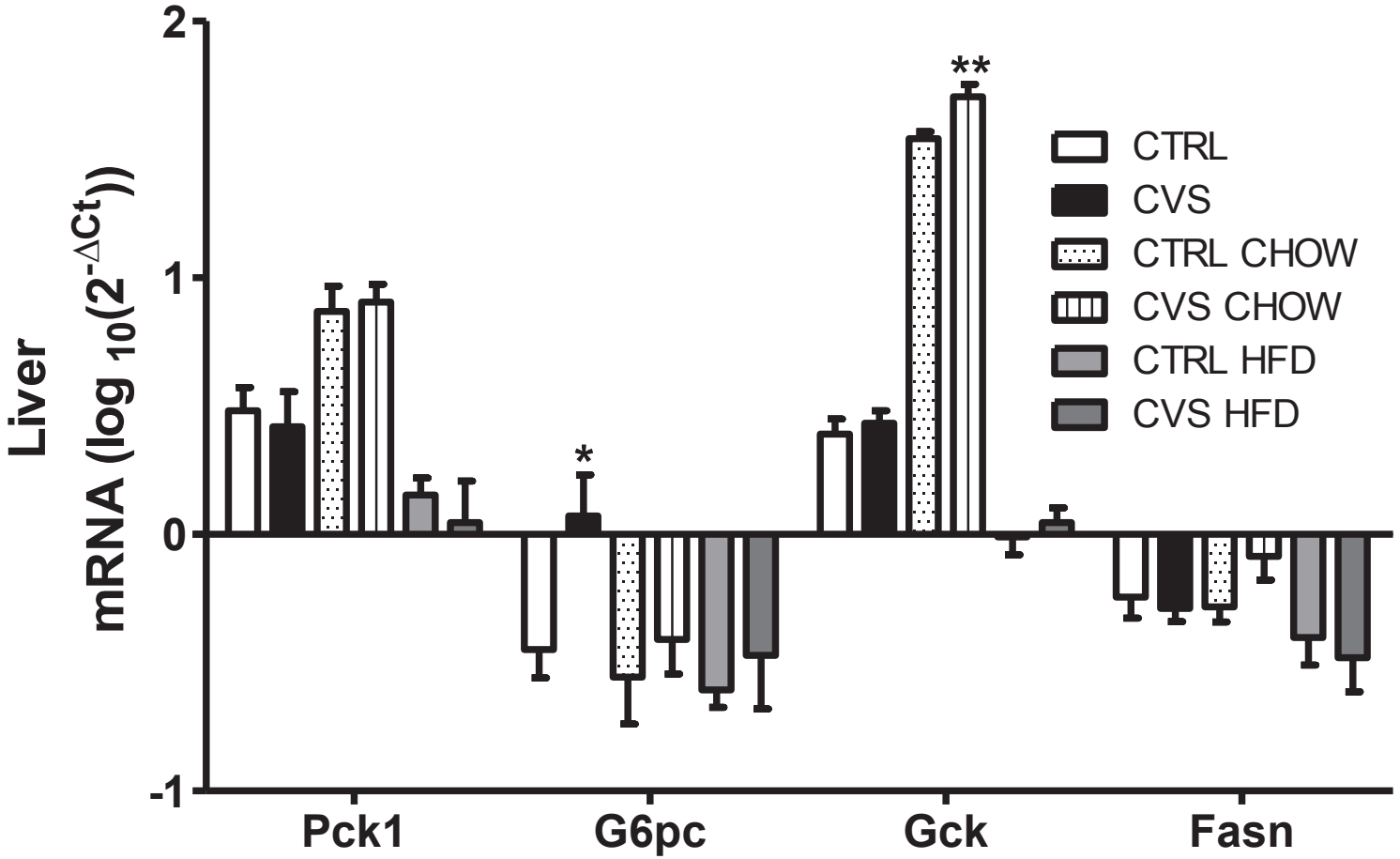


Figure 14: Gene expression profile of liver metabolism genes in response to CVS and diet. CVS increased the expression of *G6pc* (n=CTRL: CVS=9:10) and did not affect the expression of *Pck1* (n=10:10), *Gck* (n=10:10) and *Fasn* (n=10:10). Mice subjected to CVS followed by CHOW consumption showed increased expression of *Gck* (n=11:9) and no differences in *Pck1* (n=11:10), *G6pc* (n=11:10) and *Fasn* (n=10:9). Mice that underwent CVS followed by HFD consumption did not show differences in *Pck1* (n=8:9), *G6pc* (n=8:9), *Gck* (n=8:9) and *Fasn* (n=8:9). Results are presented as mean ± SEM, two-tailed unpaired t-test, *p<0.05, **p<0.01.

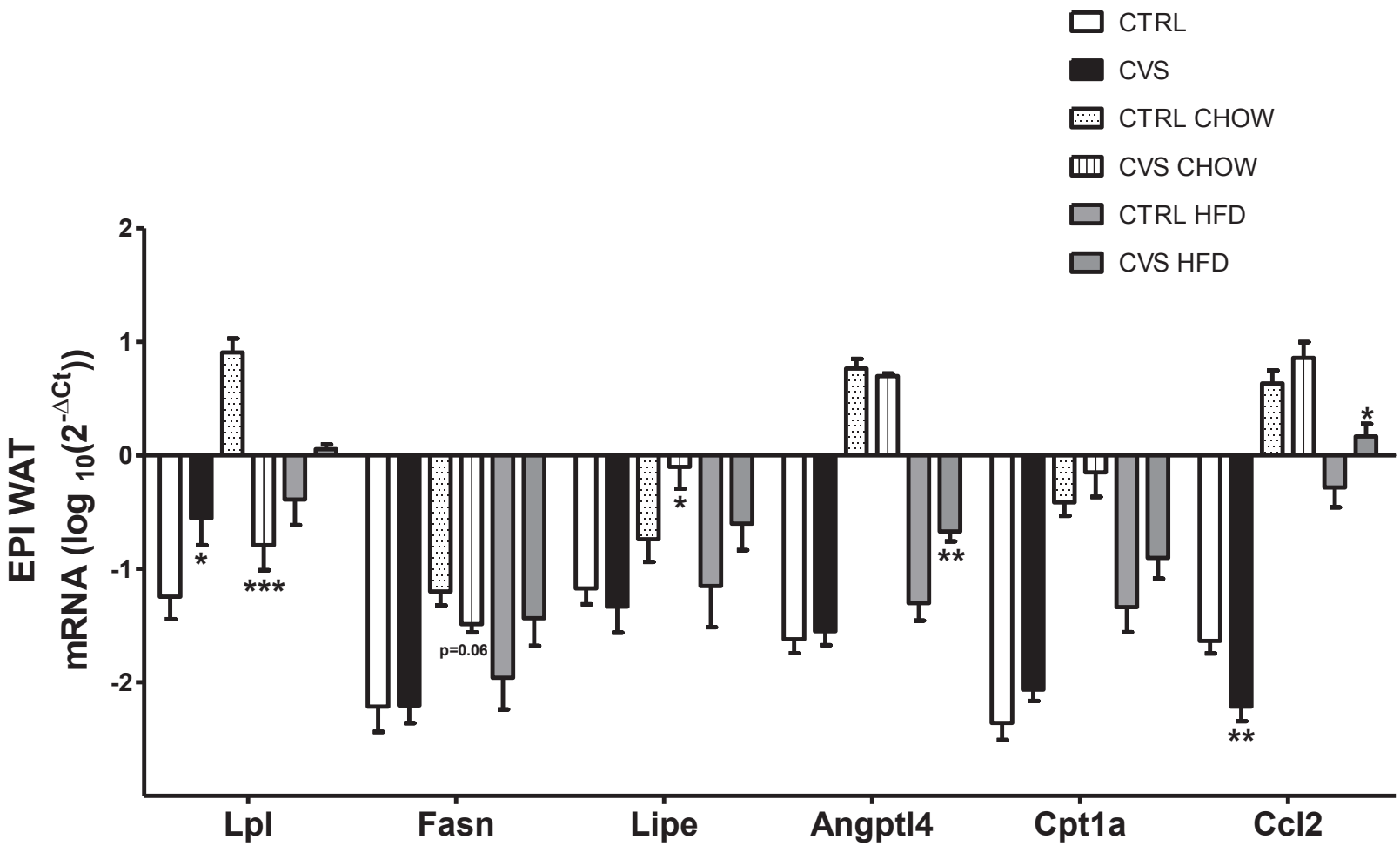


Figure 15: Gene expression profile of epididymal (EPI) WAT metabolism genes in response to CVS and diet. CVS increased *Lpl* (n=CTRL:CVS= 9:9) and decreased *Ccl2* (n=9:8) expression compared to CTRL, whereas *Fasn* (n=9:8), *Lipe* (n=8:8), *Angptl4* (n=9:8) and *Cpt1a* (n=9:8) expression was not affected. CVS CHOW mice showed decreased expression of *Lpl* (n=11:10) and *Fasn* (n=11:10) and increased expression of *Lipe* (n=11:10) whereas expression of *Angptl4* (n=11:8), *Cpt1a* (n=11:10) and *Ccl2* (n=11:10) was not altered compared with CTRL CHOW. CVS HFD mice showed higher expression of *Angptl4* (n=8:9) and *Ccl2* (n=8:9), whereas the expression of *Lpl* (n=8:8), *Fasn* (n=8:9), *Lipe* (n=8:9) and *Cpt1a* (n=8:9) was unaltered compared with CTRL HFD. Results are presented as mean ± SEM, two-tailed unpaired t-test, *p<0.05, **p<0.01, ***p<0.001.

3.7.3 *Gastrocnemius* muscle

The *gastrocnemius* muscle is a type of skeletal muscle containing both glycolytic and oxidative fibers. Therefore, the expression of hexokinase 2 (*Hk2*) as an enzyme implicated in glucose metabolism (glycolysis) and Carnitine palmitoyltransferase 1 (*Cpt1b*) as an enzyme implicated in β-oxidation of long-chain fatty acids was analyzed. As well, *Ucp3* analyzed in relation to SNS activity as presented above has been linked to fatty acid oxidation [306, 307] and mitochondrial biogenesis [311].

After CVS, *Cpt1b* expression was not affected compared to CTRL (n=10), whereas expression of *Ucp3* was increased (Figure 13b, see above). In CVS CHOW mice, expression of *Cpt1b* was increased ($p<0.05$, n=9-11) compared with CTRL CHOW mice whereas in CVS HFD mice, the expression was decreased ($p<0.001$, n=7-8; Figure 16) compared to CTRL HFD.

GCs have also been shown to directly affect muscle glucose metabolism by impairing insulin signaling [78-81] and glucose uptake [91, 92] . To investigate in our model how the gene expression pattern of muscle in response to CVS may reflect actual observations from the clamp, the expression of *Hk2* as the muscle-specific isoform of glucokinase in the liver was determined. *Hk2* expression in *gastrocnemius* muscle neither differed between CVS and CTRL mice (n=10) nor between CVS HFD and CTRL HFD mice (n=7-8). However, CVS CHOW mice showed higher *Hk2* expression compared to CTRL CHOW ($p<0.05$, n=9-10; Figure 16).

Therefore, increased *Ucp3* expression with CVS could indicate higher lipid β-oxidation in muscle besides higher SNS activity although *Cpt1b* expression was unaffected in that group. In CVS CHOW mice, an increase in *Cpt1b* expression was observed and as well could be an indicator for increased lipid β-oxidation in muscle of those mice. Furthermore, in accordance with higher *Gck* expression in the liver, increased expression of *Hk2* in CVS CHOW compared to CTRL CHOW mice suggests higher glycolytic activity with low fat consumption following the CVS intervention. In contrast, the decrease in *Cpt1b* in CVS HFD mice compared to CTRL HFD could indicate lower lipid beta-oxidation with HFD consumption following the CVS intervention.

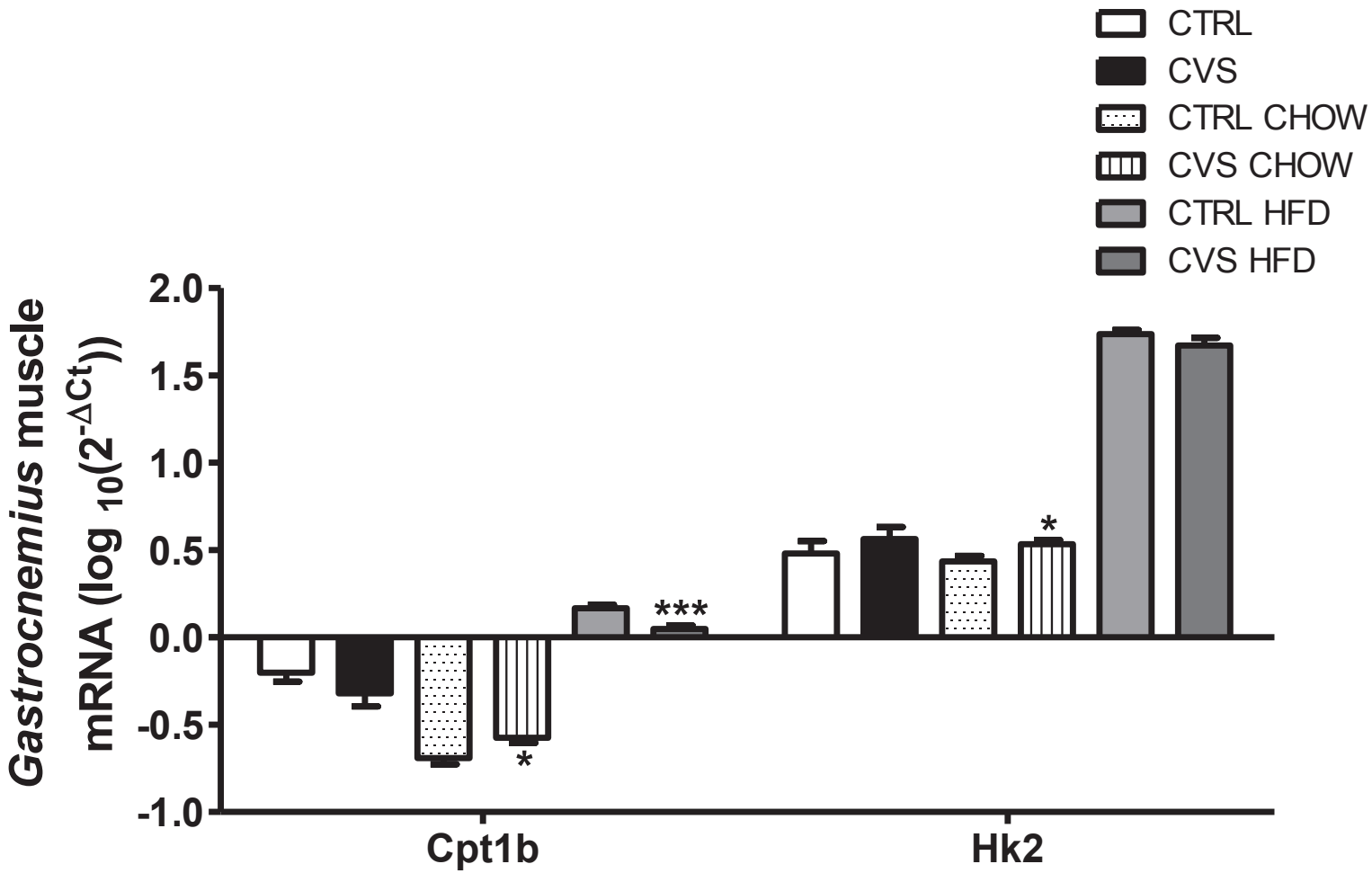


Figure 16: Gene expression profile of skeletal muscle (*gastrocnemius*) metabolism genes in response to CVS and diet. CVS did not affect the expression of *Cpt1β* (n=10:10) and *Hk2* (n=10:10). Mice subjected to CVS followed by CHOW consumption showed increased expression of *Cpt1β* (n=11:9) and *Hk2* (n=10:9) compared to CTRL CHOW mice. Mice subjected to CVS followed by HFD consumption showed decreased expression of *Cpt1β* (n=7:8) and no differences in *Hk2* (n=7:8). Results are presented as mean ± SEM, two-tailed unpaired t-test, *p<0.05, ***p<0.001.

3.8 Analysis of protein markers for nutrient sensing, insulin signaling and glucose metabolism

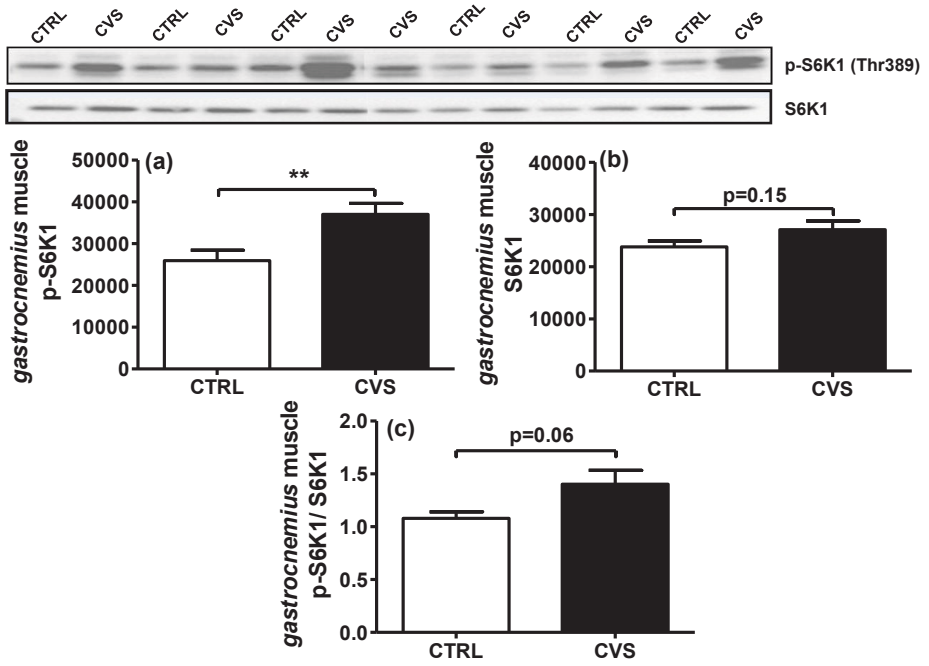
3.8.1 S6K1

The mammalian target of rapamycin (mTOR) and its effector the serine-threonine kinase S6K1 represent important components of insulin signaling. Besides its positive anabolic role in insulin mediated cell growth S6K1 also acts as a rheostat (sensing changes in the activation level of the pathway) by suppressing insulin signaling under conditions of hyperactivation of the mTOR complex 1 (mTORC1) pathway. The mTORC1/S6K1 pathway has not only been suggested to link nutrient sensing with the molecular control of energy metabolism [312] but has also been linked to stress with inconsistent results: Chronic restraint stress has been shown in the hippocampus to increase protein levels of mTORC1 [313] whereas on the contrary, dexamethasone decreased mTORC1 signaling *in vitro* in muscle cells (L6 myoblasts) [314].

In order to investigate in the present experimental model how the mTORC1/S6K1 pathway may be involved in the alterations of insulin sensitivity with CVS and diet, the protein levels of S6K1 were analyzed in skeletal muscle.

On the short-term, CVS increased the total levels (raw data after quantification without normalization to any other protein) of p-S6K1 ($p<0.01$, n=6-7; Figure 17a) and the levels of p-S6K1 in relation to S6K1 ($p=0.06$, n=6-7; Figure 17c) with no changes in the total levels of S6K1 (n=6-7; Figure 17b) compared with CTRL mice. When consuming CHOW following the CVS intervention, the levels of p-S6K1 in relation to GAPDH were not altered compared to CTRL CHOW (n=6; Figure 17d), whereas the levels of S6K1 in relation to GAPDH showed a trend to increase ($p=0.08$, n=6; Figure 17e) and levels of p-S6K1 in relation to S6K1 and GAPDH showed a trend to decrease ($p=0.08$, n=6; Figure 17f). When consuming HFD subsequent to the CVS intervention, neither the levels of p-S6K1 in relation to GAPDH (n=7; Figure 17g), nor the levels of S6K1 in relation to GAPDH (n=7; Figure 17h) nor the levels of p-S6K1 in relation to S6K1 and GAPDH (n=7; Figure 17i) were altered compared to CTRL HFD mice.

Since the mTORC1/S6K1 pathway is implicated in cell growth, the increase in p-S6K1 levels with CVS on the short-term may reflect the high need for muscle rebuilding in response to elevated levels of corticosterone during the CVS intervention. In contrast, the decrease in p-S6K1 with CHOW consumption following the CVS intervention may suggest increased insulin sensitivity since S6K1 negatively regulates insulin receptor substrate 1 in order to protect the system from an excess of insulin-stimulated nutrient uptake and therefore from a nutrient overload [312].



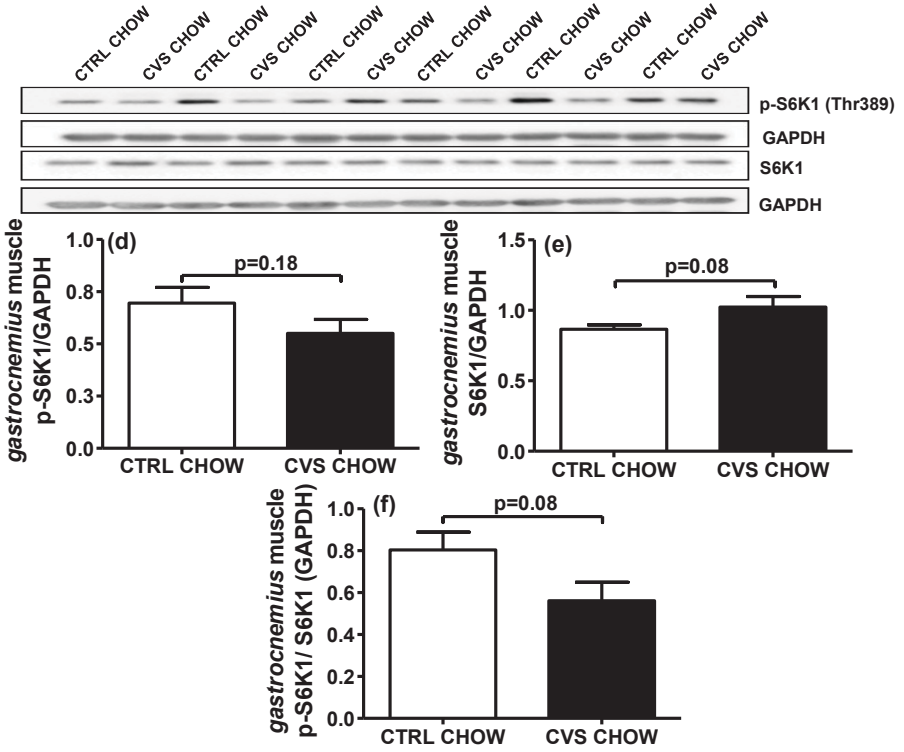
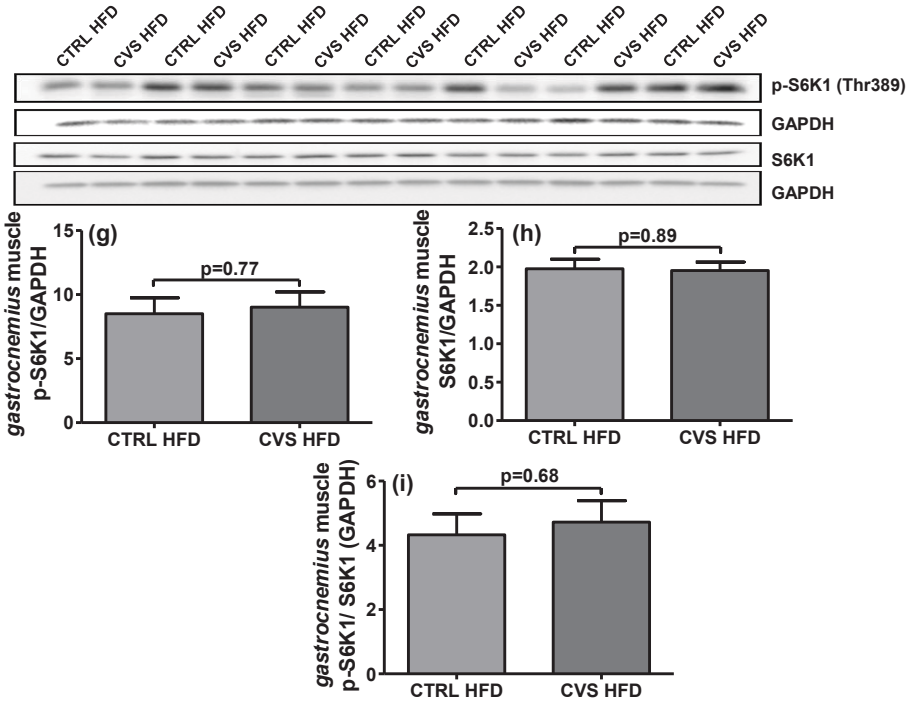


Figure 17: Abundance and phosphorylation (Thr389) of S6K1 in *gastrocnemius* muscle in response to CVS and diet. CVS (n=CTRL: CVS=6:7) increased the total levels of p-S6K1 (**a**) without altering the total levels of S6K1 (**b**) whereas the levels of p-S6K1 in relation to S6K1 tended to increase **(p=0.06)** (**c**). CVS following CHOW consumption (n=CTRL: CVS=6:6) did not alter the total levels of p-S6K1 (**d**) but tended to increase the total levels of S6K1 in relation to GAPDH **(p=0.08)** (**e**) and to decrease the levels of p-S6K1 in relation to S6K1 and GAPDH **(p=0.08)** (**f**). CVS following HFD consumption (n=CTRL:CVS=7:7) did not alter the levels of p-S6K1 in relation to GAPDH (**g**) likewise the levels of S6K1 in relation to GAPDH (**h**) and the levels of p-S6K1 in relation to S6K1 and GAPDH (**i**). Results are represented as mean ± SEM, two-tailed unpaired t-test, **p<0.01.

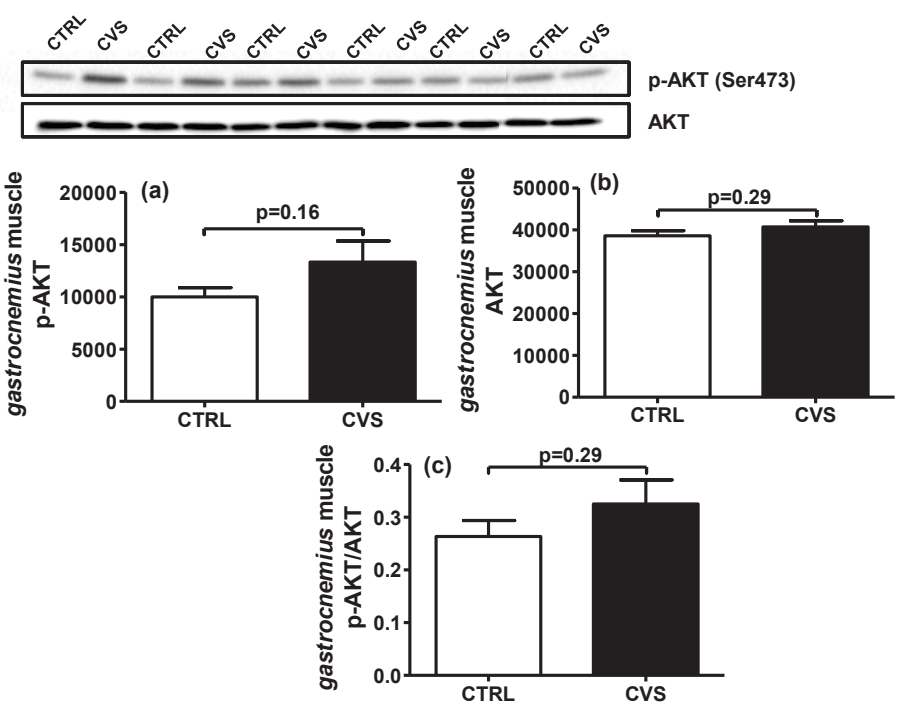


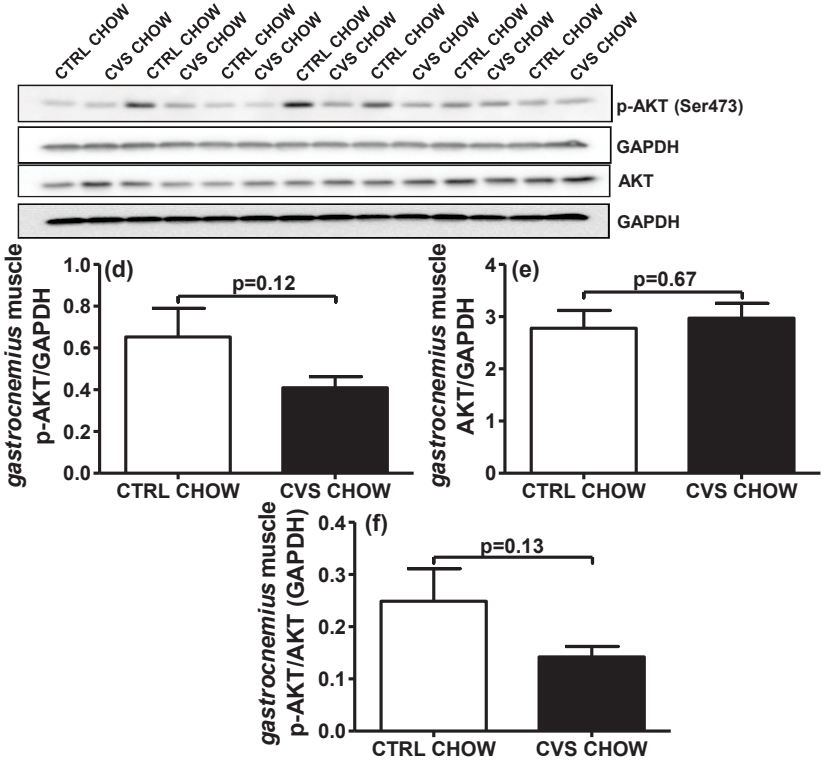
3.8.2 AKT

The protein kinase B/AKT is a serine/threonine-specific protein with a key role in metabolic processes like cell growth, survival, proliferation and metabolism [315]. One key function of AKT in metabolism is the regulation of insulin-stimulated glucose uptake in both muscle and adipose tissue via activation of AS160 and subsequent translocation of GLUT4 to the cell membrane [315]. Decreased insulin-stimulated AKT activity has been shown to lead to impaired GLUT4 translocation and therefore insulin resistance in muscle and adipose tissue of db/db mice and insulin-stimulated maximal AKT activity has been described to be reduced in type 2 diabetic patients [316]. However, also AKT-independent pathways for insulin action have been proposed [317, 318].

Considering the key role of AKT in functional insulin signaling and glucose metabolism in muscle and potential alterations with CVS and diet, levels of AKT were analyzed in the three experimental groups.

On the short-term, CVS did not alter the total levels (raw data after quantification without normalization to any other protein) of p-AKT (n=6; Figure 18a) likewise the total levels of AKT (n=6; Figure 18b) and the levels of p-AKT in relation to AKT (n=6; Figure 18c). When consuming CHOW following the CVS intervention, the levels of p-AKT in relation to GAPDH were not altered compared with CTRL CHOW mice (n=7; Figure 18d), as well as the levels of AKT in relation to GAPDH (n=7; Figure 18e) and the levels of p-AKT in relation to AKT and GAPDH (n=7; Figure 18f). Also with CVS and subsequent HFD consumption, p-AKT in relation to GAPDH was unaltered compared with CTRL HFD mice (n=7; Figure 18g), likewise the levels of AKT in relation to GAPDH (n=7; Figure 18h) and the levels of p-AKT in relation to AKT and GAPDH (n=7; Figure 18i).





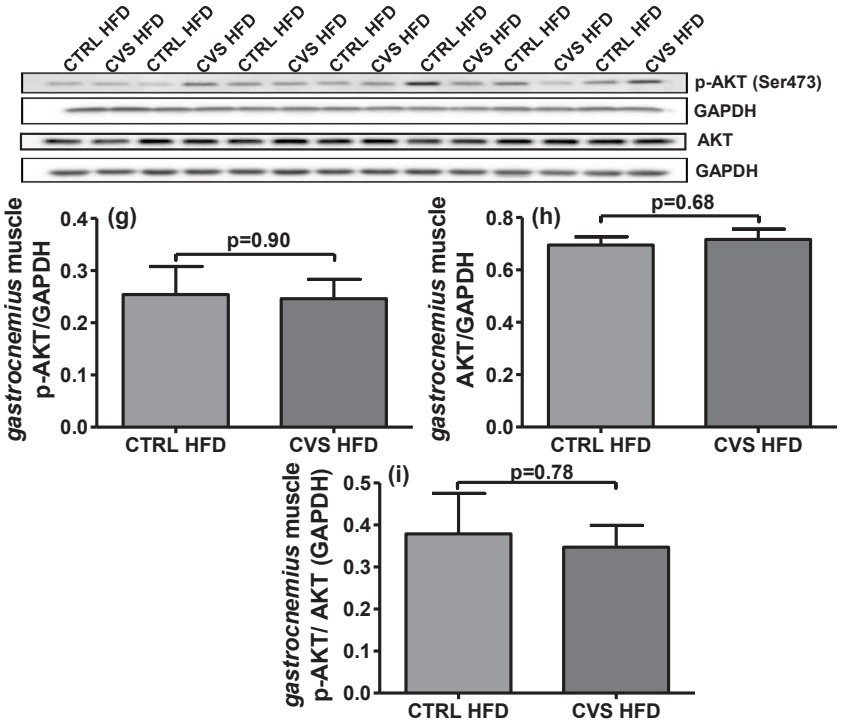


Figure 18: Abundance and phosphorylation (Ser473) of AKT in *gastrocnemius* muscle in response to CVS and diet. CVS (n=CTRL:CVS=6:6) did not alter the total levels of p-AKT (**a**) likewise the total levels of AKT (**b**) and the levels of p-AKT in relation to AKT (**c**) compared with CTRL mice. CVS following CHOW consumption (n=CTRL:CVS=7:7) did not alter the levels of p-AKT in relation to GAPDH (**d**) likewise the levels of AKT in relation to GAPDH (**e**) and the levels of p-AKT in relation to AKT and GAPDH (**f**). CVS following HFD consumption (n=CTRL:CVS=7:7) did not alter the levels of p-AKT in relation to GAPDH (**g**) likewise the levels of AKT in relation to GAPDH (**h**) and the levels of p-AKT in relation to AKT and GAPDH (**i**). Results are presented as mean ± SEM, two-tailed unpaired t-test.

3.8.3 Glucose transporters

Finally, the basal cellular distribution of GLUT4 and GLUT1 as mediators of glucose transport was analyzed in the three experimental conditions.

On the short-term, the total levels (raw data after quantification without normalization to any other protein) of plasma membrane GLUT4 were not altered compared to CTRL mice (n=7, Figure 19a) likewise microsomal GLUT4 in relation to GAPDH (n=7; Figure 19c). However, the total levels of plasma membrane 1α Na^+K^+ ATPase tended to increase compared to CTRL mice (p=0.09, n=7; Figure 19b). The total levels of plasma membrane GLUT1 were not altered compared to CTRL mice (n=6-7; Figure 19d) as well as microsomal GLUT1 in relation to GAPDH (n=6-7; Figure 19f) and the total levels of plasma membrane 1α Na^+K^+ ATPase (n=6-7; Figure 19e).

When the animals were consuming CHOW following the CVS intervention, plasma membrane GLUT4 in relation to 1α Na^+K^+ ATPase was not altered compared with CTRL CHOW mice (n=6-7; Figure 20a). Likewise, microsomal GLUT4 in relation to GAPDH was not altered compared to CTRL CHOW mice (n=6-7; Figure 20b). However, the GLUT4 plasma membrane:microsome ratio showed a trend to be increased compared to CTRL CHOW mice (p=0.06, n=6-7; Figure 20c).

Plasma membrane GLUT1 in relation to 1α Na^+K^+ ATPase was not altered compared to CTRL CHOW mice (n=5-6; Figure 20d) as well as microsomal GLUT1 in relation to GAPDH (n=5-6; Figure 20e) and the GLUT1 plasma membrane:microsome ratio (n=5-6; Figure 20f).

When the animals were consuming HFD following the CVS intervention, plasma membrane GLUT4 in relation to 1α Na^+K^+ ATPase was not altered compared to CTRL HFD mice (n=7; Figure 21a) likewise microsomal GLUT4 in relation to GAPDH (n=7; Figure 21b) and the GLUT4 plasma membrane:microsome ratio (n=7; Figure 21c).

Plasma membrane GLUT1 in relation to 1α Na^+K^+ ATPase was not altered compared to CTRL HFD mice (n=6-7; Fig. 21d) likewise microsomal GLUT1 in relation to GAPDH (n=6-7; Fig. 21e) and the GLUT1 plasma membrane:microsome ratio (n=6-7; Fig. 21f).

In summary, CVS did not cause striking changes in skeletal muscle glucose transport whereas alterations could be found in the levels of 1α Na^+K^+ ATPase, probably due to SNS activity. Similarly, on the long-term and independently from the diet, no striking changes in glucose transport were observed with the exception of a trend to higher GLUT4 plasma membrane distribution in CVS CHOW mice. Therefore, in the present physiological model for CVS in contrast to pharmacological studies, glucose metabolism at the level of skeletal muscle glucose transport may not play a key role regarding changes in insulin sensitivity.

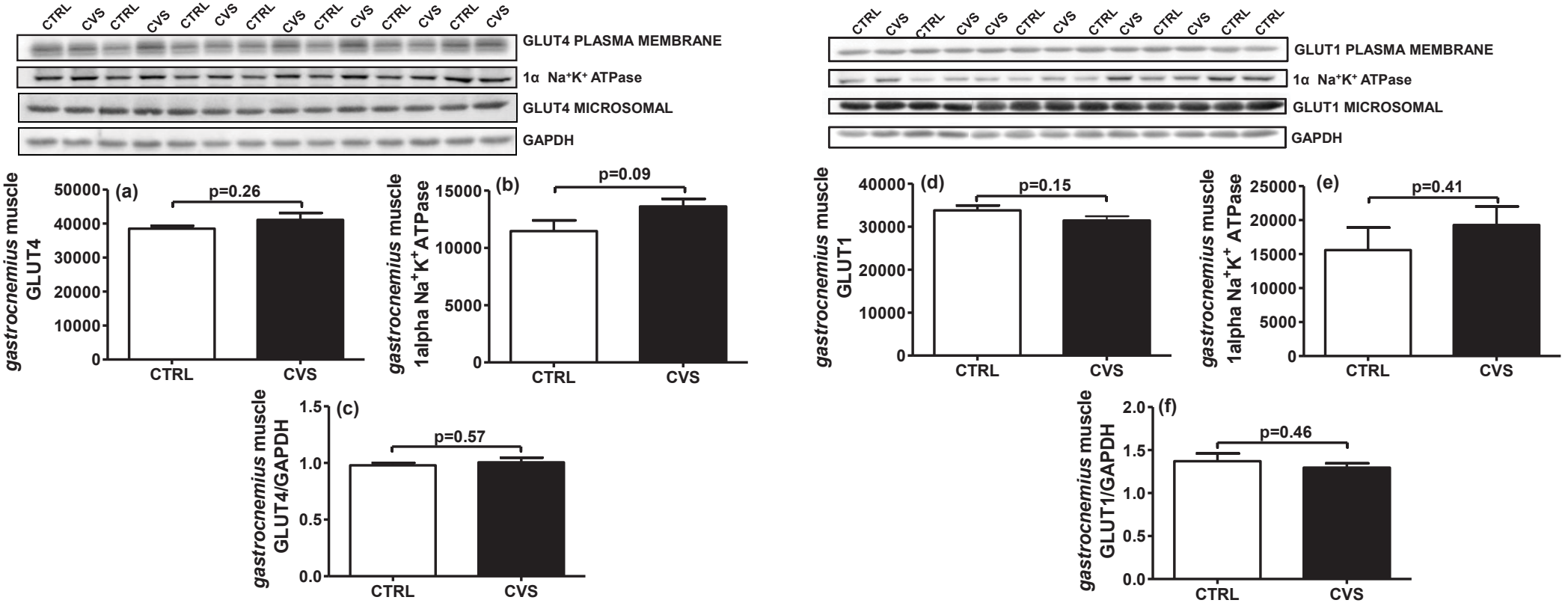


Figure 19: Basal GLUT4 and GLUT1 cellular distribution in *gastrocnemius* muscle in response to CVS. CVS (n=CTRL: CVS=7:7) did not alter in the plasma membrane the total levels of GLUT4 (**a**), slightly increased the total levels of plasma membrane 1α Na^+K^+ATPase **(p=0.09)** (**b**) and did not alter in the microsomal fraction the levels of GLUT4 in relation to GAPDH (**c**). CVS (n=CTRL:CVS=7:6) did not alter in the plasma membrane the total levels of GLUT1 (**d**) and of 1α Na^+K^+ATPase (**e**) and as well did not alter in the microsomal fraction the levels of GLUT4 in relation to GAPDH (**f**). Results are represented as mean ± SEM, two-tailed unpaired t-test.

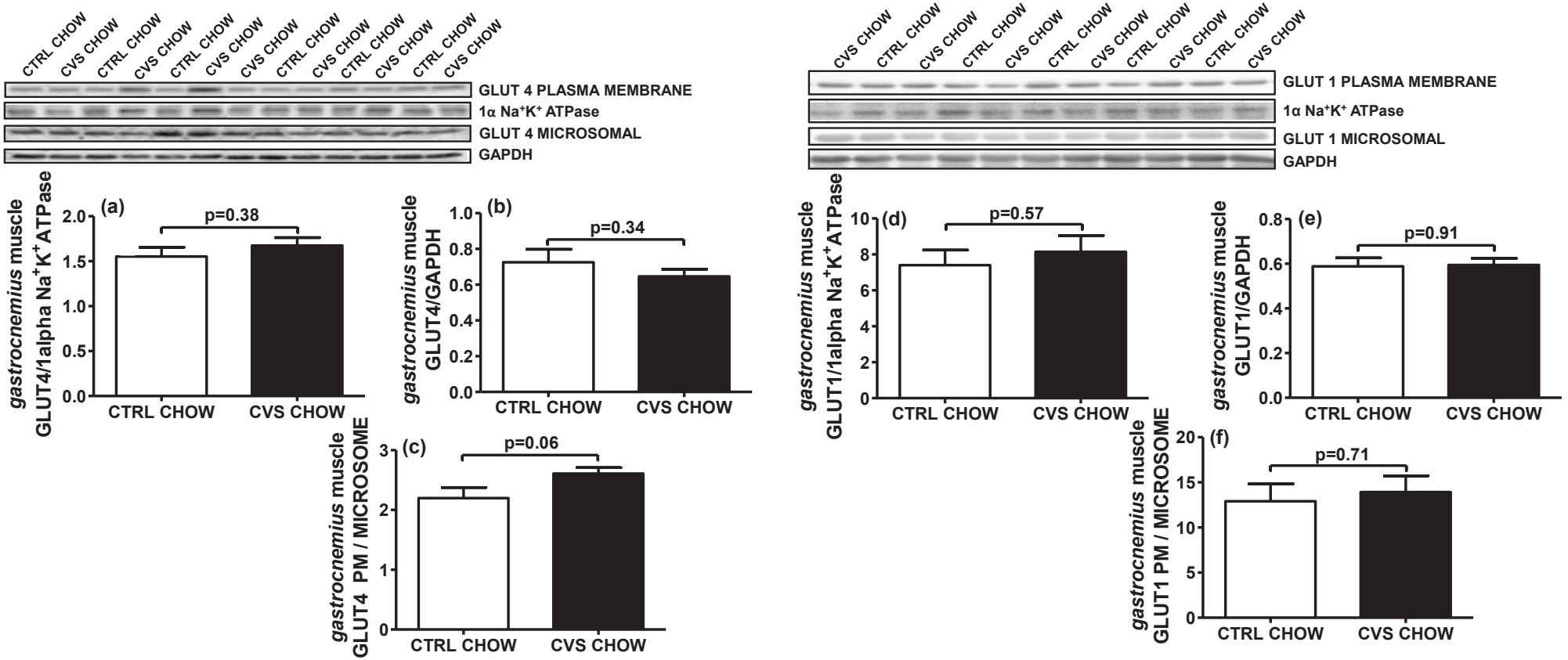


Figure 20: Basal GLUT4 and GLUT1 cellular distribution in *gastrocnemius* muscle in response to CVS and CHOW consumption. CVS following CHOW consumption (n=CTRL:CVS=6:7) did not alter in the plasma membrane the levels of GLUT4 in relation to 1α Na^+Ka^+ATPase (**a**) likewise in the microsomal fraction the levels of GLUT4 in relation to GAPDH (**b**) but slightly increases the GLUT4 plasma membrane (**PM**) : microsome ratio (**p=0.06**) (**c**). CVS and CHOW consumption (n=CTRL:CVS=5:6) did not alter in the plasma membrane the levels of GLUT1 in relation to 1α Na^+Ka^+ATPase (**d**) likewise in the microsomal fraction the levels of GLUT1 in relation to GAPDH (**e**) and the GLUT1 PM : microsome ratio (**f**). Results are represented as mean ± SEM, two-tailed unpaired t-test.

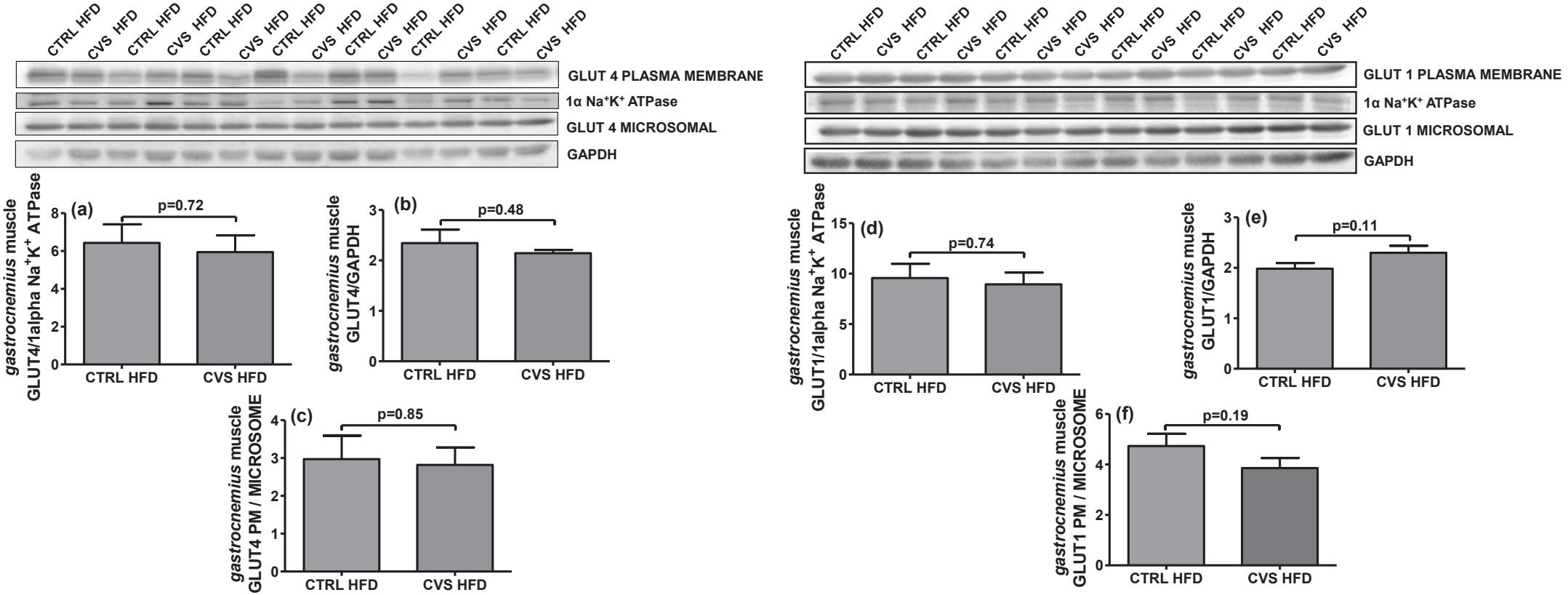


Figure 21: Basal GLUT4 and GLUT1 cellular distribution in *gastrocnemius* muscle in response to CVS and HFD consumption. CVS following HFD consumption (n=CTRL:CVS=7:7) did not alter in the plasma membrane the levels of GLUT4 in relation to 1α Na^+Ka^+ATPase (**a**) as well as in the microsomal fraction the levels of GLUT4 in relation to GAPDH (**b**) and the GLUT4 plasma membrane (PM) : microsome ratio (**c**). CVS and HFD consumption (n=CTRL:CVS=6:7) did not alter in the plasma membrane the levels of GLUT1 in relation to 1α Na^+Ka^+ATPase (**d**) likewise in the microsomal fraction the levels of GLUT1 in relation to GAPDH (**e**) and the GLUT1 PM : microsome ratio (**f**). Results are represented as mean ± SEM, two tailed unpaired t-test.

3.9 Analysis of protein markers of fatty acid oxidation

Although various studies have been analyzing the effects of stress hormones on glucose and lipid metabolism with emphasis on the adipose tissue, GCs were also shown to affect mitochondrial function [261]. Since mitochondria are more abundant in skeletal muscle than in WAT, the effects of GCs and catecholamines on lipid metabolism can be extended to that tissue, which contribution to insulin sensitivity regulation is a current discussion topic [319, 320].

AMPK is the key regulator of lipid metabolism in skeletal muscle and is involved in the regulation of fatty acid transport into mitochondria [107] along with acetyl-CoA carboxylase (ACC) and carnitinepalmitoyl-transferase 1 beta (CPT1β) [107] for subsequent β-oxidation. More in detail, AMPK phosphorylates and inactivates ACC which results in a decrease in the levels of malonyl-CoA as an allosteric inhibitor of CPT1β [321]. In consequence, fatty acid uptake into mitochondria for subsequent β-oxidation is enhanced [107]. Dysregulation of lipid metabolism in skeletal muscle may contribute to metabolic disorders like obesity [322] and insulin resistance [323], also stress may be an environmental factor to contribute to a dysregulation of fatty acid metabolism.

To determine how CVS and diet may affect key enzymes of fatty acid oxidation and therefore to go beyond the gene expression analysis, protein levels of (phosphorylated) AMPK and ACC were investigated.

3.9.1 AMPK

On the short-term, CVS did not significantly alter the total levels (raw data after quantification without normalization to any other protein) of p-AMPK compared with CTRL mice (n=7; Figure 22a). The total levels of AMPK were increased compared with CTRL mice ($p<0.001$, n=7; Figure 22b). The levels of p-AMPK in relation to AMPK were unaltered by CVS (n=7; Figure 22c). With CHOW consumption following the CVS intervention, neither the levels of p-AMPK in relation to GAPDH (n=7; Figure 22d) nor the levels of AMPK in relation to GAPDH (n=7; Figure 22e) nor the levels of p-AMPK in relation to AMPK and GAPDH (n=7; Figure 22f) showed differences between CVS CHOW and CTRL CHOW mice. When the animals were consuming HFD subsequent to the CVS intervention, neither changes were observed in the levels of p-AMPK in relation to GAPDH (n=6-7; Figure 22g), nor in the levels of AMPK in relation to GAPDH (n=6-7; Figure 22h) nor in the levels of p-AMPK in relation to AMPK and GAPDH (n=6-7; Figure 22i).

3.9.2 ACC

On the short-term, CVS increased the total levels (raw data after quantification without normalization to any other protein) of p-ACC ($p<0.05$, n=7; Figure 23a) as well as of ACC ($p<0.01$, n=7; Figure 23b). Like for the GLUTs in the membrane fraction, the 1α Na^+K^+ ATPase was tested as a housekeeping protein. However, like for the GLUTs in that experimental condition, total levels of that protein were increased compared to CTRL mice ($p<0.001$, n=7; Figure 23c and d) potentially due to SNS activity. For that reason, 1α Na^+K^+ ATPase was not used for normalization same as unphosphorylated ACC being increased by CVS as well (Figure 23b). When the animals were consuming CHOW following the CVS intervention, neither the levels of p-ACC in relation to GAPDH (n=7; Figure 23e) nor the levels of ACC in relation to GAPDH (n=7; Figure 23f) nor the levels of p-ACC in relation to ACC and GAPDH (n=7; Figure 23g) differed between CVS CHOW and CTRL CHOW mice. As well with HFD consumption subsequent to the CVS intervention, neither the levels of p-ACC normalized by GAPDH (n=6-7; Figure 23h) nor the levels of ACC normalized by GAPDH (n=6-7; Figure 23i) nor the levels of p-ACC normalized by ACC and GAPDH (n=6-7; Figure 23j) were altered in CVS HFD compared with CTRL HFD mice.

The results in p-ACC/ACC and AMPK analysis with CVS on the short-term suggest an increase in lipid β-oxidation in muscle. On the long-term independently from the diet, no changes between CVS and CTRL mice were observed in the levels of those proteins. However, changes in *Cpt1b* expression and plasma triglycerides suggest as well changes in lipid β-oxidation being increased with CHOW consumption and decreased with HFD consumption, potentially through alternative routes of lipid metabolism regulation by GCs such as peroxisome proliferator-activated receptors (PPARs) [324, 325].

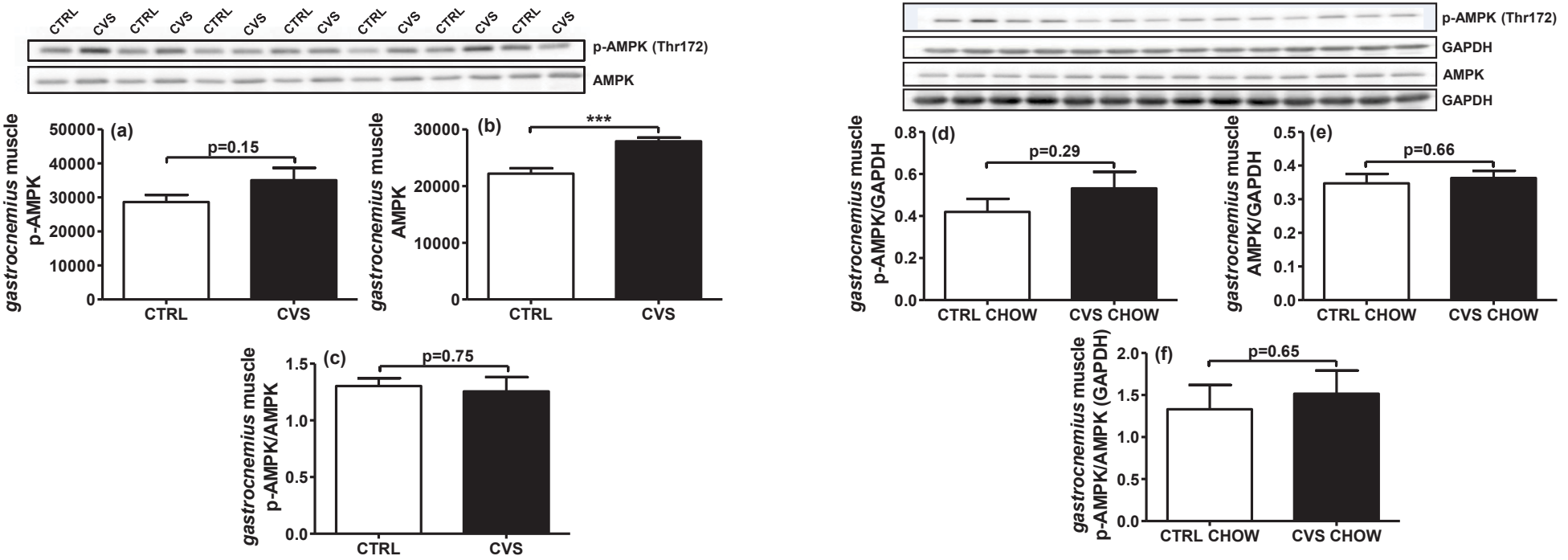


Figure 22: Abundance and phosphorylation (Thr172) of AMPK in *gastrocnemius* muscle in response to CVS and diet. CVS (n=CTRL:CVS=7:7) did not alter the total levels of p-AMPK (**a**) but increased the total levels of AMPK (**b**) with no changes in the levels of p-AMPK in relation to AMPK (**c**). CVS following CHOW consumption (n=CTRL:CVS=7:7) did not alter the levels of p-AMPK in relation to GAPDH (**d**) as well as the levels of AMPK in relation to GAPDH (**e**) and the levels of p-AMPK in relation to AMPK and GAPDH (**f**). CVS following HFD consumption (n=CTRL:CVS=7:6) did not alter the levels of p-AMPK in relation to GAPDH (**g**) likewise the levels of AMPK in relation to GAPDH (**h**) nor the levels of p-AMPK in relation to AMPK and GAPDH (**i**). Results are represented as mean ± SEM, two-tailed unpaired t-test, ***p<0.001.

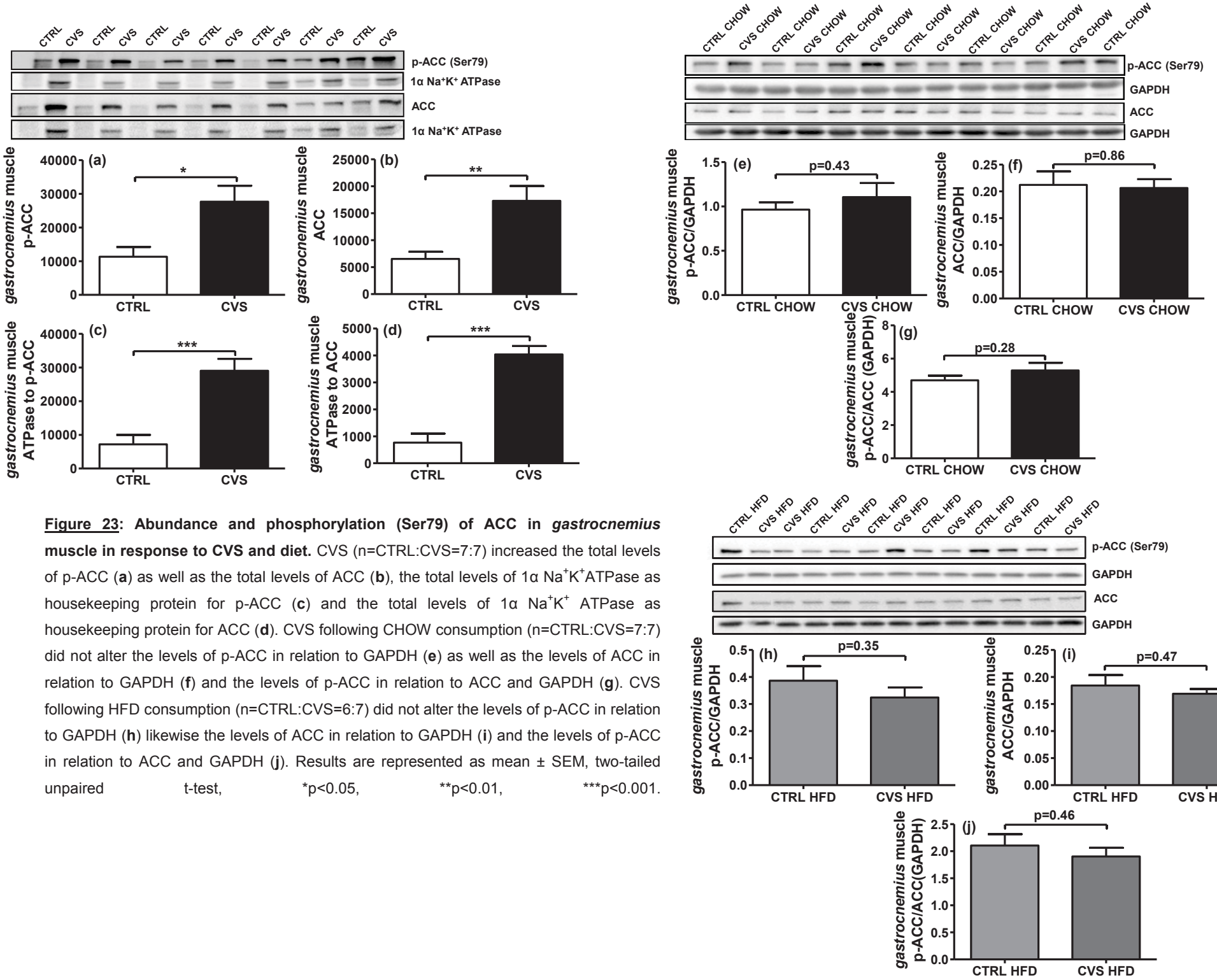


Figure 23: Abundance and phosphorylation (Ser79) of ACC in *gastrocnemius* muscle in response to CVS and diet. CVS (n=CTRL:CVS=7:7) increased the total levels of p-ACC (**a**) as well as the total levels of ACC (**b**), the total levels of 1α Na^+K^+ATPase as housekeeping protein for p-ACC (**c**) and the total levels of 1α Na^+K^+ ATPase as housekeeping protein for ACC (**d**). CVS following CHOW consumption (n=CTRL:CVS=7:7) did not alter the levels of p-ACC in relation to GAPDH (**e**) as well as the levels of ACC in relation to GAPDH (**f**) and the levels of p-ACC in relation to ACC and GAPDH (**g**). CVS following HFD consumption (n=CTRL:CVS=6:7) did not alter the levels of p-ACC in relation to GAPDH (**h**) likewise the levels of ACC in relation to GAPDH (**i**) and the levels of p-ACC in relation to ACC and GAPDH (**j**). Results are represented as mean ± SEM, two-tailed unpaired t-test, *p<0.05, **p<0.01, ***p<0.001.

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4 Discussion

Although stress hormones have an impact on glucose and lipid metabolism and stress has been discussed as a risk factor for metabolic disorders, the physiology of stress on metabolism beyond its acute effects is still poorly understood. Epidemiological studies in humans link chronic stress and post-traumatic stress disorder (PTSD) with diabetes onset but those studies are restricted regarding analysis of the overall mechanisms behind. Studies in animal models to analyze the effects of chronic stress on glucose homeostasis and insulin sensitivity are as well limited and often refer to just one stressor and acute effects. Furthermore, the studies are done with synthetic corticosteroids, such as dexamethasone, which are not subjected to the physiological regulatory mechanism applicable to endogenous GCs [326, 327]. Factors like time after the stress has ceased or dietary composition are rarely considered although this approach would closer resemble the situation in humans.

Therefore, the aim of the present research project was to analyze for the first time changes in insulin sensitivity *in vivo* with hyperinsulinemic-euglycemic clamps in a PTSD mouse model and possible potential mechanisms:

1) **Just after the CVS intervention.**
2) **Three months after the CVS intervention with subsequent low- or high-fat diet consumption.**

I hypothesized that CVS would cause whole-body insulin resistance that would not be carried in time with subsequent low fat diet consumption. On the contrary, HFD consumption would act as a synergistic factor prolonging CVS insulin resistance effects in time.

4.1 A PTSD mouse model for the study of chronic stress effects

Since there is increasing interest in stress as a risk factor for metabolic disorders besides low physical activity and high caloric food, former studies in animal models have been carried out to study the effects of stress on metabolism. Although a variety of effects has been observed, no consensus was achieved and there were a lot of limitations regarding for instance the durations of the stress intervention and the kind of stressors.

On the one hand, it is noteworthy that many studies are restricted to acute effects of one single stressor, like an increase in circulating FFA in rats in response to a 6 h mild electric shock [251] or in dogs in response to 120 min cold exposure [252]. As well, short-term (60 min) water avoidance stress [275] and immobilization (6h) [213] have been used as single

stressors in rodent models to investigate effects on circulating hormones like ghrelin or adiponectin or on gene expression levels of neuropeptides in the CNS [52, 53, 55, 56] (hypothalamus, pituitary gland). Therefore, those studies do not consider prolonged stress challenge to the organism as well as potential long-term regulatory changes in metabolic function after the stress has ceased. Moreover, although there are as well studies to analyze the effects of chronic stress (total time of stress intervention between 5 days and 5 weeks) on metabolism very often just one single stressor is used like immobilization/restraint [154, 265, 273] or social defeat stress [153, 245, 278]. Since just single stressors have been applied for a prolonged period like several weeks, a constant activation of the HPA cannot be ensured and adaptation to the stressor may be considered. These studies, like the acute ones, refer to just one metabolic outcome and/or tissue (liver, skeletal muscle, adipose tissue, CNS, gastrointestinal tract, skin, heart) during the time the stress intervention is taken place.

Therefore, there are hardly mouse models for the analysis of the long-term effects of chronic stress on glucose tolerance and insulin sensitivity and its relation to diet composition. Specifically, some studies show that chronic social stress in combination with HFD consumption caused insulin resistance in subordinate mice as measured with HOMA, but these analyses were done during the stress intervention period [328]. Other studies showed that chronic variable stress (CVS) and subsequent consumption of a HFD leaded to glucose intolerance 3 months after the stress intervention ended [214]. However, the study mainly focused on the adipose tissue and did not take into account the potential contribution of other key tissue in glucose homeostasis (liver, skeletal muscle). Furthermore, glucose tolerance measured by GTT and not actual insulin sensitivity *in vivo* was analyzed [214]. Therefore, there is still a need for an animal model that resembles human (unpredictable) chronic stress conditions, to elucidate how they may affect overall insulin sensitivity beyond the level of one single tissue, how the potential impairment can last in time and how dietary fat content can be a regulator. Most of the epidemiological studies in humans identify the previous presence of chronic stress early in life through diagnoses of PTSD years later (war veterans, victims of natural disasters etc) [5-10].The subsequent associations between metabolic impairment and chronic stress in human subjects are therefore established through the concomitant presence of PTSD.

In order to reproduce these conditions, I worked with an animal model for PTSD. A mouse model for PTSD is achieved through the application of 5 randomly alternating stressors, twice per day for a total duration of 15 days, therefore in a chronic variable fashion also known as chronic variable stress (CVS) with the aim to keep the HPA axis active and to avoid adaptation [290, 291]. The nature of this specific protocol and the analysis 3 months later after diet consumption allows reproducing *in vivo* in a mouse model a situation that

resembles the one observes in war veterans (return to a hyper-energetic environment after extreme conditions) [5, 7].

4.2 Plasma corticosterone and body composition

Plasma corticosterone levels and body composition were analyzed in CVS and CTRL animals just after the stress intervention and 3 months later. As expected from the HPA axis activation plasma corticosterone was elevated in the CVS mice at the end of the intervention [290, 329, 330]. Also at this time point and in line with the proteolytic activity of prolonged exposure to GCs, lean mass was lower in the CVS mice confirming the stressed state. Due to the loss of lean mass, body weight was lower as well [214, 290].

Although changes in fat mass were not uniform in all animals, fat mass average was increased after the CVS intervention in accordance with the GC-mediated up-regulation of enzymes like LPL, FAS or hyperplasia [18, 127].

Three months after the CVS intervention, mice consuming a HFD showed higher BW and fat mass compared with the CHOW group as expected. However, no changes regarding CVS were observed between the groups in body composition independently of the diet.

The HPA axis/GC system sensitivity decreases with age [331, 332] and in consequence plasma corticosterone levels were higher 3 months later independently of the diet. No differences were observed between the CTRL CHOW and CTRL HFD groups. Former studies on HFD effects on basal corticosterone levels are not consistent showing either an increase or decrease [333, 334], probably due to the research model (rats and mice), the fat percentage of the diet (20-45 %) and time of consumption (1-12 weeks). Therefore the lack of differences in the present study could be due to the experimental conditions. However, corticosterone levels were higher with CVS and HFD suggesting GC resistance with these two factors together.

4.3 Insulin sensitivity regulation by CVS and diet

It is observed in the present research project that CVS has consequences for metabolic regulation on the short-term as well as on the long-term. CVS acutely affects insulin sensitivity in a tissue-dependent manner impairing endogenous glucose production (liver) and increasing glucose disposal in peripheral tissues with no changes in whole-body insulin sensitivity. On the long-term, when fat consumption is low (CHOW) following the CVS intervention, glucose disposal in peripheral tissues is still increased as well as whole-body insulin sensitivity whereas with high fat consumption (HFD) following the CVS intervention,

peripheral glucose disposal as well as whole-body insulin sensitivity is decreased. Therefore, long-term effects of CVS are regulated by dietary fat content. Changes in systemic inflammation, plasma hormones, hypothalamic neuropeptides, sympathetic activity and tissue glucose and lipid metabolism were analyzed as potential mechanisms behind these effects.

4.3.1 Acute/short-term effects of CVS on insulin sensitivity

On the short-term CVS affects insulin sensitivity in a tissue-dependent manner impairing hepatic glucose production and increasing glucose disposal in peripheral tissues. As a consequence of these two opposing effects, whole-body insulin sensitivity is not altered by CVS as measured by the glucose infusion rate (GIR) with clamps. Those results are in line with studies where the administration of dexamethasone in the arcuate and paraventricular nuclei of the hypothalamus of rats decreased the suppression of EGP and increased Rd, respectively [243], suggesting that in our physiological model acute CVS effects could be CNS-mediated.

4.3.1.1 The sympathetic nervous system as a mediator in insulin sensitivity regulation by CVS

The decrease in endogenous glucose production (EGP) under basal conditions could be explained by the observed lower circulating levels of the pancreatic hormone glucagon known for stimulating gluconeogenesis [335]. However, under hyperinsulinemic conditions, suppression of EGP was impaired in those animals indicating hepatic insulin resistance. These results are in accordance with the gluconeogenic action attributed to GCs [20]. Although this effect in stress is highly protective in order to cope with a perceived threat, an overstimulation may lead to hepatic insulin resistance, as shown in hepatoma cells [66, 67] as well as under chronic stress (restraint and forced swimming (1-4 h/day for 2,4 or 24 weeks) in rats [241] and in clinical models of Cushing syndrome [242]. Since GCs exert many of their effects through changes in gene expression, high hepatic glucose output in response to glucocorticoid treatment or release can usually be explained by increased expression of gluconeogenic genes like *Pck1* and *G6pc* [30, 62]. Indeed, in the present studies, gene expression levels of *G6pc* were increased in the liver with CVS, in line with the clamp findings.

Changes in sympathetic activity seem to be the mechanism behind hepatic insulin resistance by GCs, as the impairment of hepatic insulin sensitivity with hypothalamic

dexamethasone administration in rats was lost after hepatic sympathetic denervation [243]. Specifically, NPY and POMC in the hypothalamus modulate sympathetic activity in the liver and adipose tissue [139, 243, 297, 300, 336] and the expression of these neuropeptides as well as agouti-related protein (*Agrp*) is increased by GCs and stress [47, 48, 52, 53, 55, 56]. In accordance with these results increased expression of *Npy, Pomc* and *Agrp* was observed with CVS. A former study in rats showed that hypothalamic NPY administration induces hepatic insulin resistance and that effect is reversed by sympathetic denervation of the liver [300]. Therefore, it is possible that NPY in the present experimental model is the factor to mediate hepatic insulin resistance by CVS through sympathetic output to the liver.

Furthermore, in BAT, expression of *Ucp1* and *Adrb3* were decreased with CVS, probably due to the inhibitory effect of NPY [301] and GCs [304, 305] on adrenergic stimulation in this tissue. Nevertheless, *Ucp3* expression and protein levels of 1α Na^+K^+ATPase were increased in muscle suggesting SNS activation [337, 338]. This could be due to a differential tissue innervation of the SNS [302, 303] by NPY and as well POMC multisynaptic pathways. Although both neuropeptides are activated by stress and GCs, they exert differential actions on sympathetic activity. Based on the present results, it is possible that NPY plays a more predominant role in the liver [243, 300] although a contribution of POMC cannot be excluded [339].

4.3.1.2 Systemic inflammation as a mediator in insulin sensitivity regulation by CVS

Inflammation is a contributor to visceral obesity and insulin resistance [293], as well HFD promotes inflammation [340]. Although potent anti-inflammatory effects are ascribed to GCs, chronic stress can overcome those short-term effects [137-139]. Therefore, systemic as well as WAT inflammation (*Ccl2*) was analyzed as a potential mechanism behind CVS and diet effects on insulin sensitivity.

Although the body composition analysis in the present project shows higher fat mass after the CVS intervention, increased plasma levels of the anti-inflammatory cytokine IL-10 are also observed. IL-10 is produced by immune cells in the WAT and its plasma levels positively correlate with insulin sensitivity [293, 341, 342]. IL-10 levels are increased by GCs as shown in humans under GC therapy [343] and by adiponectin [344] which shows a higher trend in the present mouse model after the CVS intervention. It was also observed a decrease with CVS in plasma levels of the pro-inflammatory cytokines IL-1α, Eotaxin (CCL11), MIP-1β (CCL4) and RANTES (CCL5) being components of the neuroimmune axis (IL-1α) [345] or involved in eosinophil recruitment (Eotaxin) [346], leukocyte activation and trafficking (MIP-

1β) [347] and T-cell accumulation in the WAT of obese humans (RANTES) [348]. Moreover, *Ccl2* expression in the epididymal WAT was decreased in CVS mice in accordance with the described properties of endogenous as well as exogenous GCs to suppress peripheral *Ccl2* expression [349]. Therefore based on our results, on the short-term CVS mice display a systemic anti-inflammatory state that is in line with the anti-inflammatory effects described for GCs that could contribute to the higher peripheral glucose disposal measured by the rate of disappearance (Rd %) with clamps.

4.3.1.3 Hormones as mediators in insulin sensitivity regulation by CVS

Adipokines can also contribute to insulin/glucose homeostasis regulation in relation to GCs and stress as already shown in previous studies [214]. Therefore, I proceeded to the analysis of these hormones, and extended it to other hormones that may as well play a role in relation to insulin sensitivity regulation by CVS and diet. Studies with GC administration *in vitro* in adipocytes [350, 351] and *in vivo* in adrenalectomized animals [202, 203] show a decrease in adiponectin secretion and expression (adipose tissue). However, in the experimental mouse model used in the present project, CVS leads to a trend to higher adiponectin levels in line with studies in rats showing increased plasma adiponectin in response to treatment with prednisolone or dexamethasone [204, 352]. Adiponectin has been described to suppress inflammation, same as GCs classically do, antagonizing TNF-α [187, 191] and inducing anti-inflammatory cytokines like IL-10 (as previously mentioned) and IL-1RA [344]. Adiponectin also suppresses hepatic gluconeogenesis [187, 353, 354] improving liver insulin sensitivity [187]. Since the present experimental model shows insulin resistance at the level of the liver, increased adiponectin levels could be a way of the organism to counteract that metabolic impairment. Furthermore, adiponectin enhances fatty acid oxidation in liver and muscle [355-357]. Likewise suppression of hepatic gluconeogenesis, enhanced fatty acid oxidation by adiponectin is attributed to the activation of AMPK and ACC in liver and muscle [355-357]. In line with these effects, CVS mice displayed increased protein levels of p-ACC and ACC in *gastrocnemius* muscle as a key player of fatty acid oxidation [358] downstream of AMPK that was increased as well. Supporting higher fatty acid oxidation, *Ucp3* expression was also enhanced [306, 307] and plasma levels of triglycerides were lower in the CVS compared with the CTRL mice. Therefore, adiponectin just after CVS may be a relevant regulatory signal to enhance lipid β-oxidation at the level of the muscle through AMPK and ACC and therefore may contribute to the higher Rd observed with clamps.

I further analyzed the adipokines resistin, plasminogen activator inhibitor-1 (PAI-1) and leptin, also linked to insulin sensitivity regulation [215, 219, 220, 296, 359]. Although administration of dexamethasone has been described to increase expression and secretion of resistin [240] and PAI-1 [205, 285], we did not observe differences in these hormones after CVS. Leptin levels show a higher trend in accordance with the gain in fat mass observed after CVS intervention [360, 361].

In addition to adipokines, gastrointestinal hormones have also been related with GC and stress [103, 151-154, 294]. In the current study, ghrelin levels were decreased with CVS on the short-term in contrast to studies in rodents showing an increase in ghrelin levels upon acute and chronic stress [151-154]. Differences between my results and the data from other studies could be related to the model itself (rats and mice of different genetic backgrounds), to the kind of stress intervention (acute versus chronic stress with just one stressor) and the time the measurements were taken. Lower levels of ghrelin in the present model in response to CVS could also be explained considering a role of ghrelin in impairing glucose-stimulated insulin secretion [156-161] and insulin sensitivity [163, 164, 168, 169]. Lower levels of ghrelin with CVS may reflect that those animals did not display an overall insulin resistant state that is restricted to the liver while glucose disposal in peripheral tissues is increased (Rd %). To further elucidate the role of ghrelin in CVS-induced insulin sensitivity changes, further studies could be done in ghrelin /GHSR knockout mice.

No differences were found between CVS and CTRL mice in the incretins GIP and GLP-1. Studies on the effects of GCs on those hormones are limited with the exception of one study in humans showing that GLP-1 levels were increased by dexamethasone administration [294].

4.3.1.4 Adipose tissue and skeletal muscle metabolic alterations as a mechanism in insulin sensitivity regulation by CVS

Given that WAT and skeletal muscle play a relevant role in glucose disposal (Rd %) and therefore in insulin sensitivity, these tissues were further analyzed with special emphasis in skeletal muscle as a relevant glucose and fatty acids user.

GCs have been described to exert both lipolytic and adipogenic effects. In the present mouse model, changes in the expression of *Lipe* with CVS were not observed in line with previous pharmacological studies in humans where GCs were administered for several days

[115-117]. Adipogenic effects by GCs are potentially mediated on the one hand by hypertrophy of adipocytes through increased *Lpl* as shown in the adipose tissue of rats and humans [127, 128] and through increased expression of *Fasn* [131]. Although the expression of *Lpl* was increased after CVS no changes in *Fasn* were observed compared with the CTRL mice. Therefore, the present results indicate that in the WAT the adipogenic properties of GCs play a more relevant role with CVS than the lipolytic properties in line with the gain in fat mass.

Although the expression of *Angptl4* is induced by GCs [126, 308] and its expression is high in adipose tissue of diabetic mouse models [310], we did not observe differences in the expression of *Angptl4* between the CVS and the CTRL mice in line with the lack of differences in whole body insulin sensitivity.

I further analyzed gene and protein markers for nutrient sensing, glucose and lipid metabolism in skeletal muscle.

Since the mTORC1/S6K1 pathway is key in nutrient sensing, insulin signaling and cell growth and has been linked to GCs with chronic stress increasing mTORC1 levels in the hypothalamus and dexamethasone decreasing mTORC1 signaling in skeletal muscle cells [313, 314], I aimed to investigate the potential role of this pathway in the periphery with CVS. In my experimental model, the activation of S6K1 as a downstream target of mTORC1 was increased with CVS in skeletal muscle. Considering the great proteolytic effect of GCs with CVS, the role of the mTORC1/S6K1 pathway could be mainly related to its actions in protein synthesis and cell growth [362]. Therefore, an activation of this pathway may be a way of the organism to counteract muscle wasting and to contribute to its rebuilding.

Also in relation to insulin signaling/glucose metabolism, I analyzed under basal conditions protein levels of GLUT4 and GLUT1 in the plasma membrane and microsomal fraction with no changes. Since the absolute levels of 1α Na^+K^+ ATPase as the housekeeping protein for the plasma membrane fraction show a trend for an increase with CVS (p=0.09), membrane GLUT4 and GLUT1 were not normalized to that protein and no calculations of the plasma membrane:microsome ratio for GLUT4 and GLUT1 were done. No changes in *Hk2* expression in muscle as a marker for glycolysis were either observed with CVS. Therefore, although *ex vivo* studies with dexamethasone indicate muscle insulin resistance [78-80, 91, 92], endogenous GCs in our mouse model for CVS do not seem to cause dramatic changes in muscle glucose metabolism.

Besides glucose metabolism, there is increasing interest in the contribution of skeletal muscle lipid metabolism to insulin sensitivity regulation [319, 320]. Actually, impaired fatty acid oxidation and mitochondrial function in muscle are contributors to ectopic lipid deposition and can induce insulin resistance by mechanisms summarized as lipotoxicity [363]. GCs have been shown to affect lipid metabolism as well as mitochondrial function in several cell types [261, 364, 365]. Since mitochondria are highly abundant in muscle, changes in lipid metabolism may play a relevant role regarding insulin sensitivity regulation by CVS.

Although changes in *Cpt1β* expression were not observed with CVS, *Ucp3* expression was higher in line with higher protein levels of p-ACC, ACC and AMPK suggesting an increase in lipid β-oxidation and potentially mitochondrial biogenesis by CVS. Supporting this possibility, basal plasma triglycerides levels were lower with CVS. Those results are in line with studies showing upon chronic GC administration a stimulation of mitochondrial biogenesis in skeletal muscle of rats [261] (dexamethasone, 6 mg/kg/day, s.c.) and an increase in energy metabolism in humans [366] (methyprednisolone, 125 mg/ 30 min., i.v. or 40 mg/day, orally). However, fatty acid oxidation was decreased in response to dexamethasone in cultured myoblasts and skeletal muscle of broiler chickens [367]. In relation to chronic stress in rats, 15 days of crowding stress led to disturbed mitochondrial activity in the intestine [264] and 6 h restraint for 3 weeks decreased mitochondrial lipid metabolism enzymes (CPT2, ACOT1) in cardiomyocytes [265]. Based on our results and the limited number of previous studies, GCs affect muscle lipid metabolism. However, it is possible that the higher mitochondrial biogenesis observed with dexamethasone is due to lower lipid oxidation as shown *ex vivo* and *in vitro* [367] and not actually related to higher lipid utilization.

4.3.2 Chronic/long-term effects of CVS and diet on insulin sensitivity

While CVS does not have short-term effects on whole-body insulin sensitivity, CVS affects whole-body insulin sensitivity on the long-term in a dietary fat-dependent way as another main finding in the present doctoral thesis. Specifically, CVS is diabetes-protective with low dietary fat (CHOW) subsequent to the CVS intervention as a result of increased glucose disposal in peripheral tissues and whole-body insulin sensitivity. On the contrary, when the animals consume a HFD, CVS is diabetogenic, decreasing glucose disposal in peripheral tissues and whole-body insulin sensitivity. The results go in line with previous studies showing an impairment of glucose homeostasis in relation to CVS and HFD due to imbalanced adiponectin/resistin ratio [214], but they go one step further measuring actual

insulin sensitivity changes *in vivo* and giving further insides into potential mechanism behind the effects observed.

4.3.2.1 Role of the sympathetic nervous system in chronic/long-term effects of CVS and diet on insulin sensitivity

The lower basal EGP observed after CVS was still present after 3 months in the CVS CHOW mice but not in the CVS HFD mice compared with their corresponding control groups. However, changes in the suppression of EGP were not found between CVS and CTRL mice on the long-term independently of the diet. These findings could suggest a recovery of the hepatic insulin resistance observed after CVS attributed to the SNS [243]. In accordance with no alterations in the suppression of EGP by CVS and diet on the long-term, no changes in the expression of sympathetic markers were found in CVS CHOW and CVS HFD mice compared to their respective CTRL although the expression of *Adrb3* showed a trend to be increased in CVS CHOW. Therefore, hepatic insulin resistance by CVS is a short-term effect in line with the acute/fast actions ascribed to the SNS.

4.3.2.2 Role of systemic inflammation in chronic/long-term effects of CVS and diet on insulin sensitivity

Although we observed a decrease in systemic inflammation after CVS, it was not prolonged in time as CVS CHOW mice just showed higher levels of one single pro-inflammatory cytokine, granulocyte colony-stimulating factor (G-CSF) whose levels actually have been shown to be increased in mononuclear cells after dexamethasone stimulation [368]. Although classically GCs are known as anti-inflammatory hormones, GCs can also promote inflammation under chronic stress conditions through an increase of pro-inflammatory factors like IL-6 and TNF-α due to GC or GR resistance [137-139]. Likewise, HFD consumption has been shown to promote inflammation [293] as also observed in the results here presented. Therefore, we might consider a synergistic effect of CVS and HFD increasing inflammation and the subsequent decrease in insulin sensitivity under our experimental conditions. However, no changes in systemic cytokines were observed with HFD 3 months after CVS although we observed higher expression of *Ccl2* in WAT in accordance with previous studies [214]. Therefore, systemic inflammation does not seem to be the answer to the long-term effects of CVS and diet on insulin sensitivity although inflammation at the tissue level cannot be ruled out.

4.3.2.3 Role of hormones in chronic/long-term effects of CVS and diet on insulin sensitivity

Independently of the diet, adiponectin levels were decreased 3 months after the CVS intervention. For CVS HFD, this observation is in line with previous studies [214] and the decrease in insulin sensitivity observed with clamps. For CVS CHOW, this finding could be difficult to interpret considering improved whole-body insulin sensitivity in that group. Nevertheless, other effects parallel to decreased adiponectin can occur with CVS promoting insulin sensitivity. Actually, HIF-1α has been described to increase basal glucose uptake [369] and reduce adiponectin [370]. As indirect evidences of this possibility a trend to higher GLUT4 [371] plasma membrane:microsome ratio was observed as well as low p-S6K1 protein levels [372] in muscle and low PAI-1 plasma levels [370] in the CVS CHOW mice; these results are later discussed.

Resistin levels with CVS CHOW and CVS HFD reflected the profile of whole-body insulin sensitivity (GIR). In many aspects, the actions of resistin oppose those of adiponectin by decreasing AMPK leading to increased glucose output by the liver [216, 227, 230, 231] and in muscle to inhibit insulin-mediated glucose uptake via down-regulation of GLUT4 [228]. In line with the lower resistin levels in the CVS CHOW group, GLUT4 distribution towards the plasma membrane (plasma membrane:microsome ratio) in skeletal muscle was higher. No significant changes were observed in AMPK and ACC activation although *Cpt1β* expression was increased. In contrast, with HFD consumption, *Cpt1β* expression in muscle was decreased with no changes either in AMPK and ACC activation.

Leptin was decreased with CHOW consumption in line with lower fat mass and increased with HFD consumption independent of the CVS intervention in accordance with previous studies [214].

PAI-1 was decreased 3 months after CVS with CHOW consumption. Since higher plasma levels of PAI-1 have been linked to obesity and the metabolic syndrome [296] reduced levels of PAI-1 with CVS and CHOW are in accordance with the improved insulin sensitivity of that group. No changes were observed with HFD following the CVS intervention.

Ghrelin was unaltered in CVS CHOW mice and increased in CVS HFD mice compared with their corresponding CTRL groups. As mentioned before, ghrelin has been shown to be increased with acute as well as with chronic stress in rodents [151-154] and can activate the HPA axis [146, 147, 149] same as HFD [334]. Since CVS HFD mice also show elevated

corticosterone levels compared with CTRL HFD mice, higher levels of ghrelin may be associated with elevated corticosterone. Furthermore, ghrelin has been linked to impaired glucose tolerance/insulin sensitivity [163, 164, 168, 169] also observed in the CVS HFD group. Further studies with CVS and diet in ghrelin KO mice could contribute to investigate those possibilities. Changes in GIP and GLP-1 were not observed on the long-term independently of the diet.

4.3.2.4 Role of adipose tissue and skeletal muscle metabolism in chronic/long-term effects of CVS and diet on insulin sensitivity

Like in the short-term group, the contribution of the WAT and skeletal muscle to effects on whole-body insulin sensitivity and glucose disposal (Rd) and therefore to insulin sensitivity was further analyzed with special emphasis in muscle.

4.3.2.4.1 CHOW diet

Animals that underwent CVS and subsequently consumed CHOW showed an increase in whole-body insulin sensitivity through high peripheral glucose disposal. In line with that observation, *Hk2* expression was higher in skeletal muscle of CVS CHOW compared with CTRL CHOW mice. That finding along with the increased *Gck* expression in liver suggests a general higher glycolytic activity (liver and skeletal muscle) in these animals.

Furthermore, a trend to higher basal plasma membrane:microsome ratio of GLUT4 was observed pointing towards increased basal glucose uptake. This trend to increased basal glucose uptake could as well be an indicator of hypoxia in muscle [369], along with the decreased protein levels of p-S6K1 observed in that tissue since hypoxia has been related to a decrease in the kinase activity of mTORC1 [372-374]. To follow up with that possibility, protein levels of HIF-1α as a marker for hypoxia [375, 376] could be analyzed in skeletal muscle in future studies.

When analyzing the gene expression profile of the WAT, *Lpl* and *Fasn* expression were decreased while *Lipe* expression was increased in CVS CHOW mice compared with CTRL CHOW. These data suggest lower adipogenic activity [377] that could as well be linked to higher lipid β-oxidation in accordance with the lower fat mass, lower plasma TGs and increased expression of *Cpt1β* in skeletal muscle as a marker for fatty acid oxidation. However, no changes in p-ACC and p-AMPK protein levels were observed in that tissue. It is

important to consider that the amount of protein levels detected by WB does not reflect the activity of these proteins as measured in *ex vivo* functional studies. Furthermore, the lack of changes in AMPK and ACC may suggest alternative routes of lipid metabolism regulation by GCs such as peroxisome proliferator-activated receptors (PPARs) [324, 325].

No changes were observed between CVS CHOW and CTRL CHOW mice in the expression of *Angptl4* and *Ccl2*.

4.3.2.4.2 HFD

In mice consuming a HFD for 3 months after the CVS intervention, whole-body insulin sensitivity as well as peripheral glucose disposal was decreased. However, no relevant effects regarding glucose metabolism were detected with gene expression and protein levels analysis in skeletal muscle which is unexpected at first view regarding the observations of the clamps. However, it is possible that players in glucose metabolism beyond the ones investigated in the present thesis (like for instance other enzymes of glycolysis) play a role in glucose metabolism/insulin sensitivity with CVS and HFD.

Furthermore, as with CHOW, changes in lipid metabolism could play a more relevant role. In line with that assumption, the expression of *Cpt1β* in WAT was decreased in CVS HFD mice compared to CTRL HFD mice suggesting lower lipid β-oxidation. As in CVS CHOW mice, changes in protein levels of p-AMPK and p-ACC were not observed.

In the WAT, *Angptl4* expression was increased as seen in adipose tissue of diabetic mouse models [310]. Moreover, *Ccl2* expression was increased as in previous studies [214] suggesting that inflammation at the tissue level (in contrast to systemic inflammation) may play a relevant role in insulin resistance with CVS and HFD due to synergistic effects of those two factors [293].

Therefore, changes in *Angptl4* and *Ccl2* along with a decrease adiponectin/resistin ratio [214] underline the relevance of the WAT in insulin sensitivity regulation with CVS and HFD consumption.

4.4 Concluding remarks

In summary the present results show that exposure to CVS impairs hepatic glucose production and improves peripheral glucose disposal without changing whole body insulin sensitivity. Furthermore, dietary fat content determines the long-term effects of CVS enhancing (low fat) or impairing (high fat) insulin sensitivity. Changes at the level of the WAT (*Lipe*, *Lpl,* adipokines) and muscle gene markers for lipid metabolism (*Ucp3, Cpt1β*) suggest changes in lipid metabolism as the main mechanism in relation to CVS and dietary fat effects on insulin sensitivity regarding time. Therefore, the present experimental animal model for PTSD proves for the first time that chronic stress, as observed in human subjects, has effects on insulin sensitivity that can be manifested late in time. Furthermore, lifestyle factors such as diet would determine the onset of the pathological state.

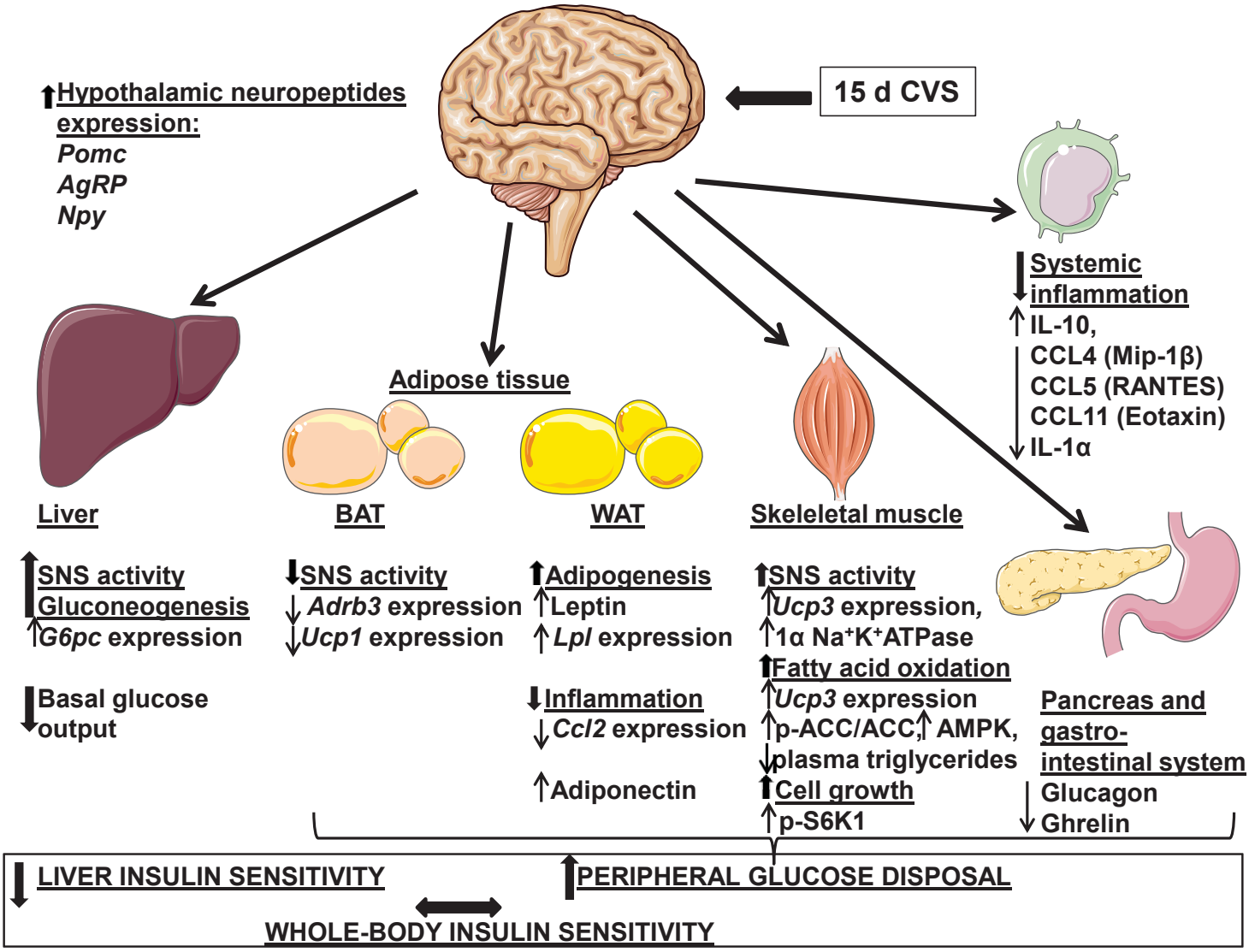

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Figure 24: Summary - Effects of CVS on insulin sensitivity. CVS impairs hepatic glucose production and improves peripheral glucose disposal whereas whole-body insulin sensitivity is unaffected. Hepatic insulin resistance is characterized by diminished glucose output under basal conditions and elevated glucose output under hyperinsulinemia mediated through the sympathetic nervous system (SNS), also in line with increased *Npy* expression in the hypothalamus and *Ucp3* expression and 1α Na^+K^+ ATPase protein levels in skeletal muscle. In BAT, SNS activity is inhibited as indicated by decreased *Adrb3* and *Ucp1* expression. In WAT, CVS promotes adipogenesis through increased *Lpl* expression in line with increased fat mass and leptin levels. In line with a systemic anti-inflammatory state characterized by increased IL-10 and decreased CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (Eotaxin) and Il-1α in plasma, *Ccl2* expression in WAT is decreased. Furthermore, plasma adiponectin is increased in line with higher protein levels of p-ACC, ACC and AMPK, increased *Ucp3* expression and decreased plasma triglycerides (ß-oxidation). Lower glucagon and ghrelin levels could play a role in the low basal glucose output and increased peripheral glucose disposal, respectively.

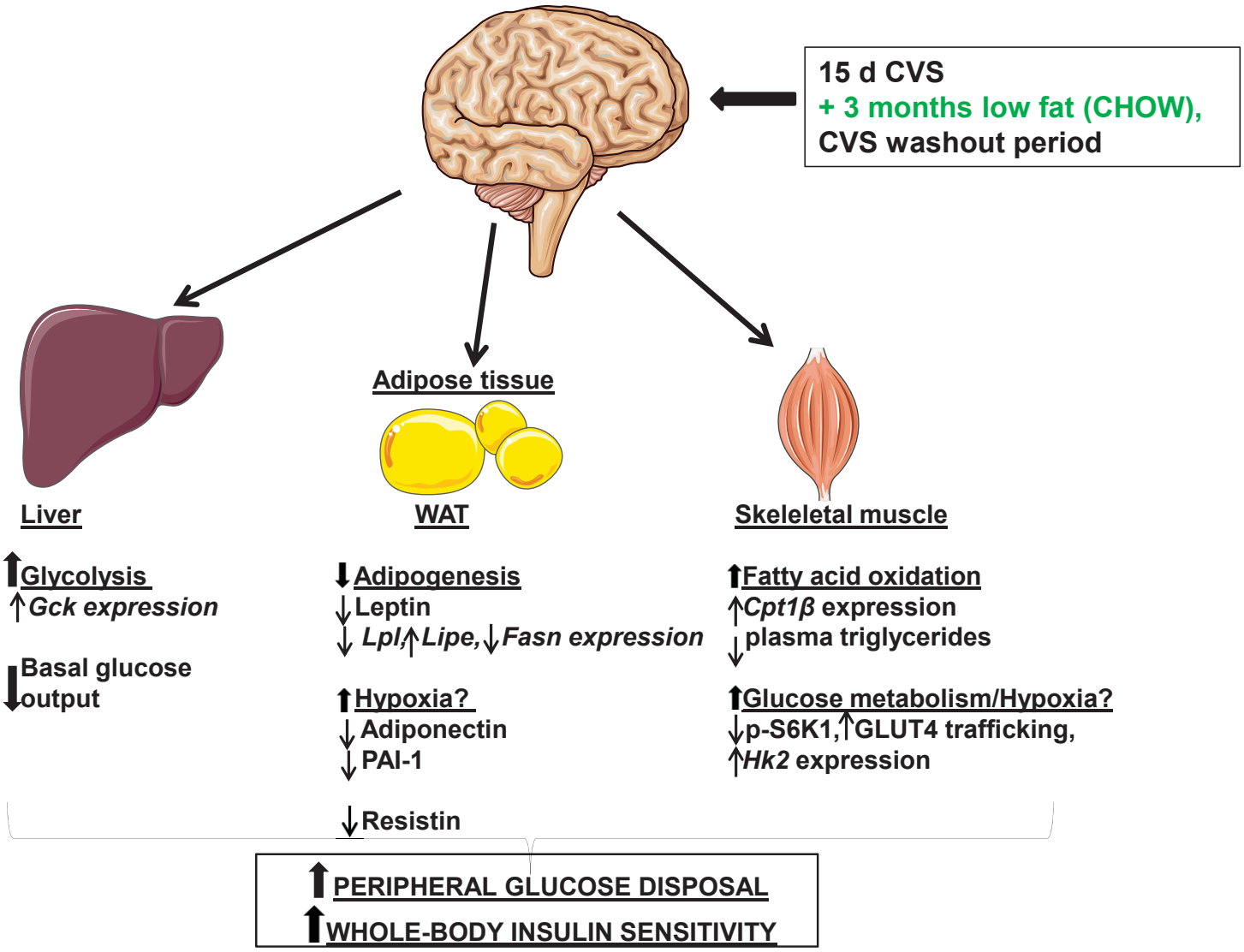

Figure 25: Summary - Effects of CVS on insulin sensitivity after 3 months of low fat consumption (CHOW). On the long-term with CHOW consumption following the CVS intervention, whole-body insulin sensitivity as well as peripheral glucose disposal is improved compared with the CTRL. Although decreased basal glucose output observed with CVS on the short-term is kept, the liver recovered from impaired insulin sensitivity and *Gck* expression is increased suggesting higher glycolytic activity. In line with that observation, *Hk2* expression is increased in skeletal muscle. Low p-S6K1 protein levels and basal GLUT4 trafficking in skeletal muscle together with decreased adiponectin and PAI-1 plasma levels suggest higher glucose uptake and insulin sensitivity in relation to hypoxia in the CVS mice. In line with improved insulin sensitivity, resistin levels are lower. Furthermore, *Cpt1β* expression in skeletal muscle is increased and plasma triglycerides are decreased suggesting maintenance of the higher fatty acid oxidation observed with CVS on the short-term. Conversely, adipogenesis is decreased as suggested by lower fat mass, lower leptin levels and decreased expression of *Lpl* and *Fasn* in WAT while *Lipe* expression is increased.

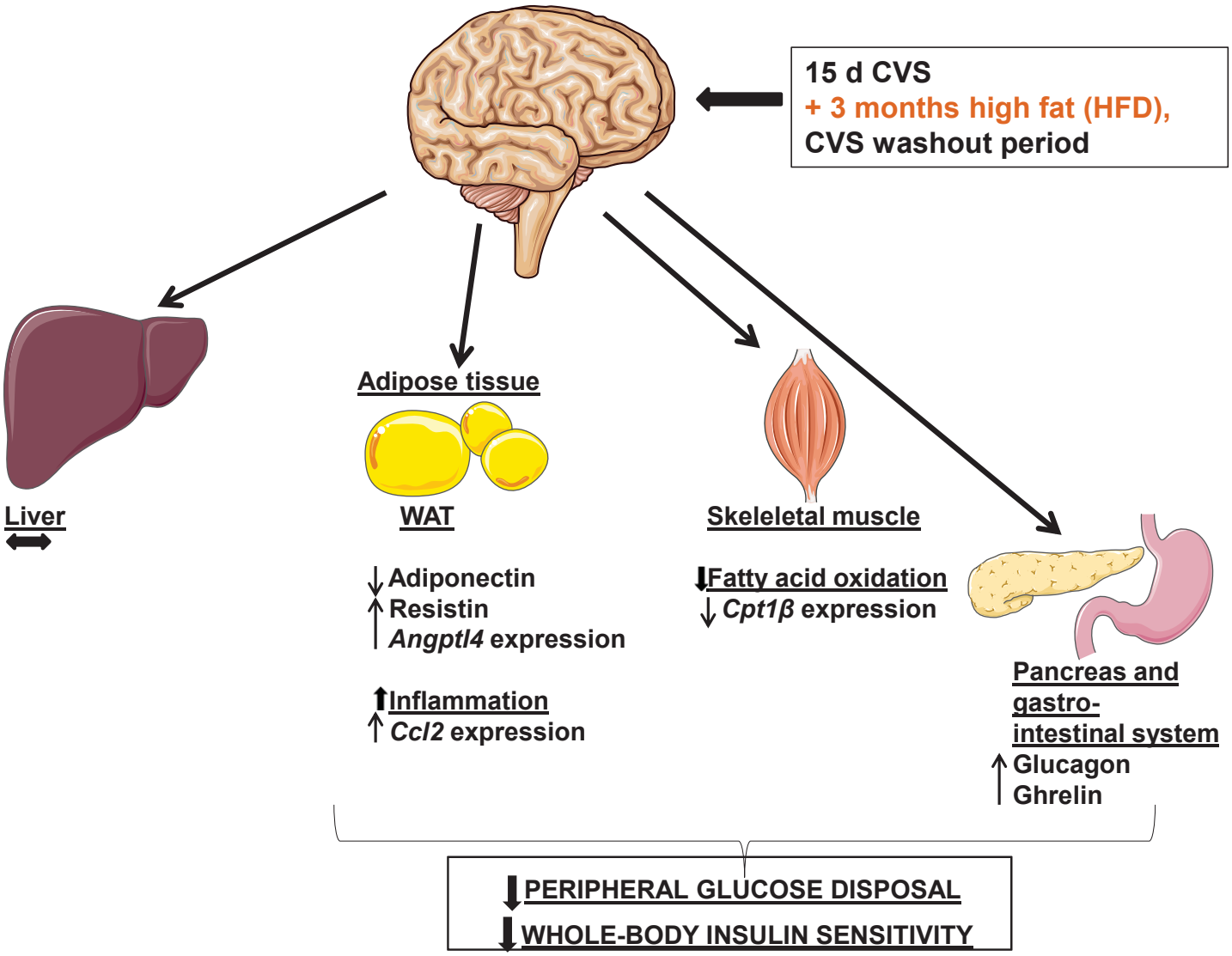

Figure 26: Summary - Effects of CVS on insulin sensitivity after 3 months of HFD consumption. On the long-term with HFD consumption following the CVS intervention, whole-body insulin sensitivity is impaired and peripheral glucose disposal is decreased. No effects on the liver are observed under these conditions while fatty acid oxidation in skeletal muscle is decreased as suggested by lower *Cpt1β* expression. In line with previous studies, the adiponectin:resistin ratio is decreased and WAT inflammation is increased as suggested by high *Ccl2* expression. Like in WAT of diabetic mice, *Agptl4* expression is high as well with CVS. Glucagon and ghrelin levels are increased potentially contributing to impaired insulin sensitivity.

5 Summaries

5.2 Summary (English)

Background and aim: Although glucocorticoids (GCs) and catecholamines have an impact on glucose and lipid metabolism and actually stress has been suggested to contribute to insulin resistance, the metabolic effects behind the physiology of stress are still poorly understood. Most of the studies have been performed with synthetic GCs like dexamethasone and are often focused just in one tissue, with the observations limited to acute effects and the interpretation subjected to the research model, tissue and time of exposure. Epidemiological studies in humans link chronic stress and PTSD with late diabetes onset; however, those studies do not allow the analysis of the overall mechanisms behind the observed effects. Therefore, the aim of the present doctoral thesis was to elucidate in a mouse model for PTSD whether chronic stress may affect whole-body insulin sensitivity, whether those effects last in time (after the stress situation ceased) and how they can be regulated by dietary fat. Furthermore, potential factors that could drive and orchestrate those metabolic alterations were also analyzed in the present doctoral thesis.

Methods: Three-months old, body weight-matched male C57BL/6 mice were exposed to a random series of stressors for 15 days (CVS), the unstressed controls (CTRL) were housed separately during that time. After CVS, body composition was analyzed with nuclear magnetic resonance *(*NMR) and blood was collected (8-12 a.m.) for corticosterone (radioimmunoassay, RIA) analysis. Subsequently, insulin sensitivity was analyzed *in vivo* with hyperinsulinemic-euglycemic clamps in one group of animals (short-term effects of CVS). Another group of mice was divided into two subgroups either consuming a low fat diet (CHOW, 9 % fat) or a high fat diet (HFD, 43 % fat) for 3 months without further stress intervention before the analysis of insulin sensitivity (long-term effects of CVS). In addition to the analysis of insulin sensitivity, lipid metabolites, hormones and cytokines in plasma and gene expression (real time PCR, RT-PCR) and protein levels (western blot, WBs) in hypothalamus, muscle, liver and adipose tissue were done at the same time points.

Results: Exposure to CVS increased plasma corticosterone and fat mass and decreased lean mass and body weight. On the short-term, CVS impaired endogenous glucose production (EGP, liver) and increased peripheral glucose disposal (Rd %) without changing whole body insulin sensitivity (glucose infusion rate, GIR). In line with Rd % and GIR, respectively, adiponectin showed a trend to be increased while resistin was unchanged. The protein levels of muscle markers for fatty acid oxidation (AMPK, p-ACC, ACC) were

increased while plasma triglycerides (TGs) were decreased. No changes were observed in the expression of the fatty acid oxidation marker *Cpt1b* in skeletal muscle. On the long-term with CHOW consumption, CVS increased GIR and Rd % compared to CTRL CHOW. On the contrary, GIR as well as Rd % were decreased in CVS HFD mice compared to CTRL HFD mice. CVS CHOW mice showed decreased plasma adiponectin and resistin, whereas CVS HFD mice showed decreased plasma adiponectin and increased plasma resistin. *Cpt1b* expression in skeletal muscle was increased in CVS CHOW and decreased in CVS HFD mice compared to the respective CTRL mice suggesting higher or lower fatty acid oxidation, respectively. In line with that observation, CVS CHOW mice showed lower plasma TGs and lower adipogenic activity in WAT as suggested by decreased *Lpl* and *Fasn* expression and increased *Lipe* expression. With HFD, no changes between CVS and CTRL mice were observed in plasma TGs levels and in WAT *Lpl*, *Fasn* and *Lipe* expression. No differences were found in the protein levels of muscle markers for fatty acid oxidation on the long-term.

Conclusions: Exposure to CVS impairs hepatic insulin sensitivity and improves peripheral glucose disposal without changing whole-body insulin sensitivity. On the long-term, CVS improves insulin sensitivity and increases peripheral glucose disposal when dietary fat is kept low. On the contrary, HFD consumption decreases peripheral glucose disposal increasing insulin resistance. Therefore, dietary fat content determines the long-term effects of CVS enhancing (low fat) or impairing (high fat) insulin sensitivity. Changes at the level of the WAT and skeletal muscle gene markers for fatty acid oxidation suggest changes in lipid metabolism as the main mechanism in relation to CVS and dietary fat effects on insulin sensitivity regarding time. Therefore, the present experimental animal model for PTSD proves for the first time that chronic stress, as observed in human subjects, has effects on insulin sensitivity that manifest late in time. Furthermore, lifestyle factors such as diet would determine the onset of the pathological state.

5.2 Zusammenfassung (deutsch)

Fragestellung: Obwohl Glukokortikoide und Katecholamine Einfluss auf den Glukose- und Lipidmetabolismus nehmen und Stress als ein Risikofaktor für Insulinresistenz diskutiert wird, sind die metabolischen Effekte, denen die Stressphysiologie zugrunde liegt, noch größtenteils unbekannt. Der Großteil bereits vorhandener Studien stützt sich auf den Einsatz synthetischer Glukokortikoide wie Dexamethason mit Schwerpunkt auf der Untersuchung einzelner Gewebe. Des Weiteren bleiben in diesen Studien die Beobachtungen oftmals auf akute Effekte beschränkt und die Interpretation der Daten ist stark dem jeweiligen Forschungsmodell, dem Gewebetyp und der Expositionszeit unterworfen. Epidemiologische Studien im Menschen haben bereits einen Zusammenhang zwischen chronischem Stress bzw. posttraumatischer Belastungsstörung und spätmanifestiertem Diabetes aufgedeckt, jedoch erlauben diese Studien keine umfassende Analyse der zugrundeliegenden Mechanismen.

Zur weiteren Beschreibung dieser Vorgänge war das Ziel der vorliegenden Dissertation die Aufklärung der potenziellen Auswirkungen von chronischem Stress auf die Ganzkörper-Insulinsensitivität in einem Mausmodell für posttraumatische Belastungsstörung sowie die Identifizierung möglicher Mediatoren stress-induzierter metabolischer Veränderungen. Des Weiteren sollte als wesentlicher Aspekt in dieser Fragestellung untersucht werden, ob die potenziellen Auswirkungen von chronischem Stress auf die Insulinsensitivität langfristig bestehen bleiben (nach Abschluss der Stressintervention) und wie sie in Abhängigkeit vom Fettgehalt der Ernährung reguliert werden könnten.

Material und Methoden: Drei Monate alte, männliche C57BL/6J-Mäuse wurden für 15 Tage einer randomisierten Reihe verschiedener Stressoren ausgesetzt, wobei die Kontrolltiere (CTRL) für diesen Zeitraum in einem separaten Raum gehalten wurden. Nach der Stressintervention wurde die Körperzusammensetzung (*nuclear magnetic resonance*, NMR) analysiert und die Corticosteron-Spiegel (08:00-12:00 Uhr) im Plasma bestimmt (RIA). Die Insulinsensitivität wurde *in vivo* mittels eines hyperglykämischen-euglykämischen Clamps 1) unmittelbar nach der Stressintervention (Kurzzeiteffekte von CVS) und 2) drei Monate nach der Stressintervention mit Niedrigfett (CVS CHOW) (9 % Fett) - oder Hochfett (CVS HFD) (43 % Fett) - Fütterung bestimmt (Langzeiteffekte von CVS). Die Analysen der Kurz- und Langzeiteffekte von CVS auf die Insulinsensitivität wurden durch Messungen der Spiegel von Fettmetaboliten, Hormonen und Cytokinen im Plasma und durch die Bestimmung der Genexpression (*real-time-PCR*, RT-PCR) und Proteinspiegel (Western Blots, WBs) in Hypothalamus, Skelettmuskel, Leber und Fettgewebe ergänzt.

Ergebnisse: CVS-Mäuse wiesen eine Verringerung der fettfreien Masse und des Körpergewichts auf, während die Corticosteron-Spiegel im Plasma und die Fettmasse erhöht waren. Die endogene Glukoseproduktion (EGP, Leber) wurde durch CVS herabgesetzt. Die

Ganzkörperinsulinsensitivität gemessen an der Glukoseinfusionsrate (GIR) blieb durch CVS unverändert, jedoch war der Insulin-stimulierte Glukoseumsatz in peripheren Geweben (*rate of glucose disposal*, Rd %) im Vergleich mit den CTRL-Mäusen erhöht. In Einklang mit der gesteigerten Rd % bzw. der unveränderten GIR waren die Adiponektin-Spiegel im Plasma tendenziell erhöht, während die Resistin-Spiegel unverändert blieben. Des Weiteren waren die Proteinspiegel der Fettsäureoxidations-Marker AMPK, p-ACC, ACC im Skelettmuskel erhöht und die Triglycerid-Spiegel im Plasma verringert. Unterschiede in der Expression des Fettsäureoxidations-Markers *Cpt1b* im Skelettmuskel wurden nicht gefunden. Drei Monate nach der Stressintervention wiesen CVS CHOW-Mäuse im Vergleich mit CTRL CHOW-Mäusen eine erhöhte GIR und Rd % auf. Im Gegensatz dazu wiesen CVS HFD-Mäusen im Vergleich mit den CTRL HFD-Mäusen eine verminderte GIR und Rd % auf. CVS CHOW-Mäuse zeigten verringerte Adiponektin- und Resistin- Spiegel, während CVS HFD-Mäuse verringerte Adiponektin- und erhöhte Resistin-Spiegel aufwiesen. Die *Cpt1b*-Expression im Skelettmuskel war im Vergleich mit den entsprechenden CTRL-Mäusen in CVS CHOW-Mäusen erhöht und in CVS HFD-Mäusen verringert, was auf eine erhöhte bzw. verminderte Fettsäureoxidation hinweist. In Einklang damit zeigten CVS CHOW-Mäuse verringerte Triglycerid-Spiegel im Plasma und eine verminderte adipogene Aktivität im weißen Fettgewebe (WAT) gemessen an der verringerten Expression von *Lpl* und *Fasn* und der gesteigerten Expression von *Lipe*. CVS HFD-Mäuse wiesen im Vergleich mit den CTRL HFD-Mäusen keine Unterschiede in Triglycerid-Spiegeln und in der *Lpl*, *Fasn* and *Lipe*-Expression im WAT auf. CVS zeigte keine Langzeiteffekte auf die Proteinspiegel von Fettsäureoxidations-Markern im Skelettmuskel.

Schlussfolgerungen: CVS vermindert die hepatische Insulinsensitivität, während der Glukoseumsatz in peripheren Geweben erhöht ist. Als Folge dieser komplementären Regulation stellt sich die Ganzkörper-Insulinsensitivität (GIR) durch CVS als unverändert dar. Langfristig steigert CVS bei fettarmer Ernährung die Insulinsensitivität und den Glukoseumsatz in peripheren Geweben, während CVS in Verbindung mit nachfolgender Hochfetternährung die Insulinsensitivität und den Glukoseumsatz in peripheren Geweben herabsetzt. Demnach reguliert der Fettgehalt der Nahrung die Langzeiteffekte von CVS als Steigerung (Niedrigfett) oder Verminderung (Hochfett) der Insulinsensitivität. Metabolische Veränderungen auf der Ebene des WAT ebenso wie die veränderte Gen- und Proteinexpression von Fettsäureoxidations-Markern im Skelettmuskel deuten auf Modifikationen des Lipidmetabolismus als wesentlicher Mechanismus hinter den Langzeiteffekten von CVS und Ernährungsintervention auf die Insulinsensitivität hin. Daher konnte im vorliegenden Mausmodell für posttraumatische Belastungsstörung zum ersten Mal gezeigt werden, dass chronischer Stress Auswirkungen auf die Insulinsensitivität hat, die sich spät im Leben in Abhängigkeit von Lebensstilfaktoren wie Ernährung manifestieren.

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7 Appendix

7.1 Abbreviations

1,3BGP	1,3-bisphospho-glycerate
11βHSD1	11-β-hydroxysteroid dehydrogenase type 1
2-[3H]DG	(2-[3H])deoxyglucose
2PG	2-phosphoglyceric acid
3PG	3-phosphoglyceric acid
4AAP	4-aminoantipyrine
a-AA	4-amino-antipyrine
ACC	Acetyl-CoA carboxylase
ACOD	Acetyl-CoA oxidase
ACOT1	Acyl-CoA thioesterase 1
ACS	Acetyl-CoA synthase
ACTB, *Actb*	Beta-actin
ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
AgRP	Agouti-related protein
AKT	Protein kinase B
AMPK	Adenosine-monophosphate-activated protein kinase
ANGPTL4, *Angptl4*	Angiopoietin-like protein 4
AP	Activator protein-1
APS	Ammonium persulfate
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
$Ba(OH)_2$	Barium hydroxide
BAT	brown adipose tissue

BCA	Bicinchoninic acid
BMI	body mass index
BSA	bovine serum albumin
BW	body weight
cAMP	Cyclic adenosine monophosphate
CAP-1	Adenylyl cyclase-associated protein 1
CART	Cocaine-amphetamine related transcript
CCL2, *Ccl2*	Chemokine (C-C motif) ligand 2
CoaA	Coenzyme A
CORT	Corticosterone
CPT	Carnitine palmitoyltransferase
CPT1α, *Cpt1a*	Carnitinepalmitoyl-transferase 1 alpha
CPT1β, *Cpt1b*	Carnitinepalmitoyl-transferase 1 beta
CRH	Corticotropin-releasing hormone
Ct	cycle threshold
CTRL	control
CVS	Chronic variable stress
d	day
DHAP	Dihydroxyacetone phosphate
dl	deciliter
DNA	Desoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGP	endogenous glucose production
EGTA	Ethylene glycol tetraacetic acid
EPI	epididymal
et al.	*et alii* (and others)
F-1,6-BP	Fructose-1,6-bisphosphatase
F-6-P	Fructose-6-phosphate
FAS, *Fasn*	Fatty acid synthase

FFA	free fatty acids
G3P	Glyceraldehyde-3-phosphate
G-6-P	Glucose-6-phosphate
G6Pase, *G6pc*	Glucose-6-phosphatase
GAPDH, *Gapdh*	Glyceraldehyde 3-phosphate dehydrogenase
GCK, *Gck*	Glucokinase
GCs	glucocorticoids
G-CSF	Granulocyte colony stimulating factor
GHSR	Growth hormone secretagogue receptor
GIP	Gastric inhibitory polypeptide
GIR	glucose infusion rate
GK	Glycerol kinase
GLP-1	Glucagon-like-peptide 1
GLUT1	Glucose transporter 1
GLUT4	Glucose transporter 4
GLUTs	glucose transporters
GM-CSF	Granulocyte macrophage colony stimulating factor
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive element
GRU	Glucocorticoid response unit
GTT	Glucose tolerance test
h	hour
H_2O_2	Hydrogen peroxide
HFD	High fat diet
HK2, *Hk2*	Hexokinase 2
HOMA	Homeostasis model assessment
HPA axis	Hypothalamus-pituitary-adrenal axis
HSL	Hormone-sensitive lipase

HSP	Heat shock protein
IFN-γ	Interferon gamma
IL	Interleukin
IRS-1	Insulin receptor substrate 1
IRS-2	Insulin receptor substrate 2
ITT	Insulin tolerance test
kg	kilogram
KO	knockout
KOH	Potassium hydroxide
l	liter
LPL	Lipoprotein lipase
M	mole
MC4R	Melanocortin 4 receptor
MCP-1	Monocyte chemotactic protein 1
MCP-1β	macrophage inflammatory protein 1 beta
MEHA	3-methyl-N-mthyl-N-(beta-mydroxyethyl)-aniline
mg	milligram
MGL	Monoacylglycerol lipase
min	minute
MIP-1α	Macrophage inflammatory protein alpha
Ml	milliliter
mM	millimole
mTORC1	Mammalian target of rapamycin complex 1
mU	milliunits
NaCl	Sodium chloride
NEFA	Non-esterified fatty acids
NFDM	Non-fat dry milk
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

nM	nanomole
NMR	Nuclear magnetic resonance
NPY	Neuropeptide Y
NSB	Non-specific binding
o.n.	over night
PAI-1	Plasminogen activator inhibitor-1
PC	Pyruvate carboxylase
PEG	Polyethylene glycol
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PEPCK, *Pck1*	Phosphoenolpyruvate carboxykinase 1
PGC-1α	Peroxisome proliferator-activated receptor co-activator 1 alpha
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
POD	Peroxidase
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator-activated receptor
PPi	Pyrophosphoric acid
PTSD	posttraumatic stress disorder
PVN	Paraventricular nucleus
RANTES	Regulated on activation, normal T cell expressed and secreted)
Rd	rate of disposal/disappearance
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPS18, *Rps18*	40S ribosomal protein S18
RT	room temperature
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean

SNS	sympathetic nervous system
TAGs, TGs	triglycerides
TEMED	Tetramethylethylenediamine
T_m	melting temperature
TNF-α	Tumor necrosis factor alpha
TRIS	Tris(hydroxymethyl)aminomethan
U	units
UCP1, *Ucp1*	Uncoupling protein 1
UCP3, *Ucp13*	Uncoupling protein 3
v	volume
V	volt
w	weight
w/v	weight per volume
WAT	white adipose tissue
WB	Western Blot
x g	gravitation
Y2R	NPY receptor 2
$ZnSO_4$	Zinc sulfate
α-MSH	Alpha-melanocyte stimulating hormone
µg	microgram
µl	microliter

7.2 List of figures

7.3 List of tables

8 Acknowledgements

I would like to thank my thesis director Prof. Dr. Hadi Al-Hasani, Head of the Institute for Clinical Biochemistry and Pathobiochemistry at the German Diabetes Center (DDZ), to give me the valuable opportunity to do this doctoral thesis in his lab and to make possible to interact with the scientific community through the attendance to congresses and meetings. Also I would like to thank Prof. Dr. Eckhard Lammert, Head of the Institute of Metabolic Physiology at the Heinrich-Heine-University and of the Institute for Beta Cell Biology at the DDZ to co-direct this thesis from the Faculty of Mathematics and Natural Sciences.

I would especially like to thank my project supervisor Dr. Tamara R. Castañeda who gave me the opportunity to write my thesis within her very interesting research project and who advised me through all my thesis work with her cheerful and cordial disposition and her motivational strength. She always invested a lot of time not only in excellent scientific support that helped me to gain experience of immeasurable value also beyond the scope of this project but also in our personal very good relation that made my time working in this project deeply precious and enjoyable. Therefore, thanks a lot for the outstanding thesis supervision and the friendship that I will always continue cherishing in the future.

Moreover, a million thanks to the other members of our small nice group starting with Elisabeth Brandt who wrote her bachelor thesis within this project. Thanks a bunch for your great work, your friendship and again for these lovely farewell words that you wrote to me and I will never forget! As well, a lot of thanks to Christian Binsch and Matthias Dille who write their master theses in our group. I really had the time of my life with the two of you! Christian, thank you so much for the delicious meals, the funny bets, great discussions and last but not least all the creative nick names ☺. All of you were the best colleagues and friends that one would wish with your reliability, motivation, smartness and support in every possible way. Thank you for all the laughs, guys. I am going to miss you so much and hope we will never lose sight of each other!

As well I would like to give very special thanks to Dr. Tomas Jelenik from the Institute for Clinical Diabetology of the DDZ who supported this work with his excellent expertise in hyperinsulinemic-euglycemic clamps. His expert knowledge, his admirable patience and strength and his friendliness were major contributors in the success of this research project.
Many thanks as well to Daniela Seeger and Peter Nowotny, Head of the Technical Laboratory, for the professional and efficient clamp sample analysis within this collaboration.

Moreover, I would like to give all my thanks to Prof. Dr. Michael Roden, Head of the Institute for Clinical Diabetology at the DDZ, who made possible this excellent and exciting clamps collaboration.

Furthermore, I would like to give many thanks to Waltraud Passlack, Dr. Sonja Hartwig and Dr. Stefan Lehr from the Proteome Analysis Unit of the Institute for Clinical Biochemistry and Pathobiochemistry at the DDZ for their collaboration with the Bioplex analyses.

Many thanks as well to the members of the animal facilities of the DDZ that facilitated the experimental animal work with their energetic and foresighted way of being. Special thanks to Dr. H.-J. Partke, who introduced me to the DDZ community at the very beginning and trained me to work *in vivo*. Many thanks for the great time; I will never forget all the fun that we had!

I would also like to thank all the other colleagues from the Institute for Clinical Biochemistry and Pathobiochemistry not only for inspiring scientific discussions but as well for the very pleasant and amicable atmosphere, also beyond work. Special thanks to Antonia Osmers, Annette Schober, Anette Kurowski, Angelika Horrighs and Ilka Römer. And it would not be possible to forget these wonderful people, Sandra Lebek, Christian de Wendt, Tanja Schallschmidt, Torben Stermann, Christian Springer, Tim Benninghoff, Simon Göddeke and David Barbosa. Thank you guys for all those great Karneval celebrations, barbecues and birthday parties! I hope we will always stay in contact!

Furthermore, I would like to thank all my former colleagues from the Institute for Clinical Diabetology, especially PD Dr. Volker Burkart, Ulrike Partke and Fariba Zivehe who were always willing to give a hand at the different stages of the development of this research work. Thanks a lot for your kindness, your help and your friendliness!

Last but not least I would like to give all my thanks to my parents Claudia and Jürgen, my sister Laura and to my boyfriend Jürgen. They supported me in all those years in every possible way that I could always look forward and never got discouraged. Many things would not have been possible without your love and motivational strength. I am deeply grateful for all your consideration, patience and interest in my work and I am so happy that I could always rely on you. Thank you so much!